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Rodrigo Pereira Martins

EFEITOS DOS RECURSOS TERAPÊUTICOS SOBRE MARCADORES BIOQUÍMICOS NA LESÃO MUSCULAR POR ESTIRAMENTO EM RATOS

Santa Maria, RS, Brasil
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Dissertação apresentada ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica do Centro de Ciências Naturais e Exatas da Universidade Federal de Santa Maria (UFSM,RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Profº. Dr. Gustavo Orione Puntel

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Aprovado em 17 de agosto 2018:

_____

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

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Santa Maria, RS
2018

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.

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RESUMO

Dissertação de mestrado

Universidade Federal de Santa Maria

Programa de Pós-graduação em Ciências Biológicas:

Bioquímica Toxicológica

EFEITOS DOS RECURSOS TERAPÊUTICOS SOBRE MARCADORES BIOQUÍMICOS NA LESÃO MUSCULAR POR ESTIRAMENTO EM RATOS

AUTOR: RODRIGO PEREIRA MARTINS
ORIENTADOR: GUSTAVO ORIONE PUNTEL
Data e Local da Defesa: Santa Maria, 17 de agosto de 2018.

A prática esportiva pode induzir lesões nas fibras musculares, gerando um dano oxidativo e um processo inflamatório. Práticas terapêuticas são utilizadas com o intuito de reduzir estes danos, como a injeção de plasma rico em plaquetas (PRP) e a imersão em água fria (CWI). Assim, o presente estudo teve como objetivo investigar através de biomarcadores a influência do tratamento com o frio terapêutico e/ou PRP em modelo de lesão muscular por estiramento em ratos. Métodos: Ratos machos *Wistar adultos* foram submetidos à lesão muscular por estiramento e imediatamente, os animais receberam uma aplicação intramuscular de PRP e/ou foram submetido a sessões de CWI. As análises bioquímicas como: Espécies reativas ao ácido tiobarbitúrico (TBARS), espécies reativas a diclorofluorescêina (DCFRS), grupos tiois (-SH), redução de MTT, glutationa reduzida (GSH), glutaiona oxidada (GSSG), superóxido dismutase (SOD), catalase (CAT), mieloperoxidase (MPO), foram realizadas 1, 3, 5, 7 dias após o primeiro dia de tratamento. A lesão por estiramento no *gastrocnêmio* dos animais causou um aumento nos marcadores de dano oxidativo, tais como a formação de espécies reativas a diclorofluroscêina e peroxidação lipídica no músculo e no sangue. A combinação do tratamento com PRP e CWI parece ser o mais efetivo, em comparação aos tratamentos isolados, em prevenir o dano induzido pela lesão muscular possivelmente devido a sua capacidade em modular os danos à estrutura da célula muscular e também a intensidade da resposta inflamatória que segue a lesão músculo esquelética.

Palavras-chaves: Plasma Rico em Plaquetas, Imersão em água fria, dano oxidativo, Musculoesquelético.

ABSTRACT

Masters dissertation

Federal University of Santa Maria

Graduate Program in Biological Sciences:
Toxicological Biochemistry

EFFECTS OF THERAPEUTIC RESOURCES ON BIOCHEMICAL MARKERS IN INJURY STRETCH MUSCLE IN RATS

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Date and Location of Defense: Santa Maria, 17 of August 2018

The sports practice can induce injuries in the muscular fibers, generating an oxidative damage and inflammatory process. Therapeutic practices are used to reduce these damages, such as platelet-rich plasma (PRP) and cold water immersion (CWI). Thus, the present study aims to investigate through biomarkers the influence of treatment with CWI and / or PRP in muscle stretch model by stretching in rats. Methods: Adult male *Wistar* rats were subjected to stretch muscle injury and immediately, the animals received an intramuscular application of PRP and / or subjected to CWI. Biochemical analyzes such as: thiobarbituric acid reactive species (TBARS), dichlorofluorescine reactive species (DCFRS), thio groups (-SH), reduction of MTT, reduced glutathione (GSH), oxidized glutamine (GSSG), superoxide dismutase (SOD) , catalase (CAT), myeloperoxidase (MPO), were performed 1, 3, 5, 7 days after the first day of treatment. Stretch lesion in the gastrocnemius of the animals caused an increase in the markers of oxidative damage, such as the formation of reactive species of dichlorofluorescein and lipid peroxidation in muscle and blood. Combinad of PRP and CWI treatment appears to be the most effective in preventing muscle injury, compared to treatments isolated, possibly due to its ability to modulate damage to muscle cell structure and also the intensity of the inflammatory response following skeletal muscle injury.

Keywords: Platelet-rich plasma, cryotherapy, oxidative damage, musculoskeletal.

LISTA DE FIGURAS

Dissertação

Manuscrito cientifico

LISTA DE ABREVEATURAS

ERs- Espécies reativas de oxigênio

ERO- Espécie reativa de oxigênio

O2- -Ânions superóxido

OH- -Radicais hidroxila

H_2O_2- Peróxido de hidrogênio

HOCL- Ácido hipocloroso

O2 – Oxigênio

SOD- Superóxido Dismutase

CAT- Catalase

GPx- Glutaiona Peroxidase

GSH- Glutationa reduzida

GSSG- Glutationa oxidada

AA- Ácido Ascórbico

NADPH- Nicotinamida-adenina-dinucleótido-fosfato-oxidase

EO- Estresse oxidativo

PRP- Plasma Rico em Plaquetas

PDGF- Fator de crescimento derivado de plaquetas (do inglês *Platelet-derived growth factor*)

TGF-β- Fator de crescimento transformante-β (do inglês *Transforming growth factor beta*)

VEGF- Fator de crescimento endotelial vascular (do inglês *Vascular endotelial growth factor*)

EGF- Fator de crescimento epidérmico (do inglês *Epdermal growth factor*)

FGF- Fator de crescimento de fibroblastos (do inglês *Fibroblast growth factor*)

HGF- Fator de crescimento de hepatócitos (do inglês *Hepatocyte growth factor*)

CWI – Imersão em água fria (do inglês *Cold water immersion*)

SUMÁRIO

APRESENTAÇÃO

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

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de um manuscrito científico, os quais se encontram alocado no item **MANUSCRITO CIENTÍFICO**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontra-se no respectivo manuscrito e representa a íntegra deste estudo.

O item **CONCLUSÃO**, encontrado no final desta monografia, apresenta interpretações e comentários gerais sobre o manuscrito científico contido neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** se referem somente às citações que aparecem no item **INTRODUÇÃO** desta dissertação de mestrado.

1- INTRODUÇÃO

1.1- Lesão muscular

O músculo esquelético pode ser considerado um órgão composto de dois componentes principais, as fibras musculares e os tecidos envoltórios conjuntivos. As fibras musculares, com sua inervação, são responsáveis pela função contrátil do músculo, enquanto que o tecido conjuntivo fornece a estrutura que interliga as células musculares individuais durante a contração muscular e envolve os capilares e nervos no interior da estrutura muscular (BASSEL-DUBY & OLSON, 2006; JARVINEN et al., 2005).

Aproximadamente 45% da massa do corpo humano adulto é formado por tecido muscular. Em geral, os músculos desempenham um papel importante na locomoção, preensão, mastigação, movimentos oculares e outros eventos dinâmicos (OSTROVIDOV et al., 2013). O tecido muscular esquelético é composto por células que apresentam estabilidade como característica, em outras palavras, a morte destas células é relativamente rara e este evento é geralmente associado a lesões musculares (BASSEL-DUBY & OLSON, 2006).

As lesões musculares estão entre as mais comuns em diferentes tipos de esportes, a sua frequência variando entre 10-55% de todas as lesões. Neste contexto, é importante determinar qual o tipo e magnitude de lesão, sendo as contusões e os estiramentos musculares os mais frequentes. Entre essas lesões relacionadas ao esporte estão às lesões por estiramento, causadas quando indiretamente é produzida uma contração intensa combinada com o alongamento forte levando a um esforço excessivo ou estresse no musculoesquelético (PACHIONI et al., 2009). Em alguns casos, ocorre não somente o rompimento de fibras musculares, mas também um possível dano à estrutura óssea, sendo estas consideradas o tipo de lesão mais incomum na área esportiva (JARVINEN et al., 2005). A gravidade desses tipos de lesões musculares podem ser medidas pela incapacidade funcional do atleta para treinar e competir, e aumento do risco de lesões recorrentes. Em muitos casos, essa perda funcional pode durar de 30 a 40 dias (SÁNCHEZ et al., 2014).

A cura de um músculo esquelético lesionado segue um padrão constante, independentemente da causa subjacente. O processo do reparo tecidual apresenta-se dividido em três fases. Na 1ª fase ocorre à destruição/inflamação, caracterizada pela formação de hematoma, necrose e pelos sinais cardinais das reações inflamatórias (hipertermia, hiperemia, edema, dor e prejuízo/perda da função ou calor, rubor, tumor, dor e perda da função); na 2ª fase acontece o reparo, caracterizado pela fagocitose do tecido muscular necrosado, pela regeneração das miofibrilas, e pela proliferação de tecido conjuntivo cicatricial e de capilares sanguíneos; e na 3ª fase ocorre o remodelamento e reorganização do tecido, caracterizada pela maturação das miofibrilas regeneradas, pela contração e reorganização do tecido conjuntivo cicatricial, e pela restauração da funcionalidade do tecido muscular reparado (JARVINEN et al., 2005).

As lesões musculares já apresentadas podem ser divididas de acordo com sua magnitude, como, leve (1º grau), moderado (2º grau) e grave (3º grau) (JARVINEN et al., 2005). As lesões de 1º grau são as mais comuns e acontecem quando há um estiramento das fibras musculares, porém o individuo permanece praticando suas atividades. A sensação intensa de dor costuma aparecer no dia seguinte e o período de recuperação é de cerca de 5 dias. Nas lesões de 2º grau uma maior quantidade de fibra é rompida. Neste caso, é geralmente referida a sensação de que o músculo está “rasgando”, associada a incapacidade de manter a atividade física, sendo o período de recuperação corresponde a aproximadamente 10 dias. Já as lesões de 3º grau são os casos mais graves, nestes casos ocorre uma ruptura total da musculatura, geralmente na junção miotendínea, sendo o tempo de sua recuperação de, aproximadamente, 21 dias (KIRKENDALL & GARRETT, 2002).

1.2- Processo inflamatório e estresse oxidativo

Cada uma das fases da cura do tecido muscular apresenta uma intima dependência do grau de lesão do tecido. Nestas circunstâncias, as propriedades de elasticidade, extensibilidade e contratibilidade das células musculares podem ser comprometidas. As alterações nas propriedades mecânicas e fisiológicas do tecido muscular podem ser resultado de uma incapacidade da unidade contrátil da fibra muscular, a qual é designada como sarcômero (JARVINEN et al., 2005).

A resposta inflamatória apresenta sintomas e sinais característicos, como a formação de calor, rubor, edema, dor e por ultimo a perda da função. Em geral, o dano muscular é caracterizado por alterações do citoesqueleto dos miócitos, associada com uma perda da força e aumento da rigidez muscular, com o extravasamento de proteínas musculares, e com o processo inflamatório (GUILHEM et al., 2013).A resposta inflamatória subsequente à injúria muscular é necessária, e visa a reabilitação e regeneração tecidual.

As células inflamatórias são predominantemente neutrófilos e macrófagos, envolvidas na remoção do material danificado. Assim sendo, a fibra muscular pode responder à lesão tanto com a regeneração, quanto com a formação de fibrose na área lesada, caracterizando um processo inflamatório crônico. No entanto, este processo pode levar à inibição completa da regeneração do tecido muscular (JARVINEN et al., 2005).

O processo inflamatório em lesões musculares consiste de na quimiotaxia, na ativação e no acumulo de neutrófilos dentro da área lesada (TOUMI & BEST, 2003). Os neutrófilos são extremamente importantes durante o processo inflamatório, sendo as primeiras populações de células brancas a entrar na área traumatizada ou tecidos estressados, e sua principal função é conter e destruir o tecido danificado ou corpos estranhos através da fagocitose (BROWN et al., 2001).

O processo inflamatório desencadeado para reabilitação frequentemente é acompanhada por um aumento da formação de Espécies Reativas (ERs), as quais auxiliam na degradação e remoção de células danificadas. Toda via, este processo deve ser controlado para evitar uma excessiva produção de ERs e infiltração de neutrófilos, as quais podem exacerbar o dano tecidual (TOUMI & BEST, 2003). Dentre as principais formas de ERs estão as derivadas do metabolismo oxidativo chamadas de Espécies Reativas de Oxigênio (EROs) (HALLIWELL & CROSS,

1994). Tendo em vista que o suprimento energético do tecido muscular é especialmente aeróbico, via fosforilação oxidativa mitocondrial, é possível compreender que o aumento na atividade metabólica muscular, tal qual é observada em lesões musculares, está geralmente associada com um aumento na formação de EROs (TOUMI & BEST, 2003).

O estresse oxidativo é a consequência de um desequilíbrio de pró-oxidantes e antioxidantes que levam a danos celulares e lesão tecidual. Quando as espécies reativas de oxigênio não são removidas de forma eficaz e com segurança, o estresse oxidativo pode prejudicar a saúde. A exaustão dos sistemas antioxidantes é uma das razões para a ocorrência do estresse oxidativo, o que resulta na produção excessiva de EROs ou radicais livres, como: ânions superóxido (O2-) e radicais hidroxila (OH-) . As espécies reativas de oxigênio também incluem compostos que não são radicais livres, como: H_2O_2, ácido hipocloroso (HOCl) e ácido hipobromoso (HOBr). Estas espécies podem danificar as membranas lipídicas e produzir necrose celular (PUPPEL et al., 2014). Halliwell & Cross (1994) afirma que toxicidade por oxigênio (O_2) ocorre devido ao excesso de formação de radical O_2^-, sendo este, o produto de redução de um elétron de O_2.Todos os organismos sofrem alguma exposição ao OH-, pois este radical é gerado in vivo por fissão homolítica de títulos O-H na água. O Radical hidroxila é tão reativo com todas as moléculas biológicas que é impossível evoluir um para removedor específico de quase tudo dele em organismos vivos. Já o peróxido de hidrogénio é adicionalmente gerado in vivo por diversas enzimas oxidase, tais como glicolato oxidase, xantina oxidase e D-aminoácido oxidase (HALLIWELL & CROSS, 1994).

O processo inflamatório em lesões musculares consiste de neutrofilia, ativação de neutrófilos e acúmulo de neutrófilos dentro da área lesada (TOUMI & BEST, 2003). A presença de quantidades significativas de neutrófilos na área lesada determina a geração de ácido hipocloroso e hipoclorito ($HOCL/OCL^-$) a partir de H_2O_2 na presença de íons cloreto, a fim de promover a degradação e remoção do tecido danificado (BROWN et al., 2001).

Os dados experimentais mostram claramente que a remoção de O_{2-} e H_2O_2 por sistemas de defesa antioxidante é essencial para a vida saudável aeróbica. A fim de neutralizar as ERs continuamente formadas durante o metabolismo aeróbico, os miócitos e células dispõem de vários mecanismos de defesa endógenos coletivamente definidos como antioxidantes. As duas classes principais destes

mecanismos antioxidantes celulares são: as defesa enzimática, em especial as enzimas Superóxido Dismutase (SOD), Catalase (CAT) e Glutationa Peroxidase (GPx); e as não-enzimáticas, em especial a Glutationa Reduzida (GSH) e o Ácido Ascórbico (AA). A enzima SOD contendo manganês no seu local ativo (Mn-SOD) nas mitocôndrias e SOD com cobre e zinco no sítio ativo (Cu, Zn-SOD) presente em grande parte no citosol, remove O_2- e acelera muito a sua conversão ao H_2O_2. A enzima Catalase converte H_2O_2 em água e O_2, e ajuda a eliminar H_2O_2 gerado pelas enzimas peroxissomais. No entanto, enzima mais importante para remoção de H_2O_2 são as glutationa peroxidases (GPx), que necessitam de selénio (como um resíduo do sítio ativo da selenocisteína) para a sua ação. As enzimas GPX remove H_20_2, usando-o para oxidar GSH à glutationa oxidada (GSSG). A glutationa redutase é uma enzima flavoproteína, regenera de GSH a GSSG, com NADPH como uma fonte de poder redutor (HALLIWELL & CROSS, 1994).

O ácido ascórbico atua na fase aquosa como um excelente antioxidante sobre os radicais livres, mas não é capaz de agir nos compartimentos lipofílicos para inibir a peroxidação dos lipídeos. Por outro lado, estudos in vitro mostraram que essa vitamina na presença de metais de transição, tais como o ferro, pode atuar como uma molécula pró- -oxidante e gerar os radicais H_2O_2 e OH^-. Geralmente, esses metais estão disponíveis em quantidades muito limitadas e as propriedades antioxidantes dessa vitamina predominam in vivo (ODIN, 1997).

Sempre que a formação de ERs, em especial EROs, for além das capacidades antioxidantes celulares neutralizá-las, temos o desencadeamento de danos oxidativos a biomoléculas celulares. Esta situação é reconhecida como Estresse Oxidativo (EO), o qual vem sendo reconhecido como um evento fundamental na fisiopatologia de inúmeras doenças e também na fisiologia do envelhecimento humano. Dentre as principais alterações celulares associadas ao estresse oxidativo estão a peroxidação e comprometimento da permeabilidade de membranas lipídicas, as alterações nas funções enzimáticas, as alterações no funcionamento mitocondrial, e os comprometimentos na funcionalidade nuclear associada à síntese e renovação biomolecular, as quais podem resultar na necrose celular (HALLIWELL & CROSS, 1994).

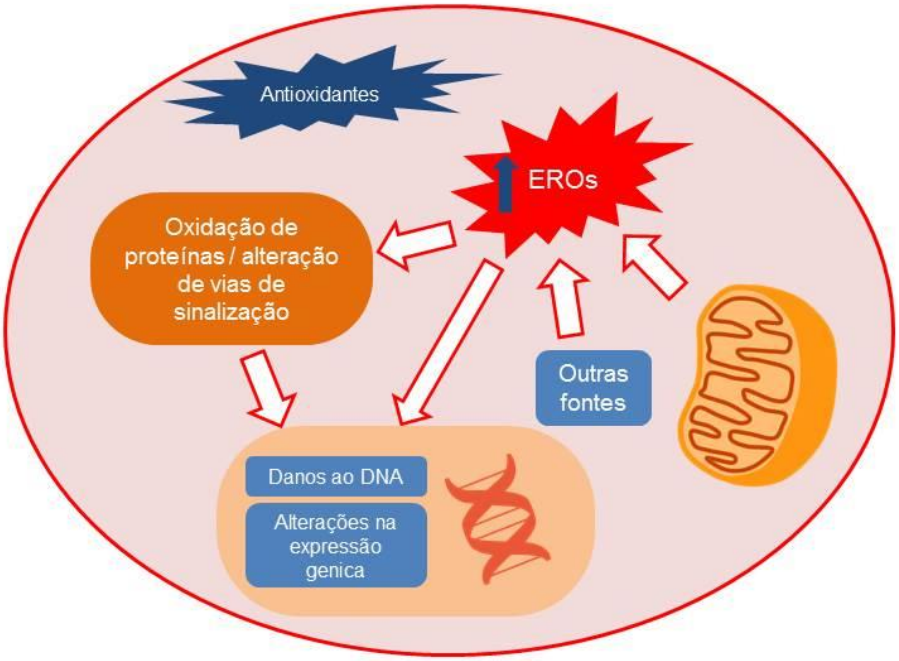

Figura 1: Esquema ilustrativo demonstrando alguns danos causados pelo aumento das espécies reativas de oxigênio (EROs).

1.3- Plasma Rico em Plaquetas

Estudos ao longo do tempo investigam variáveis que influenciam a velocidade de cicatrização frente ao processo de reparação tecidual (DIMAURO et al., 2014; ANITUA et al., 2007) sendo que o uso de Plasma Rico em Plaquetas (PRP) é um recente e promissor método adjuvante nesse contexto. O objetivo do tratamento do PRP para a lesão muscular é o de melhorar e acelerar o processo de reparação muscular e, consequentemente, permitir que o paciente possa continuar com suas atividades desportivas ou diárias, o mais rapidamente possível, sem recaídas (SÁNCHEZ et al., 2014). O PRP é um método simples, derivado do processo laboratorial de sangue autógeno ou homólogo obtido através da centrifugação, de baixo custo e minimamente invasivo, que fornece um concentrado natural de fatores de crescimento obtido através de sangue que pode ser usado para melhorar a regeneração de um determinado tecido (KON et al., 2011).

Na literatura alguns estudos investigam a eficácia do PRP perante a aceleração do processo de regeneração tecidual (PRESTES et al., 2012; Sánchez et al., 2014). O PRP proporciona um aumento de 3 a 5 vezes na concentração de plaquetas quando comparada com a concentração normal no sangue (MARX, 2004) Após sua ativação, o PRP induz a produção/liberação de inúmeros tipos de fatores de crescimento para locais lesionados, incluindo o fator de crescimento derivado de plaquetas (PDGF), divididos em PDGF αα, PDGFββ e PDGF αβ; fator de crescimento transformante-β (TGF-β), divididos em TGF- β1 e TGF- β2; fator de crescimento endotelial vascular (VEGF), fator de crescimento epidérmico (EGF), fator de crescimento semelhante a insulina-I (IGF-I), fator de crescimento de fibroblastos (FGF) e fator de crescimento de hepatócitos (HGF) (YUAN et al., 2012; MARX, 2001).

Produtos derivados de sangue autólogo transmitem além dos fatores de crescimento, citosinas e outras proteínas contidas nas plaquetas, assim como de fibrinogênio e outras proteínas plasmáticas, em um agregado biologicamente equilibrado. Estes podem ser responsáveis por duas características especiais: a resolução da inflamação e evitar a fibrose. Além de transmitir os fatores de crescimento, PRGF fornece ao tecido danificado um andaime de fibrina biológica transiente que deriva da polimerização de fibrinogênio, que é uma proteína do

sangue pleiotrópica que regula a coagulação, inflamação, e a regeneração de tecidos (SÁNCHEZ et al., 2014).

Relatos na literatura têm sugerido que os fatores de crescimento presentes nas plaquetas promovem uma potencialização da quimiotaxia, proliferação, diferenciação e secreção celular, podendo influenciar o tempo de reparação (BAUER et al., 2009).

Alguns estudos apresentaram bons resultados sobre os efeitos biológicos com uso do PRP em tecidos moles, como por exemplo, Anitua et al. (2007) que aborda um estudo randomizado para avaliar a eficácia e segurança do PRP para tratamento de úlceras cutâneas crônicas. Os seus resultados mostram que a porcentagem de área de superfície cicatrizada no grupo PRP foi significativamente maior do que no grupo de cuidados padrão durante todos os pontos de avaliação do estudo. De fato, a porcentagem da superfície cicatrizada em oito semanas foi 72,94% no PRP e 21,48% no grupo de tratamento padrão.

Há muito poucos dados sobre o efeito do PRP em lesões musculares. Um estudo recente utilizando um modelo de rato descobriu que o tratamento PRP resultou em um tempo de recuperação mais rápido de pequenas lesões musculares por esforços repetitivas (HAMMOND et al., 2009).

Em outro estudo com modelo de contusão em ratos, uma injeção local de PRP no músculo *gastrocnêmio* lesionado não resultou em diferenças significativas no resultado funcional em vários pontos da lesão, indicando que não há benefício provável para a cura. Além disso, não houve diferença significativa entre a administração de imediato ou tardio de PRP (DELOS et al., 2014).

1.4- Crioterapia

As práticas terapêuticas tem sido implementadas com o intuito de reduzir os danos sobre a musculatura esquelética, sendo uma delas a crioterapia (GUILHEM et al., 2013). A crioterapia é um dos tratamentos mais simples e mais antigos para a lesão muscular aguda, a qual anestesia a área a que se aplica e diminui o fluxo sanguíneo local e o metabolismo.

A crioterapia permanece como a modalidade terapêutica que apresenta uma grande frequência de utilização em situações de pós-lesão muscular esquelética, sendo muito aplicada por profissionais da área clínica desportiva. A grande aplicabilidade como medida terapêutica, é em função principalmente devido à sua ampla disponibilidade e baixo custo. Assim, atualmente muitos estudos descrevem a eficácia do frio usando modelos animais e também o sucesso desta modalidade terapêutica na prática desportiva para combater os efeitos posteriores a atividade física intensa e lesões musculares (BLEAKLEY et al., 2004; PUNTEL et al., 2011; MARTINS et al., 2015).

A utilização clínica da crioterapia em circunstâncias fisiopatológicas e/ou injúrias musculares, apresenta algumas características provenientes do resfriamento local, dentre essas, o efeito analgésico, a diminuição da demanda metabólica tecidual, uma redução do fluxo sanguíneo local em decorrência do efeito vasoconstritor e por atenuação da pressão hidrostática, a qual levaria a formação do edema (BLEAKLEY et al., 2004; SCHASER et al., 2007; PUNTEL et al., 2011).

Dentre os procedimentos utilizados, pode ser citada a Imersão em Água Fria (CWI), aplicada em condições variadas em relação à forma, tempo ou temperatura (PASTRE et al., 2009). De acordo com Almeida et al., (2016) a CWI é uma das estratégias de recuperação pós-esforço mais utilizadas no contexto esportivo e tem sido demonstrada um beneficio na redução de marcadores de dano muscular. Entretanto, a literatura ainda é controversa quanto aos protocolos adotados, sobretudo com relação à temperatura da água, tempo e profundidade da imersão.

Neste contexto, o frio terapêutico mostra ser capaz de modular o dano oxidativo, possivelmente, pela sua capacidade de limitar a intensidade da resposta inflamatória, atenuar o comprometimento da função mitocondrial e também por preservar a morfologia do musculoesquelético. Isto pode ser explicado pelo fato da crioterapia atenuar a disfunção microvascular, diminuindo a temperatura, o

metabolismo e a demanda de O_2 na cadeia de transporte de elétrons. Além disso, é possível observar a preservação morfoanatômica e histológica do tecido lesado quando submetido ao tratamento com o frio (PUNTEL et al. 2011). Desta forma, o tratamento com Crioterapia, através do método de CWI, mostra um beneficio e auxiliaria no processo de reabilitação do tecido lesado.

1.5- Justificativa

Alguns estudos investigam, de formas isoladas, a influência que o PRP e a CWI oferecem na reparação de tecidos musculoesqueléticos em diversos achados clínicos. No entanto, até o momento, nenhum estudo investigou as alterações bioquímicas determinadas pelo uso combinado destes recursos terapêuticos em modelos experimentais de lesões musculares.

Considerando o fato de existir poucos relatos na literatura sobre os fenômenos bioquímicos envolvidos nos efeitos do tratamento com PRP e CWI sobre a lesão musculoesquelética, o conhecimento de como estas técnicas exercem seus efeitos torna-se importante para o desenvolvimento de possíveis esclarecimentos sobre as mesmas, uma vez que estes constituem uma das estratégias mais utilizadas no tratamento de lesões musculares no âmbito esportivo.

2- OBJETIVO

2.1- Objetivo geral

O estudo teve como objetivo geral investigar através de experimentos bioquímicos a influência do tratamento com o banho de imersão em água fria e/ou Plasma rico em plaquetas em um modelo de lesão induzida por estiramento muscular em ratos.

2.2- Objetivo específico

- ✓ Avaliar os efeitos do modelo de estiramento muscular sobre marcadores bioquímicos de estresse oxidativo;
- ✓ Quantificar os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) e de diclorofluoresceina oxidada (DCFRS) em amostras do tecido muscular e fração sanguínea dos animais submetidos à lesão e tratamentos;
- ✓ Analisar os níveis antioxidantes dos grupos tiois (-SH) e da glutationa reduzida (GSH) e oxidada (GSSG).
- ✓ Analisar os efeitos dos tratamentos sobre a atividade das enzimas superóxido dismutase (SOD) e catalase (CAT) em amostras do tecido muscular;
- ✓ Quantificar a resposta inflamatória no tecido muscular através da atividade enzimática da mieloperoxidase (MPO).

3- DESENVOLVIMENTO

MANUSCRITO CIENTÍFICO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de um manuscrito científico, no qual são apresentadas as seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas relacionadas ao presente estudo. O manuscrito encontra-se na formatação de submissão para a revista científica *The American Journal of Sports Medicine*.

COMBINED PLATELET-RICH PLASMA AND COLD WATER IMMERSION TREATMENT MINIMIZE THE DAMAGE FOLLOWING A SKELETAL MUSCLE STRETCH INJURY IN RATS.

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ABSTRACT

The skeletal muscle stretch injuries are commonly observed in sports. In order to stimulate tissue healing, the platelet-rich plasma (PRP) and cold water immersion (CWI) are widely used in clinical practice. This study investigated the effects of isolated or combined PRP and/or CWI on the oxidative damage determined by a stretch injury induced in *gastrocnemius* muscle of rats. PRP and CWI are applied immediately after the injury, and the biochemical analysis was performed after 1, 3, 5, or 7 days. The levels of o thiobarbituric acid reactive substances and oxidized dichlorofluorescein were significantly increased, both in skeletal muscle tissue and erythrocytes preparations, and the combined PRP and CWI minimized these parameters. Moreover, combined PRP and CWI were more effective than the isolated treatments to increase catalase activity, also the ratio of reduced/oxidized glutathione, and the non-protein thiols (-SH) group levels. In conclusion, we could infer that the combination of these regular treatments used in an isolated form shows a great potential for treatments of muscular injuries.

INTRODUCTION

Muscle injuries are common in different sports, affecting both professional and amateur athletes, with their frequency varying to 55% of all sports lesions. These muscle lesion result in the interruption of sports activity for the treatment, in this sense, the greatest challenge of rehabilitation is to promote proper muscle tissue repair in the shortest treatment period possible, enabling a quick return to the sport. This being important both for athletes in order that they do not lose performance and for amateurs so they do not stay away for prolonged periods from their healthy habits. [11, 20]

Among these, the stretch injuries are indirectly resulted from a combination of an intense contraction with a strong stretching, leading to an excessive stress on the skeletal muscle tissue structure, as observed in an experimental study of Pachioni et al., (2009) [22]. The mechanism of lesion consists on a vigorous contraction of the skeletal muscle tissue when it is in a stretching position [19].

The inflammatory response that follows a muscle injury is essential for the healing process and consequent tissue restructuring. This response is required to start tissue regeneration via infiltration of neutrophils into the injured area. The inflammatory process must be controlled to avoid an excessive production of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which can exacerbate tissue damage leading to an increased lipid peroxidation and even damage to DNA, resulting in an impaired intracellular metabolism and even cellular death. [2 11, ,28, 32]

Therapeutic cold is a condition characterized by the diminution of tissue temperature that is produced via different therapeutic modalities collectively described as cryotherapy. The therapeutic cold is one simple and classic method

used to treat acute muscle injuries and its effects are related to a reduced sensitive perception, such as nociception, and decreased blood flow and metabolism in treated tissues. This therapeutic physical agent has been considered an effective tool to treat different skeletal muscle lesions, based on its effects against the oxidative damage [15, 25]. Immersion in cold water (CWI) is a common cryotherapy method used in the treatment of skeletal muscle injuries that consist in submersion of the whole body or part of them in cooled water for few minutes. CWI has been shown to reduce the plasmatic markers of muscular damage and also to improve muscle contractile properties. [15, 24]

On the other hand, the use of platelet-rich plasma (PRP) to treat skeletal muscle injuries is recent and poorly described in the literature. PRP is derived from autologous or heterologous, whole blood and contains numerous growth factors and cytokines that have been shown to initiate and promote healing by stimulating cell migration, cell proliferation, angiogenesis, and matrix synthesis. [35] In a previous study of our group, we observed that PRP was able to modulate the oxidative damage determined by a skeletal muscle contusion possibly by reducing the impairment of myocytes mitochondrial function and improving their antioxidant defense systems. [16] However, the mechanism by which PRP exerts these effects is not fully understood.

Although both CWI and PRP show positive effects on treatment of musculoskeletal lesion, there are no studies focusing its combined effects. Therefore, the objective of this study was to evaluate the biochemical effects of treatment CWI and/or PRP on the oxidative damage determined by a stretch injury in *gastrocnemius* muscle of rats. In order to answer this question the levels of oxidative markers, such as oxidized dichlorofluorescein (DCFRS) and thiobarbituric acid reactive substance

(TBARS), and also the index of cellular viability were analyzed. Moreover, enzymatic antioxidants superoxide dismutase (SOD) and catalase (CAT) activities, and also non-protein thiol groups (-SH) levels, the ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined. As an indicator of the inflammatory response intensity, we measured myeloperoxidase (MPO) activity.

MATERIALS AND METHODS

Ethical approval

Animals were kept and used according to the institutional committee for animal care and use of the Federal University of Santa Maria, Brazil, registered and approved by the committee of ethics of animal use with the number 4694151216/2017 .

Chemical reagents

The thiobarbituric acid (TBA), Ellman (DTNB), N,N,N′,N′-tetramethylbenzidine, citric acid mono hydrate, calcium gluconate, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, 2′,7′-Dichlorodihydrofluorescein diacetate and o-phthalaldehyde reagents were provided by Sigma-Aldrich Chemical Co. (St. Louis, MO). The others were provided by local suppliers.

Animals

Seventy-two adult male Wistar rats, weighing 250–340 g, were placed (5 animals each cage) with food and water (ad libitum) in a temperature-controlled room

(22 ± 3°C) and at a ratio of 12 hours light/dark (lights were turned on by 7:00 am). The animals were divided into four main groups:

(1) Control group: injured animals injected with one saline solution (NaCl 0.9%) at every 48 h (50 µL, *i.m.*). The first injection was performed 2 h after injury (n = 18);
(2) PRP group: injured animals injected with PRP at every 48 h (50 µL, *i.m.*). The first injection was performed 2 h after injury (n = 18). The choice for this PRP protocol was based on previous studies.
(3) CWI group: injured animals submitted to CWI at every 24h (10°C ± 1ºC, 10 minutes). The first session was performed 2 h after injury (n = 18);
(4) CWI+PRP group: injured animals submitted to CWI at every 24h (10°C ± 1ºC, 10 minutes) and injected with PRP at every 48 h (50 µL, *i.m.*). The first injection and first session CWI was performed 2 h after injury (n = 18);

The animals of each of these four groups were subdivided into further four subgroups each (n = 4–5), according to the times where the analysis was performed after the stretch injury (1, 3, 5, or 7 days). In all of these subgroups, the right hind limb of the rats was submitted to skeletal muscle lesion and the left hind limb was used as uninjured and untreated control.

Skeletal muscle stretch injury

The skeletal muscle stretch injury was performed according to Ozaki et al. [19] with few modifications. Firstly, the animals were anesthetized with ketamine (50 mg/kg, ip) and xylazine (10 mg/kg, ip). After complete anesthetization, the animals were placed in dorsal position to proceed with the right *gastrocnemius* muscle injury. *Gastrocnemius* muscle was simultaneously stretching and submitted to tetanic

muscle contractions through an electrostimulatory device, with frequency of 50Hz, twenty contractions of 10 seconds with intervals of 10 seconds are also performed through self-adhesive electrodes. After the injury, the animals were left in the coop until fully anesthesia recovery [19].

PRP preparation

PRP was prepared using a protocol that highest platelet concentration according to Kaux et al. [12]. Briefly, the whole blood was first centrifuged at 180*g* for 10 min, then the supernatant fraction was removed and centrifuged again at 1000*g* for 10 min. The final pellet containing the concentrated platelets fraction was resuspended in 510 µL of S1 and activated with calcium gluconate 10%. The final concentration of platelets obtained in PRP preparation used in this study was 4.905 × 10^3 platelets/µL.

Biochemical analysis

The biochemical analyses were developed 1, 3, 5, and 7 days after lesion. The rats were euthanized by anesthetic overdose and then decapitated. The *gastrocnemius* muscles (from right and left legs) were dissected and kept in ice until tissue preparation.

Tissue preparation

The *gastrocnemius* muscles were dissected, immediately homogenized in saline solution (NaCl 0.9%) and kept in ice. After homogenization, the skeletal muscle samples were centrifuged (at 2000*g* and 4°C for 10 min) in order to obtain a slow-speed supernatant fraction (S1). The acquired S1 was used to measure

TBARS, DCFRS non-protein thiol(-SH) groups, and also to determine the CAT and SOD enzymes activities. No fracture was observed in the dissected rat's legs.

The heparinized whole blood samples were precipitated with 40% TCA (1:1) and centrifuged (2000g, at 4°C for 10 min) in order to obtain the supernatant fraction (S2) that was used for the determination of TBA-RS and non-protein thiol (-SH) groups.

Measurement of oxidative stress markers

TBARS levels

Thiobarbituric acid Reactive Substances (TBARS) levels, malondialdehyde (MDA) mainly, were determined as an index of tissue lipid peroxidation according to the method described by Ohkawa et al. [21] Aliquots of 500 µL of supernatant fraction obtained after blood sample precipitation or 200 µL of skeletal muscle S1 were added to color reaction. TBA-RS levels were measured at 532 nm using a standard curve of MDA and corrected by the protein content.

DCFRS levels

Oxidized dichlorofluorescein (DCFRS) levels were determined as an index of the peroxide production by the cellular components. Skeletal muscle S1 samples (50 µL) were added to a medium containing a Tris-HCl buffer (0.01 mM; pH 7.4) and DCFH-DA (7 µM). After DCFHDA 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). DCFRS levels were determined using a standard curve of DCF and the results were corrected by the protein content [23].

MTT reduction levels

Methyl-tetrazolium (MTT) reduction levels were determined as an index of the dehydrogenase enzymes functions, which are involved in the cellular viability [4]. 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 dimethyl sulfoxide (DMSO). The formed formazan levels were determined spectrophotometrically at 570 nm and the results were corrected by the protein content [18].

MPO enzyme activity levels

The myeloperoxidase (MPO) enzyme activity was determined in skeletal muscle S1 according to the method proposed by Grisham et al. [9], 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 color reaction was measured at 655 nm at 37°C.

Measurement of antioxidant markers

GSH and GSSG levels

The measurement of reduced (GSH) and oxidized (GSSG) glutathione levels was performed according to Hissin and Hilf (1976) [10] with few modifications. A sample of skeletal muscle S1 (400 µL) was added in a mean containing 200 µL of

trichloroacetic acid (TCA 13%) was centrifuged (4 ° C at 13,000 rpm for 10 minutes). For GSH measurement, 100 µL of the supernatant was diluted in 1,800 µL of the phosphate buffer with EDTA (sodium phosphate (100 mM) and EDTA 5 mM), pH 8) and 100 µL of O-phthalaldehyde (OPT 1 mg / mL). To measure GSSG, 250 µL of the supernatant was incubated at room temperature with 100 µL of N-ethylmaleimide (NEM 0.04 M) for 30 minutes at room temperature, after 140 µL of the mixture was added to 1760 µL of the sodium hydroxide buffer (NaOH, 0.1 N), then 100 µL of OPT was added.

The fluorescence measurement was analyzed at 420 nm emission wavelength and 350 nm excitation and the results were expressed as GSH/GSSG ratio.

Non-protein thiol (-SH) groups levels

Non-protein (-SH) groups levels were determined in S1 skeletal muscle and erythrocytes samples according to the method described by Ellman [7], with few modifications. Firstly, the skeletal muscle S1 samples (1 mL) were precipitated with TCA (5%, 0.5 mL) and centrifuged (at 2000g and 4°C, for 10 min), in order to obtain the supernatant fraction S2.

Thereafter, samples of S2 fraction (500 µL) were added to a reaction medium containing potassium phosphate buffer (TFK 0.25 mM, pH = 7.4) and DTNB (1 mM). SH non-protein levels were measured by spectrophotometry at 412 nm. The observed values were calculated according to a standard curve built with known GSH concentrations and corrected by the protein content.

CAT and SOD enzyme activities levels

Catalase (CAT) enzyme activity was measured in the skeletal muscle S1 according to the method described by Aebi [1]. A sample of skeletal muscle S1 (50 mL) was added in a mean containing potassium phosphate buffer (TFK 50 mM, pH = 7.4) and H2O2 (1 mM). The CAT kinetic analysis was initiated after the H2O2 addition; the color reaction was measured at 240 nm.

Cytosolic superoxide dismutase (Cu/Zn SOD) enzyme activity was measured in the skeletal muscle S1 according to the method described by Misra and Fridovich [17]. Different samples of skeletal muscle S1 (10 to 50 µL) were added in a mean containing glycine buffer (50 mM, pH = 10.5) and adrenaline (1 mM). The SOD kinetic analysis was initiated after adrenaline addition; the color reaction was measured at 480 nm.

Protein measurement

Protein content was measured according to the method described by Lowry et al. [13] using bovine serum albumin as the standard measure.

Statistical analysis

All statistical analyses were done using the software GraphPad Prism 6.0 for Windows. Data are expressed as mean and standard error, and differences between groups were assessed by two-way ANOVA followed by Bonfferoni Post-Hoc test and differences were considered significant when $p \leq 0.05$.

RESULTS

Oxidative damage markers analysis

Figures 1 and 2 show that the stretch lesion caused a significant increase in TBARS ($p \leq 0.05$, Figure 1), and also DCFRS levels ($p \leq 0.05$ Figure 2) compared to control conditions uninjured and untreated until the 3rd day of analysis. In both analyses, the combined treatments (PRP and CWI) determined a reduction of TBARS levels in comparison to lesion until de 5th day ($p \leq 0.05$, Figure 1), and the DCFRS levels until de 7th day ($p \leq 0.05$, Figure 2); while the isolated PRP or CWI treatments reduced TBARS and DCFRS levels only until de 3rd day of analysis ($p \leq 0.05$, Figure 1 and 2, respectively).

The MTT reduction levels reached their lowest level on the 1st day after the injury and were significantly lower than control values until the 3rd day after the injury onset. Both the isolated or combined PRP and/or CWI treatments maintained the MTT reduction values similar to control in all the time points analyzed, reaching values significantly higher than the lesion group until the 3rd day after the lesion onset ($p \leq 0.05$, Figure 3).

Further, then the increased lipid peroxidation in injured skeletal muscle tissue, we observed a similar increase of TBARS levels in erythrocytes on the 1st day after the stretch lesion onset, which was counteracted by PRP alone or CWI+PRP ($p \leq 0.05$, Figure 4). In the lesion group, lipid peroxidation lasted until the 3rd day. The combined CWI+PRP treatment significantly reduced the TBARS levels in comparison to lesion condition during all the analyzed period ($p \leq 0.05$, Figure 4).

MPO analysis

The figure 5 shows the significant increase in skeletal muscle MPO enzyme activity determined by the stretch lesion until the 3rd day ($p \leq 0.05$, Figure 5). Both isolated and/or combined CWI and PRP treatments significantly reduced the MPO enzyme activity in comparison to lesion condition in this period ($p \leq 0.05$, Figure 5). Among the treatments, only the isolated CWI did not reach control uninjured and untreated values in the 1st day after the lesion onset ($p \leq 0.05$, Figure 5).

Antioxidant defense markers analysis

The results show a significant reduction determined by the stretch lesion in the skeletal muscle GSH / GSSG ratio until the 3rd day after the injury onset ($p \leq 0.05$, Figure 6). Isolated CWI or combined CWI and PRP, in injured condition, determined a significant increase in GSH / GSSG ratio in comparison to lesion untreated group until the 7th day ($p \leq 0.05$, Figure 6). Furthermore, the combined CWI + PRP increased the GSH / GSSG ratio in comparison to control uninjured and untreated condition from the 3rd to the 5th day ($p \leq 0.05$, Figure 6).

Similar results were observed in the skeletal muscle non-protein (-SH) group levels, where the stretch lesion determined a significant reduction until the 5th day after the injury onset ($p \leq 0.05$, Figure 7). Both isolated and/or combined CWI and PRP treatments increased the non-protein (-SH) group levels in comparison to lesion untreated condition until the 5th ($p \leq 0.05$, Figure 7). Moreover, the combined CWI + PRP increased the non-protein (-SH) group levels in comparison control uninjured and untreated condition in the 5th day after the lesion onset ($p \leq 0.05$, Figure 7). The non-protein (-SH) group levels in erythrocytes were not significantly different among all tested conditions (data not show).

Figure 8 shows the SOD enzyme activity in the skeletal muscle after the stretch injury. The isolated CWI treatment significantly decreased the SOD activity in comparison to lesion group in the 1st day after the injury onset ($p \leq 0.05$, Figure 8). Furthermore, the isolated CWI or the combined CWI + PRP treatments, in uninjured conditions, significantly reduced the SOD activity in comparison to control uninjured and untreated ($p \leq 0.05$, Figure 8). On the other hand, the isolated PRP treatment increased the SOD activity in uninjured (5th day) or injured (from 3rd to 5th day) conditions when compared to control uninjured and untreated ($p \leq 0.05$, Figure 8).

The stretch injury did not alter the enzymatic activity of CAT. However, combined CWI + PRP treatment determined a significant increase in the skeletal muscle CAT enzymatic activity in relation to lesion group during all the analyzed period; and in comparison to control uninjured and untreated condition until the 3rd day ($p \leq 0.05$, Figure 9). Moreover, the isolated PRP treatment increased CAT activity in relation to lesion group until the 3rd day, and in comparison to control uninjured and untreated condition only in the 3rd day after the lesion onset ($p \leq 0.05$, Figure 9).

DISCUSSION

Taken together, the results presented in this study show that the skeletal muscle stretch injury determined a remarkable oxidative damage in both muscular and blood tissues. This is in accordance with our previous findings, but presenting a lower magnitude than that was observed after the experimental muscle contusion [16].

Muscle stretch lesions are characterized by the rupture of sarcolemma, a membrane responsible for muscle fibers structure maintenance. The damaged muscle fibers undergo an inflammatory process and necrosis, been the neutrophils

362 the first inflammatory cells to reach the site of injury, followed by macrophages [22,
363 30]. The release by neutrophils, such as NADPH-oxidase and myeloperoxidase,
364 contribute to ROS generation, which is important to promote the destruction of
365 necrotic tissue. However, the excessive ROS (over the cellular antioxidant systems
366 capacity to scavenger them) could lead to an exacerbation of the inflammatory
367 process, determining damage to healthy myofibrils [30].

368 In this study, the separate CWI or PRP treatments determined a reduction in
369 the oxidative damage resulted from a skeletal muscle stretch injury, but the
370 combination CWI + PRP resulted in similar results but earlier than the isolated
371 treatments. Previous studies show that an excessive generation of ROS determined
372 by skeletal muscle injuries could extrapolate the site of the lesion and hence results
373 in oxidative damage of blood tissue components [16, 25]. Our results are in
374 accordance with this since we observed a significant increase in erythrocytes TBARS
375 levels after the onset of the stretch injury. The positive effects of isolated and/or
376 combined PRP and CWI treatment on erythrocyte lipid peroxidation were probably
377 due to the reduced oxidative damage at the site of lesion.

378 The analysis of MTT reduction is commonly used as an index of cellular
379 dehydrogenase activities, which are mainly located in the mitochondria, and
380 consequently reflects the cellular viability [3]. In fact, we observed here that the
381 stretch injury decreased the MTT reduction of the skeletal muscle preparation, which
382 is in agreement with previous observations [16]. The effects of isolated and/or
383 combined PRP and CWI treatment are probably related with a reduced skeletal
384 muscle mitochondrial dysfunction. One possible explanation for the better effects of
385 the combined CWI + PRP could be related to the independent and different

386 mechanism of action of PRP and CWI, especially in relation to their effect on the
387 cellular antioxidant systems activity.

388 In the present study, it was observed that the stretch lesion decreased both
389 GSH/GSSG ratio and thiols group levels in skeletal muscle tissue, but does not
390 change the antioxidant enzyme activities. In fact, CWI treatment maintained the
391 levels of non-enzymatic antioxidant (GSH/GSSG ratio and -SH levels) but decreased
392 the antioxidant enzymes activity Watanabe et al. (2016) described that the increase
393 of glutathione adducts stabilize hypoxia-inducible factors-1α (HIF-1α) during a
394 process of ischemia, determining a decrease of the ROS levels and also of the
395 enzymatic antioxidant activity [34]. Since cryotherapy is able to decrease local blood
396 flow and also ROS formation [15, 25], this pathway involving the
397 activation/stabilization of HIF-1 should be further studied after CWI application to
398 highlight this possible mechanism of action.

399 The treatment of muscle injuries with PRP has been shown to promote an
400 increase in growth factors release and also the improvement of contractile function in
401 the skeletal muscle tissue [6, 33]. Studies show that epidermal growth factor (EGF)
402 and platelet-derived growth factor (PDGF) activate tyrosine kinase, which leads to
403 phosphorylation of specific tyrosine residues and results in the activation of several
404 key signal transduction pathways, promoting cell proliferation and survival [28]. In this
405 study, the treatment with PRP determined an increase in the antioxidant activity of
406 SOD and CAT, which was also observed after a skeletal muscle contusion [16].
407 Tohidnezhad et al., (2014) shown that PRGF can activate the nuclear factor
408 erythroid-related factor 2 (NRF2) and demonstrated that this transcription factor plays
409 a complex role in of the function of the antioxidant system [31]. Normally NRF2
410 interacts with Kelch-like ECH-associated protein 1 (KEAP1), but in high ROS levels

411 conditions, these radicals oxidize cysteine residues sensitive on KEAP1 resulting in
412 dissociation of KEAP1 from NRF2. The NRF2 translocate to the nucleus and binds to
413 antioxidant-responsive elements (AREs) within the regulatory region of multiple
414 antioxidant genes. Thus, NRF2 directly affects the homeostasis of ROS by regulating
415 of the antioxidant defense systems through several mechanisms, such as induction
416 of catabolism of superoxides and peroxides [14].

417 The healing of skeletal muscle tissue involves a series of phases, which
418 include the acute inflammatory phase, an intermediate phase of repair, and the
419 advanced remodeling phase [5]. In the acute phase of skeletal muscle healing, an
420 extensive infiltration of inflammatory cells, predominantly neutrophils, is observed in
421 injured tissues. The MPO enzyme is found primarily in the azurophilic granules, but, it
422 has been established that a large percentage of them are from neutrophils. Similarly
423 to other studies [16, 25, 26], the MPO enzyme activity measurement in injured
424 tissues is a well-known indicator of the intensity of acute inflammatory response. In
425 the present study, we observed a significant increase in MPO activity in the lesion
426 group in comparison to the control group after 72 hours the stretch lesion. The
427 combined CWI + PRP treatment determined a decrease in MPO activity,
428 maintaining its level similar to the control. Furthermore, Tsai et al., (2018) observed
429 that the treatment with PRP decreased the presence of neutrophils after a stretch
430 injury, which may explain the decrease in the MPO enzymatic activity. However,
431 more studies are needed to explain the mechanisms by which neutrophil infiltration
432 decreases after treatment with PRP.

433 As observed in studies previous [25, 26, 27], we believe that the benefits of
434 cryotherapy are likely to be linked to its potential to modulate the intensity of the
435 inflammatory response that follows a skeletal muscle injury. Our results show that the

CWI treatment limited the significant increase in MPO activity, which is in accordance with another study where cryotherapy reduced the inflammatory processes thought a decrease in macrophage infiltration and a consequent reduction of TNF-α, NF-κ B, TGF-β and MMP-9 mRNA levels [27]. It is believed that the effects of cryotherapy are related to the physiological decrease in blood flow in the treated tissue, which may determine a reduction in the intensity of the inflammatory response to an acute injury. Moreover, the reduced blood flow could decreased oxygen metabolism in muscle treated with cryotherapy, thus minimizing the secondary damage that follows an acute injury [25, 26].

CONCLUSION

Our study can be considered a pioneer since it brings the combination of treatments widely used in sports activity, but normally used separately. Our intention was to perform an evaluation of the changes in oxidative stress marker levels determined by CWI and PRP treatment after a skeletal muscle stretch lesion. According to our results, we could infer that the combination of treatments shows a great potential for treatments of muscular injuries. However, more studies are needed to elucidate the effects of these treatments and to clarify possible mechanisms by which they act on the musculoskeletal tissue.

DECLARATION OF INTEREST

All authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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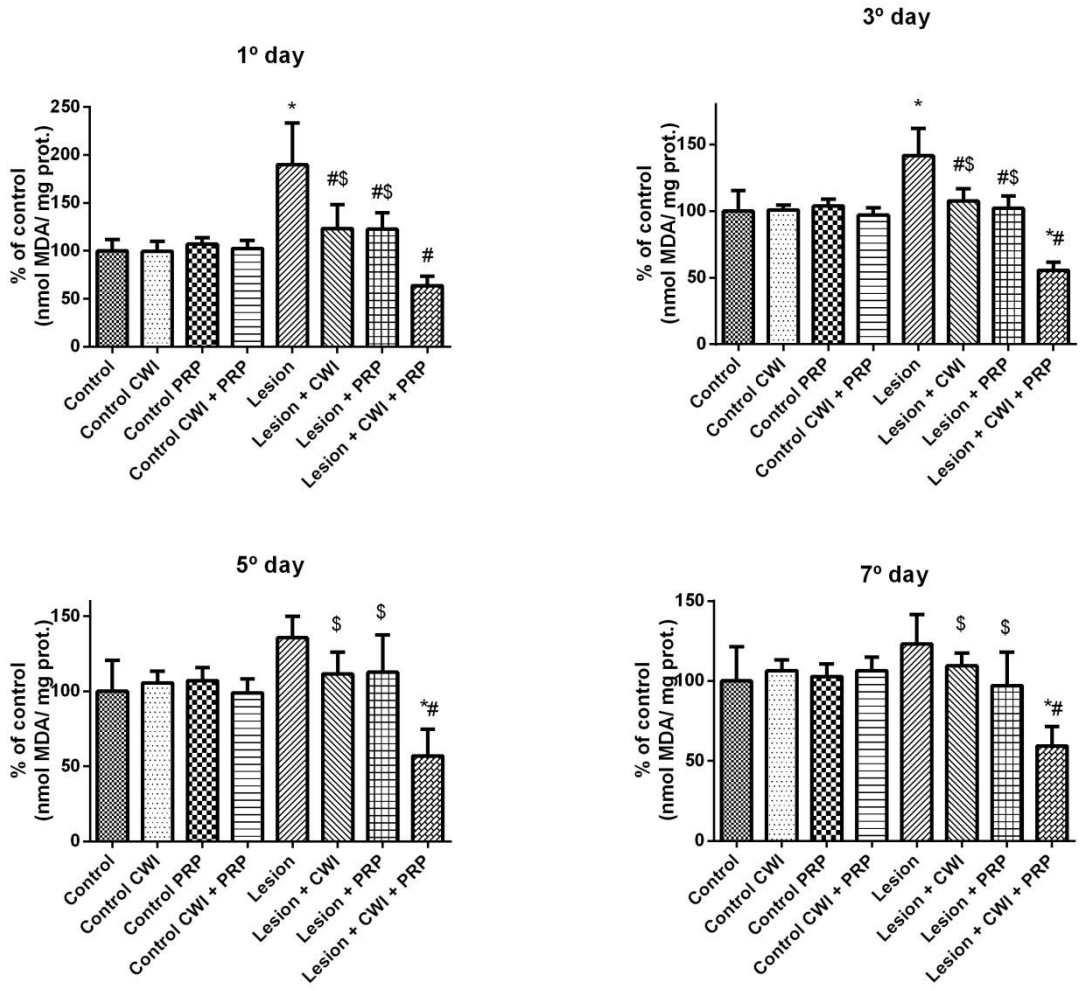
567

568 **FIGURES**

569

570 **Figure 1**



571

572 **Figure 1**: Isolated or combine PRP and/or CWI effects on the stretch injury-induced oxidative damage
573 throughout time (1, 3, 5 and 7 days after injury) assessed by means of the MDA levels and expressed in
574 percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-away),
575 followed by Bonferroni test. Differences were considered significant when $P < 0.05$. * = differences found
576 between the control of their day without injury. # = significant difference found in comparison to the lesion
577 group. $ = significant difference found in comparison to lesion + CWI + PRP group. N= 5/group.

578

Figure 2

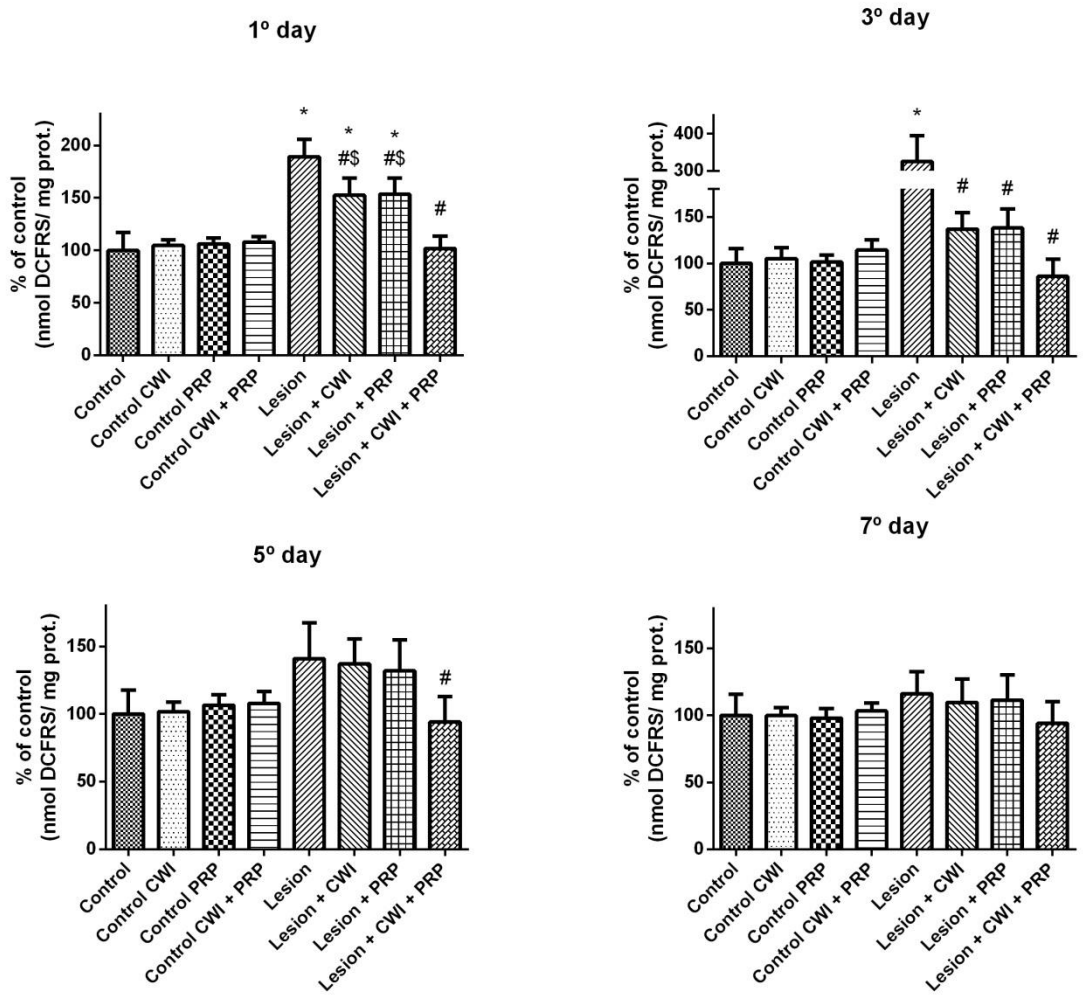


Figure 2: Isolated or combine PRP and/or CWI effects on the stretch injury-induced oxidative damage throughout time (1, 3, 5 and 7 days after injury) assessed by means of the DCFRS levels and expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test. Differences were considered significant when $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the lesion group. $ = significant difference found in comparison to lesion + CWI + PRP group. N= 5/group.

Figure 3

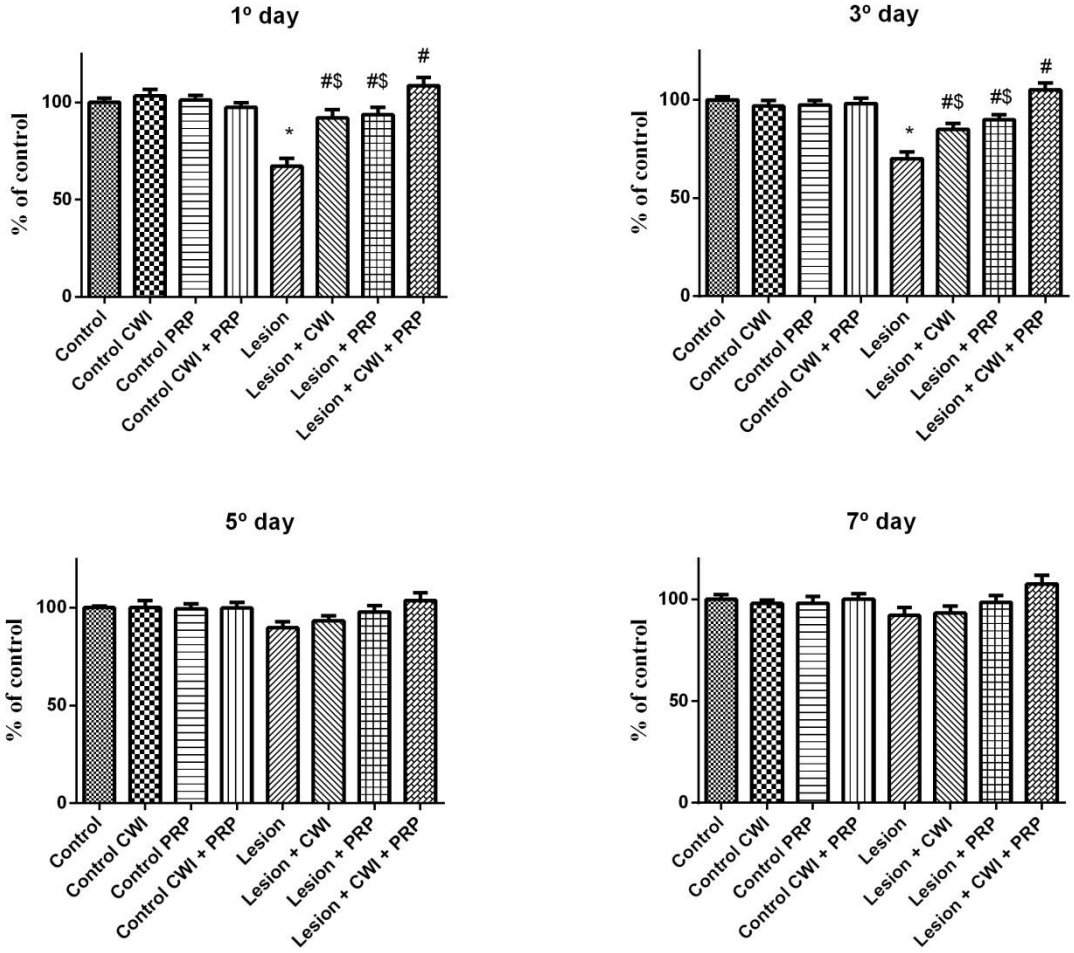


Figure 3: Isolated or combine PRP and/or CWI effects on the stretch injury-induced oxidative damage throughout time (1, 3, 5 and 7 days after injury) assessed by means of the MTT reduction levels expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test. Differences were considered significant when $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the lesion group. $ = significant difference found in comparison to lesion + CWI + PRP group. N= 5/group.

Figure 4

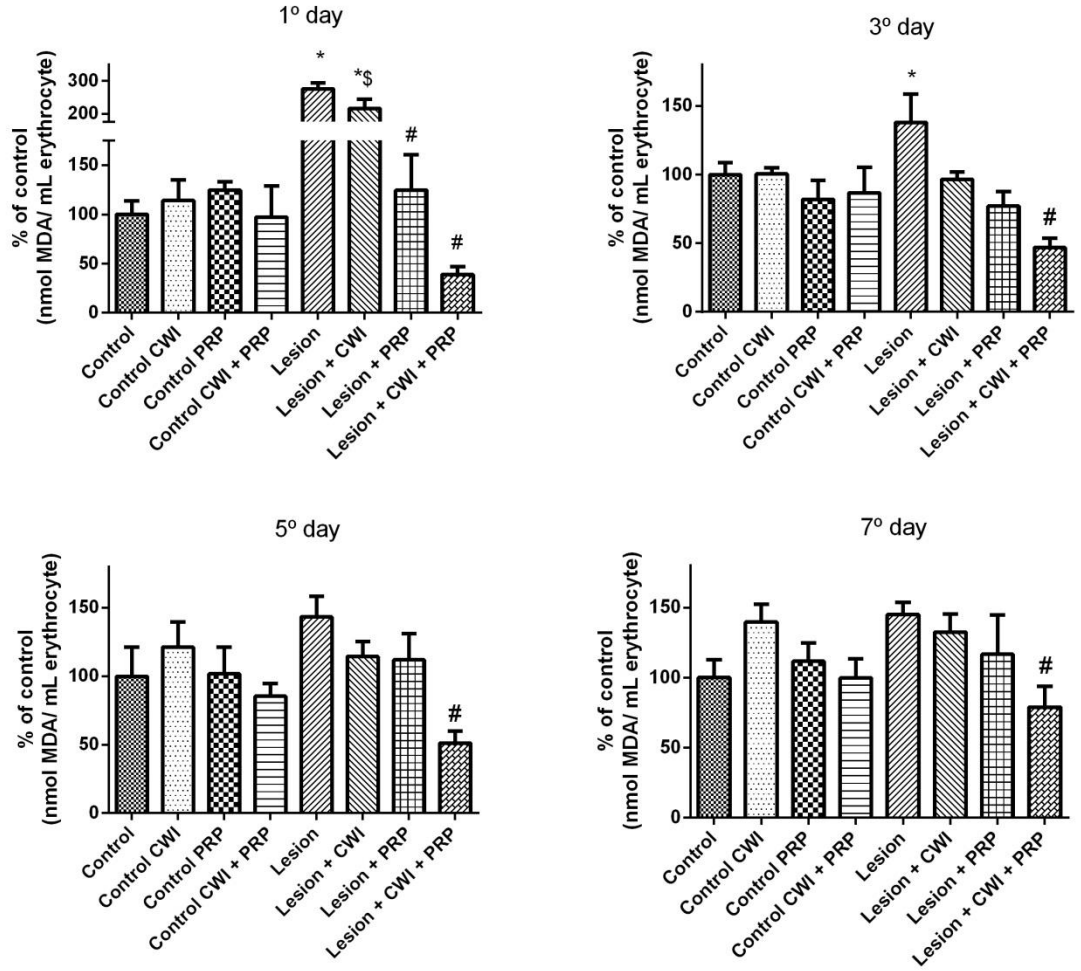
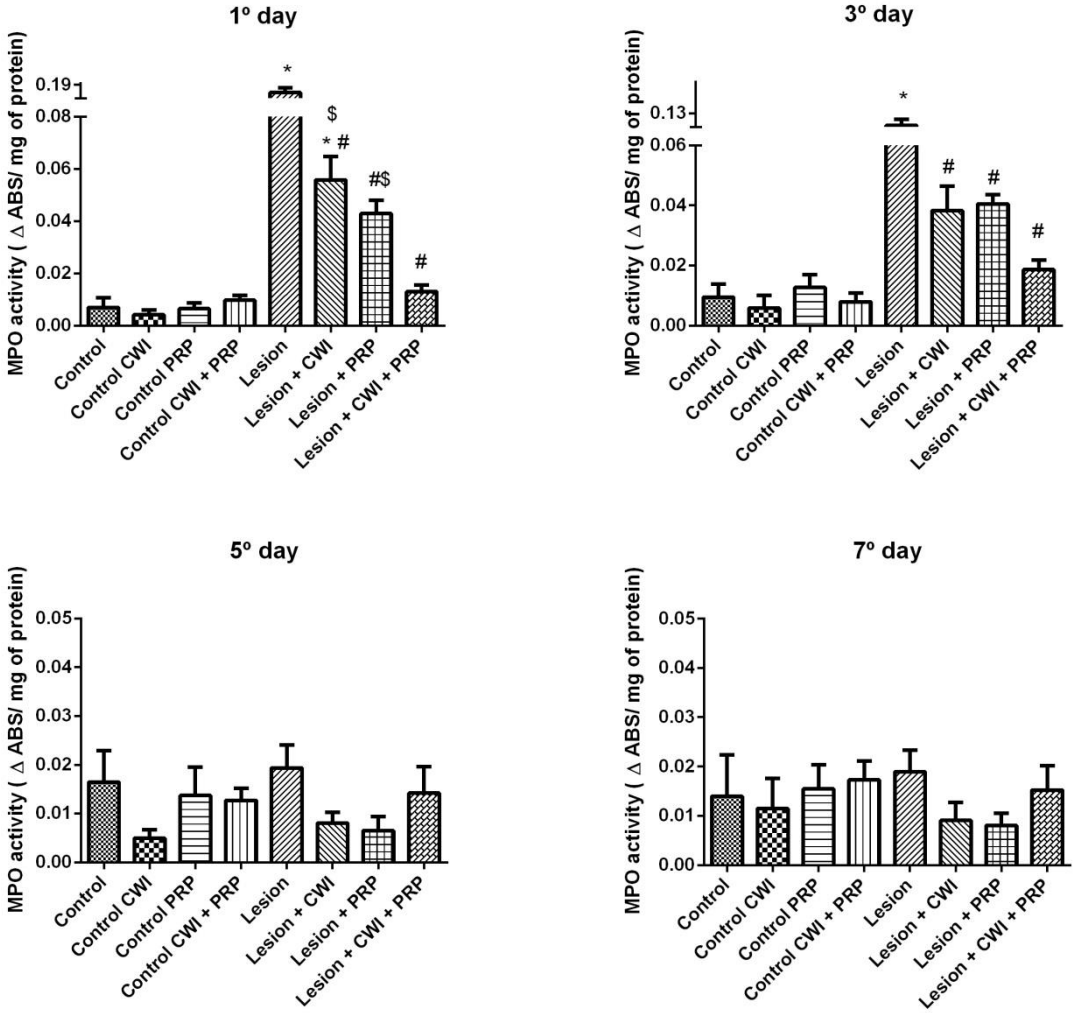


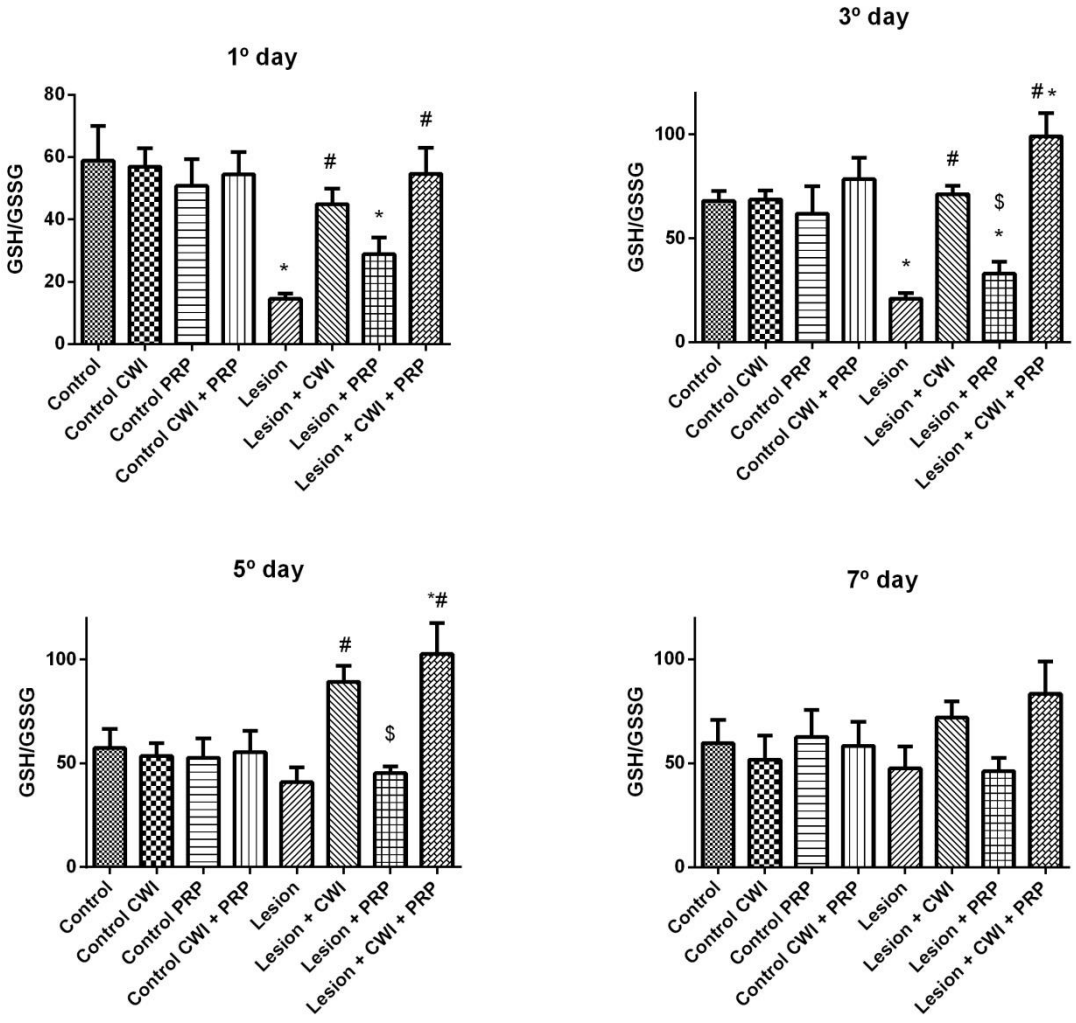
Figure 4: Isolated or combine PRP and/or CWI effects on the stretch injury-induced oxidative damage throughout time (1, 3, 5 and 7 days after injury) assessed by means of the MDA levels and expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test. Differences were considered significant when $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the lesion group. $ = significant difference found in comparison to lesion + CWI + PRP group. N= 5/group.

606 **Figure 5**



607
608 **Figure 5**: Isolated or combine PRP and/or CWI effects on the stretch injury-induced MPO enzyme activity
609 throughout time (1, 3, 5 and 7 days after injury) assessed by means of the delta absorbance/mg of protein. Values
610 are presented as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test.
611 Differences were considered significant when $P < 0.05$. * = differences found between the control of their day
612 without injury. # = significant difference found in comparison to the lesion group. $ = significant difference
613 found in comparison to lesion + CWI + PRP group. N= 5/group.
614

615 **Figure 6**



616

617 **Figure 6**: Isolated or combine PRP and/or CWI effects on the stretch injury-induced oxidative damage
618 throughout time (1, 3, 5 and 7 days after injury) assessed by means of the GSH/GSSG ratio. Values are presented
619 as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test. Differences were
620 considered significant when $P < 0.05$. * = differences found between the control of their day without injury. # =
621 significant difference found in comparison to the lesion group. $ = significant difference found in comparison to
622 lesion + CWI + PRP group. N= 5/group

623

Figure 7

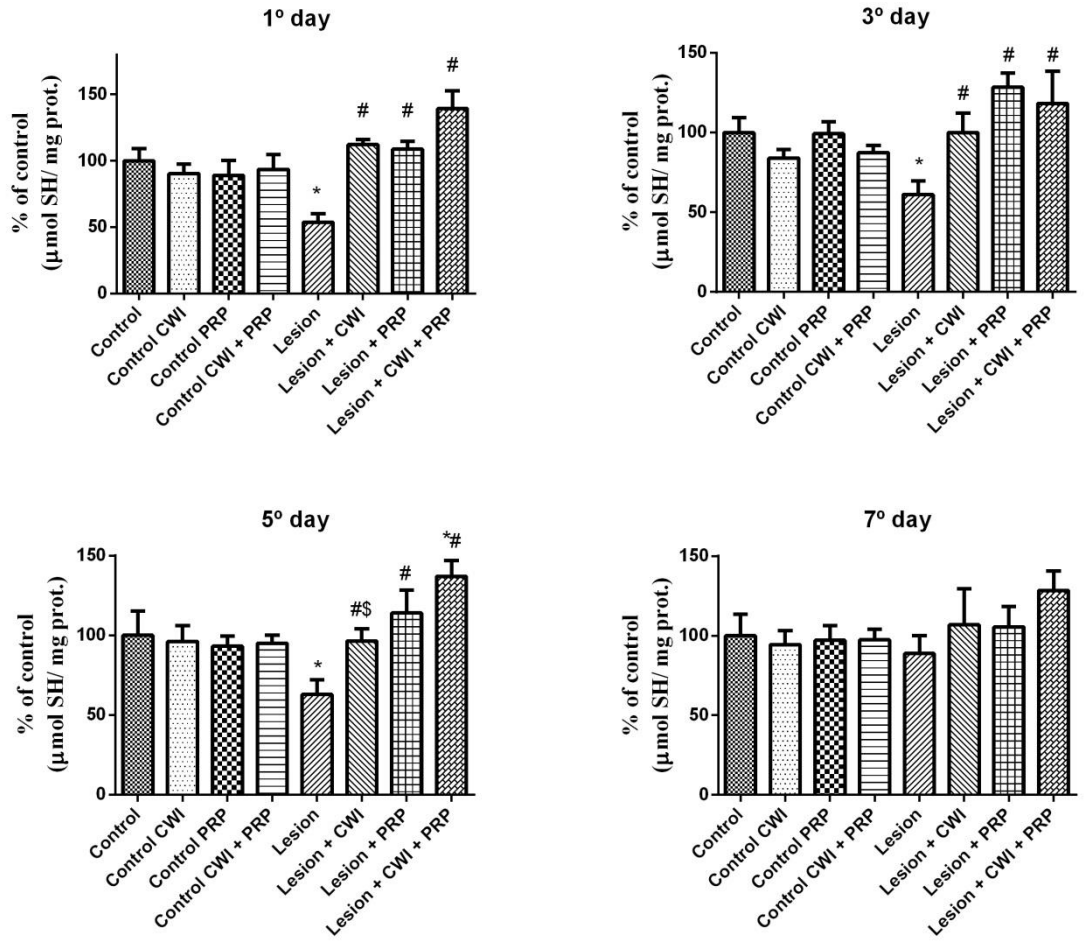


Figure 7: Isolated or combine PRP and/or CWI effects on the stretch injury-induced oxidative damage throughout time (1, 3, 5 and 7 days after injury) assessed by means of the SH levels and expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test. Differences were considered significant when $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the lesion group. $ = significant difference found in comparison to lesion + CWI + PRP group. N= 5/group

Figure 8

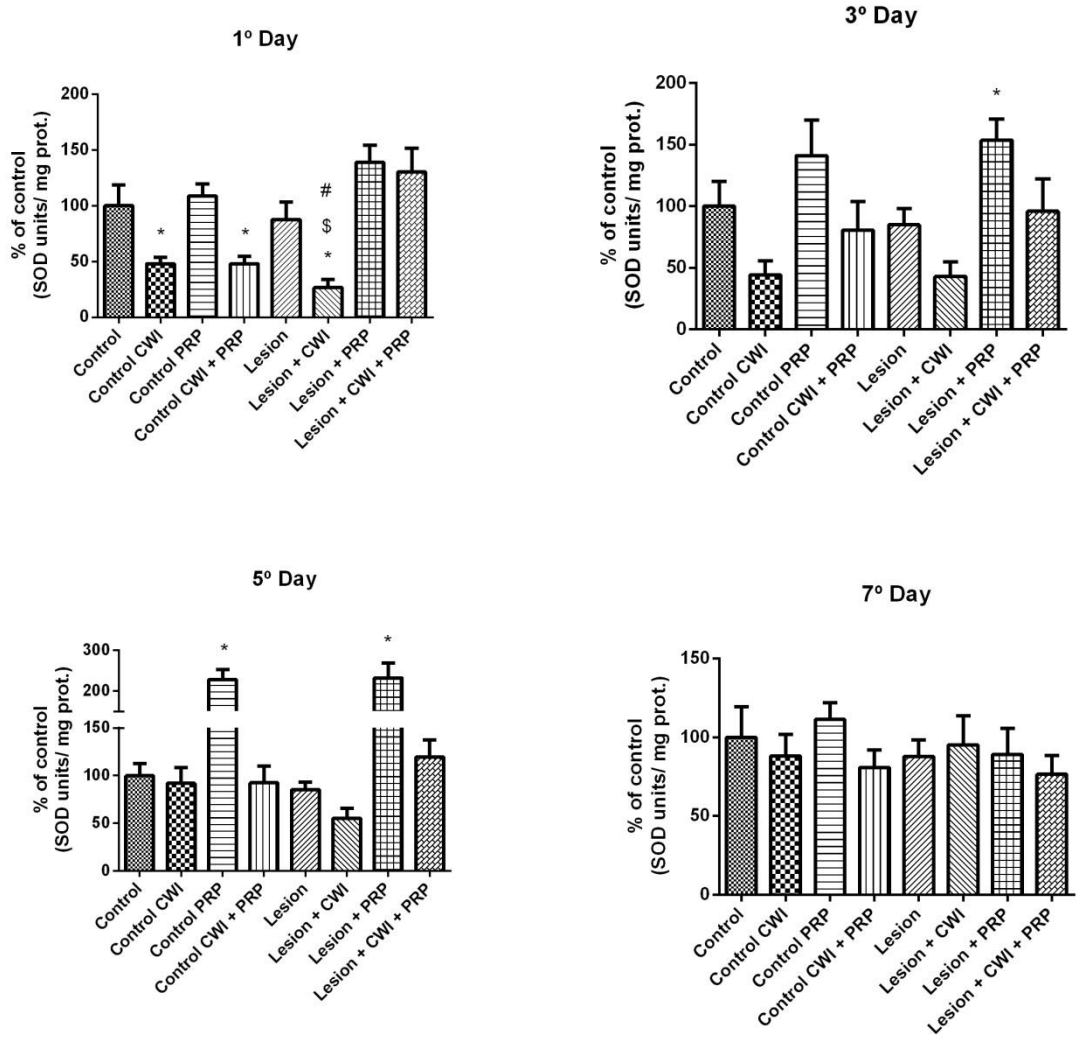


Figure 8: Isolated or combine PRP and/or CWI effects on the stretch injury-induced SOD enzyme activity throughout time (1, 3, 5 and 7 days after injury) assessed by means of the SOD units levels and expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test. Differences were considered significant when $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the lesion group. $ = significant difference found in comparison to lesion + CWI + PRP group. N= 5/group.

Figure 9

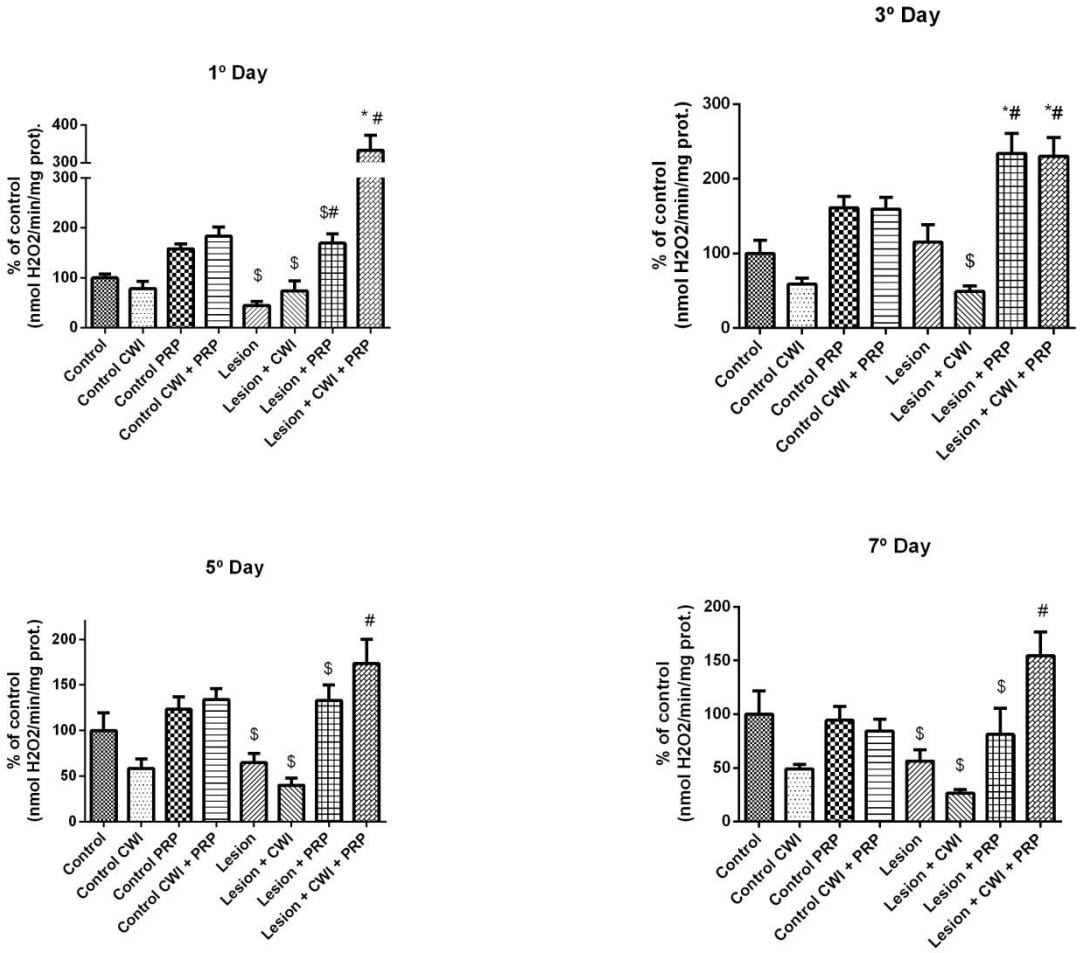


Figure 9: Isolated or combine PRP and/or CWI effects on the stretch injury-induced CAT enzyme activity throughout time (1, 3, 5 and 7 days after injury) assessed by means of the H2O2/min/mg prot. and expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-away), followed by Bonferroni test. Differences were considered significant when $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the lesion group. $ = significant difference found in comparison to lesion + CWI + PRP group. N= 5/group

4- CONCLUSÃO

A utilização dos recursos terapêuticos como o CWI e o PRP demonstrou ser eficientes no tratamento do estiramento muscular. A lesão muscular esquelética provocada pelo estiramento aumentou os marcadores de dano oxidativo. Além disso, a intensidade da resposta inflamatória parece ser também um fator envolvido na gênese do dano oxidativo nos momentos que sucedem a lesão muscular esquelética por estiramento. A aplicação terapêutica combinada entre CWI e PRP reduz o dano oxidativo, preserva a capacidade antioxidante e apresenta um efeito modulatório sobre a resposta inflamatória auxiliando na recuperação do tecido musculoesquelético.

5- PERPESCTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores são:

- ✓ Avaliar o efeito da combinação dos tratamentos com CWI e PRP sobre parâmetros comportamentais e funcionais nos ratos submetidos à lesão muscular;

- ✓ Investigar a participação de mediadores químicos pro e anti-inflamatórios, tais como interleucinas e fatores de transcrição do sistema antioxidante, durante o período da lesão muscular, assim como o efeito dos tratamentos sobre estes fatores.

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ANEXOS

ANEXO A: Registro no Gabinete de Projetos

Visualizar projeto

Dados Básicos

Título
EFEITOS DO PLASMA RICO EM PLAQUETAS SOBRE AS ALTERAÇÕES BIOQUIMÍCAS E MORFOLÓGICAS INDUZIDAS POR LESÕES MUSCULARES EM RATOS

Número do projeto
037265

Número do processo
037265

Classificação principal
Pesquisa

Data inicial
01/05/2014

Data final
31/07/2018

Resumo
Aproximadamente 45% da massa do corpo humano adulto é formado por tecido muscular. As lesões musculares estão entre as mais comuns em diferentes tipos de esportes, sendo a sua frequência variando entre 10-55% de todas as lesões. Estudos ao longo do tempo investigam variáveis que influenciam a velocidade de cicatrização frente ao processo de reparação tecidual, sendo que o uso de plasma rico em plaquetas (PRP) é um recente e promissor método adjuvante nesse contexto. Com isso, este estudo tem como objetivo avaliar os efeitos do PRP sobre as alterações bioquímicas e morfológicas induzidas por um modelo de lesão muscular em ratos. Para a realização deste estudo serão utilizados ratos wistar, divididos em 2 grupos: grupo GC (controle), grupo GPRP (PRP). As análises experimentais bioquímicas e morfológicas serão realizadas em diferentes momentos após o desenvolvimento da lesão. A partir de amostras de tecido muscular e sanguíneo, a fim de investigar a evolução cronológica das lesões bem como verificar a eficácia do tratamento com PRP.

Observação
[Não informado]

Projeto em âmbito confidencial
Não

Projeto superior
-

Palavra-chave 1
plasma rico em plaquetas

Palavra-chave 2
lesões musculares

Palavra-chave 3
[Não informado]

Palavra-chave 4
[Não informado]

Tipo de evento
Não se aplica

Carga horária do curso
[Não informado]

Situação
Em andamento

Avaliação
Não avaliado

Última avaliação
07/04/2017

Gestão do conhecimento e gestão financeira

O projeto pode gerar conhecimento passível de proteção?
Não

Propriedade Intelectual
[Não informado]

Proteção Especial
[Não informado]

Direito Autoral - Copyright
Não

O projeto contrata uma fundação? Indique a fundação
Não necessita contratar fundação

Classificações

Tipo	Classificação
Classificação CNPq	2.00.00.00-6 CIÊNCIAS BIOLÓGICAS
Linha de pesquisa	02.00.00 SAUDE
Quanto ao tipo de projeto de pesquisa	2.01 Projeto de Pesquisa Pura

Nenhum objetivo estratégico indicado

Participantes

Matrícula	▲Nome	Função	Carga Horária	Período

ANEXO B: APROVAÇÃO DO COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL – UFSM

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Efeitos dos recursos terapêuticos sobre as alterações bioquímicas e moleculares nas lesões musculoesqueléticas em ratos", protocolada sob o CEUA nº 4694151216, sob a responsabilidade de **Gustavo Orione Puntel** *e equipe; Rodrigo Pereira Martins* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 23/03/2017.

We certify that the proposal "Effects of therapeutic resources on biochemical and molecular changes in musculoskeletal injuries in rats", utilizing 160 Heterogenics rats (160 males), protocol number CEUA 4694151216, under the responsibility of **Gustavo Orione Puntel** *and team; Rodrigo Pereira Martins* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/23/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 04/2017 a 12/2018 Área: Morfologia

Origem:	Biotério Central UFSM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	2 a 3 meses	N:	160
Linhagem:	Wistar			Peso:	200 a 300 g		

Resumo: A prática esportiva pode induzir lesões nas fibras musculares, gerando um dano oxidativo e processo inflamatório. Praticas terapêutica vem sendo utilizadas com o intuito de reduzir estes danos, como o plasma rico em plaquetas (PRP) e a crioterapia. Assim, o presente estudo tem como objetivo investigar através de experimentos bioquímicos a influência do tratamento com o frio terapêutico e/ou PRP em modelos de lesões musculares. Métodos: Serão aplicados protocolos de lesão muscular por contusão, distensão ou estiramento muscular. Imediatamente, os animais receberão uma dose, intramuscular de PRP e/ou serão submetidos a sessões de terapia com gelo. As analises bioquímicas serão realizadas 1, 3, 5, 7 e 14 dias após o primeiro dia de tratamento.

Local do experimento: Laboratório de bioquímica toxicológica experimental, prédio 19 sala 3122

Santa Maria, 10 de agosto de 2018

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Avenida Roraima, 1000, Reitoria, 2º andar - CEP 97105-900 Santa Maria, RS - tel: 55 (55) 3220-9362 / fax:
Horário de atendimento: das 8:30 ás 12h e 14h ás 17hs : e-mail: ceua.ufsm@gmail.com
CEUA N 4694151216

ANEXO C: NORMAS DA REVISTA *THE AMERICAN JOURNAL OF SPORTS MEDICINE*

1. Manuscript Submission Guidelines

Manuscripts must not be under simultaneous consideration by any other publication, before or during the peer-review process. Papers presented at AOSSM meetings must be submitted to the Journal for first rights of refusal. Articles published in A*JSM* may not be published elsewhere without written permission from the publisher.

Manuscripts should cite any other work by one or more of the co-authors that is relevant to the subject matter of the current submission or that used any of the same subjects, animals, or specimens being reported in the current submission. This includes manuscripts that are currently under preparation, are being considered by journals, are accepted for publication, or already published. In any of these cases, the relationship to the current submission should be made clear.

All review articles (such as systematic review, meta-analysis) submitted will be considered for the Current Concepts section. Authors with ideas for current concepts should contact the associate editor, Timothy Foster, MD, to find out if *AJSM* has recently published a review article on that topic or if there is a similar submission in progress. Contact Dr. Foster at info@ajsm.org to inquire about your idea or submit already completed papers directly to the journal at https://submit.ajsm.org.

2. Submissions

Authors should register on our online submission site at https://submit.ajsm.org to submit manuscripts.

When manuscripts have been received by the editorial office, the corresponding author will be sent an acknowledgment giving an assigned manuscript number, which should be used with all subsequent correspondence for anything related to that particular manuscript.

The following items are required on submission:

1. Blinded manuscript including the abstract and any tables and figures where they occur in the text. No identifying information should appear in the uploaded manuscript. Please remove author names, initials, and institutions. State or country names may be used, but do not include specific locations such as cities or regions.
2. Journal Contributor Publishing Agreement and *AJSM* Author Disclosure Statement. These forms are available for download from the author area of the submission site. The corresponding author must complete the forms and return them to *AJSM* by email or upload them online as a PDF or Word file using the ‘‘upload legal documents’’ option. As an alternative to the *AJSM* disclosure form, authors may submit the ICMJE disclosure form *along with* the *AJSM* Supplemental Form available on our website.
3. A copy of the IRB or other agency approval (or waiver) if animal subjects or human subjects or tissues or health information were used. Please see further

instructions under "Text." This information should be uploaded with the disclosure and publishing forms and not as a supplemental file.

4. The original study protocol for all registered clinical trials must be included and can be uploaded as a supplemental file. This information should be blinded for peer review (remove author name and location as well as trial registration number). Use of a CONSORT flow diagram is required to illustrate the grouping and flow of patients for all randomized clinical trials. The CONSORT checklist must also be completed and uploaded as a supplemental file.

Cover letter, acknowledgments, and suggested reviewers are optional. If a paper has more than 5 authors, a cover letter detailing the contributions of all authors should be included in the appropriate box on the submission page. Only those involved in writing the paper should be included in the author line. Others should be listed as a footnote or acknowledgment. While there is no limit on the number of authors, no more than 12 will be listed on the masthead of the published article; additional authors will be listed at the end of the article.

3. Manuscript Formats

Manuscript pages should be double-spaced with consecutive page numbers and continuous line numbers. The abstract should be included with the manuscript as well as being entered in the Metadata section (except for case reports, which do not require abstracts). Manuscripts should be 6000 words or fewer (including abstract and references). There are also limitations on figures, tables, and references; see guidelines below. The system handles most common word processing formats; however, Word and PDF are preferred.

4. Manuscript Preparation

4.1 Abstract

Abstracts should summarize the contents of the article in 350 words or less. The abstract should be structured in the following format:

Background: In one or two sentences, summarize the scientific body of knowledge surrounding your study and how this led to your investigation.

Hypothesis/Purpose: State the theory(ies) that you are attempting to prove or disprove by your study or the purpose if no hypothesis exists.

Study Design: Identify the overall design of your study. See list below.

Methods: Succinctly summarize the overall methods you used in your investigation. Include the study population, type of intervention, method of data collection, and length of the study.

Results: Report the most important results of your study. Only include positive results that are statistically significant, or important negative results that are supported by adequate power. Report actual data, not just *P* values.

Conclusion: State the answer to your original question or hypothesis. Summarize the most important conclusions that can be directly drawn from your study.

Clinical Relevance: If yours was a laboratory study, describe its relevance to clinical sports medicine.

Key Terms: Include at least 4 key terms for indexing. When submitting an article, you will be asked to choose from a list of terms that are used for assigning reviewers. These terms can be used in the manuscript as well. The list can be found at https://submit.ajsm.org/submission/editexpertise.

What is known about the subject: Please state what is currently known about this subject to place your study in perspective for the reviewers.

What this study adds to existing knowledge: Please state what this study adds to the existing knowledge.

The last two items are for reviewers only and are not included in the word count, but should appear at the end of the abstract in the uploaded text.

4.2 Study Designs

Meta-analysis: A systematic overview of studies that pools results of two or more studies to obtain an overall answer to a question or interest. Summarizes quantitatively the evidence regarding a treatment, procedure, or association.

Systematic Review: An article that examines published material on a clearly described subject in a systematic way. There must be a description of how the evidence on this topic was tracked down, from what sources and with what inclusion and exclusion criteria.

Randomized Controlled Clinical Trial: A group of patients is randomized into an experimental group and a control group. These groups are followed up for the variables / outcomes of interest. **NOTE: All clinical trials started after January 1, 2016 must be registered at ClinicalTrials.gov or a similar database to be considered for publication.**

Crossover Study Design: The administration of two or more experimental therapies one after the other in a specified or random order to the same group of patients.

Cohort Study: Involves identification of two groups (cohorts) of patients, one which did receive the exposure of interest, and one which did not, and following these cohorts forward for the outcome of interest.

Case-Control Study: A study that involves identifying patients who have the outcome of interest (cases) and patients without the same outcome (controls), and looking back to see if they had the exposure of interest.

Cross-Sectional Study: The observation of a defined population at a single point in time or time interval. Exposure and outcome are determined simultaneously.

Case Series: Describes characteristics of a group of patients with a particular disease or who have undergone a particular procedure. Design may be prospective or retrospective. No control group is used in the study, although the discussion may compare the results to other published outcomes.

Case Report: Similar to the case series, except that only one or a small group of cases is reported.

Descriptive Epidemiology Study: Observational study describing the injuries occurring in a particular sport.

Controlled Laboratory Study: An in vitro or in vivo investigation in which 1 group receiving an experimental treatment is compared to 1 or more groups receiving no treatment or an alternate treatment.

Descriptive Laboratory Study: An in vivo or in vitro study that describes characteristics such as anatomy, physiology, or kinesiology of a broad range of subjects or a specific group of interest. Authors should choose the design that best fits the study.

The Editor will make the final determination of the study design and level of evidence based on the Center for Evidence Based Medicine guidelines.

4.3 Text

In general, follow the standard IMRAD (Introduction, Materials and Methods, Results, Discussion) format for writing scientific articles. The author is responsible for all statements made in the work, including copyeditor changes, which the author will have an opportunity to verify. Authors with limited fluency in English should have the paper reviewed or edited by a native English speaker to ensure clear presentation of the work.

Papers including human or animal subjects must include a statement of approval by appropriate agencies in the text, and a copy of the approval letter must be uploaded with the submission. If approval was not required, authors must upload a waiver statement from the appropriate agency. For human cadaveric specimens, please provide source (eg, donation to university anatomy program) and state if permission was obtained for use. Additionally, all studies involving animals must conform to ARRIVE guidelines. If available, please include the source of animal joint or tissue specimens. For case reports, include a letter from the patient granting permission for his/her information to be included in the publication.

Reports on surgery, except in rare instances, require a minimum follow-up of 2 years.

Use generic names of drugs or devices. If a particular brand was used in a study, insert the brand name along with the name and location of the manufacturer in parentheses after the generic name when the drug or device is first mentioned in the text.

Use metric units in measurements (centimeter vs inch, kilogram vs pound).

Abbreviations should be used sparingly. When abbreviations are used, give the full term followed by the abbreviation in parentheses the first time it is mentioned in the text, such as femur-ACL-tibia complex (FATC).

Use of a CONSORT flow diagram is required to illustrate the grouping and flow of patients in all randomized controlled trials and is recommended for all other types of clinical studies.

Statistical methods should be described in detail. Actual *P* values should be used unless less than .001. Reporting of 95% confidence intervals is encouraged.

4.4 Acknowledgment

Type the acknowledgments in the box provided on the submission page; do not include it in the manuscript. This information will be added to the accepted manuscript at the time of publication. Give credit to technical assistants and professional colleagues who contributed to the quality of the paper but are not listed as authors. Please briefly describe the contributions made by persons being acknowledged. **Note: anyone who has contributed to the preparation of the submitted text must be included on the author disclosure form, under Statement of Authorship, and his or her disclosures included there.**

4.5 References

References should be double-spaced in alphabetical order and numbered according to alphabetical listing. Except for review articles, references should be limited to 60. If references are not in alphabetical order the uploaded file will be REJECTED and will have to be resubmitted with the references in the correct form. When author entries are the same, alphabetize by the first word of the title. In general, use the Index Medicus form for abbreviating journal titles and the *AMA Manual of Style* (10th ed) for format. *Note:* References must be retrievable. Do not include in the reference list meeting presentations that have not been published. Data such as presentations and articles that have been submitted for publication but have not been accepted must be put in the text as unpublished data immediately after mention of the information (for example, ‘‘Smith and Jones (unpublished data, 2000) noted ... ’’). Personal communications and other references to unpublished data are discouraged. For review purposes, unpublished references that are closely related to the submitted paper or are important for understanding it should be uploaded as supplemental files.

References will be linked to Medline citations for the reviewers. Authors can include articles that are in Epublish mode. To ensure that these Epub references are linked correctly, please provide the PMID number from Medline at the end of the reference. For example: Emery CA, Meeuwisse WH. Injury rates and mechanisms of injury in minor hockey. *Am J Sports Med*. 2006 Jul 21; [Epub ahead of print] PMID: 16861577

4.6 Figures and Tables

Figures and tables should appear in the body of the paper near the place where they are mentioned. High-resolution images should also be uploaded separately as Figure files. The figures and tables should be cited in numeric order in the text and should not exceed 3 journal pages. One journal page equals 1 large table or figure, 2 medium-sized tables or figures, or 4 small tables or figures. Medium-sized tables and figures will be a page width and half the length of the page; small tables and figures are 1-column width and take up half the length of the page or less.

Any material that is submitted with an article that has been reproduced from another source (that is, has been copyrighted previously) must conform to the current copyright regulations. It is the author's responsibility to obtain written permission for reproduction of copyrighted material and provide the editorial office with that documentation before the material will be reproduced in the Journal.

All image files for figures should be labeled with the Figure number (label each part if figures include multiple parts, eg, 2A, 2B). The figure legend should be placed below each figure and should include descriptions of each figure part and identify the meaning of any symbols or arrows. Terms used for labels and in the legend must be consistent with those in the text. A CONSORT flow diagram should be included for all randomized clinical trials to illustrate the grouping and flow of patients.

Color will be used in the Journal where needed (eg, histology slides or surgical photographs). All other figures, such as bar graphs and charts, should be submitted in black and white.

Figures for papers accepted for publication must meet the image resolution requirements of the publisher, SAGE Publications. Files for line-based drawings (no grayscale) should ideally be submitted in the format they were originally created; if submitting scanned versions, files should be 1200 dots per inch (dpi). Color photos should be submitted at 600 dpi and black-and-white photos at 300 dpi.

Charts and graphs can be submitted in the original form created (eg, Word, Excel, or PowerPoint). Photographs or scanned drawings embedded in Word or PowerPoint are not acceptable for publication.

All photographs of patients that disclose their identity must be accompanied by a signed photographic release granting permission for their likeness to be reproduced in the article. If this is not provided, the patient's eyes must be occluded to prevent recognition.

For tables, the system accepts most common word processing formats. Tables should have a title that describes the content and purpose of the table. Tables should enhance, not duplicate, information in the text.

4.7 Videos

Use of supplementary video is encouraged. Videos may be submitted with a manuscript and, if approved by the editor, will be posted online with the article when published. Video submission is strongly encouraged for manuscripts reporting surgical, examination, or exercise techniques or injury mechanisms. For more information about the format requirements for videos, please review the Video Format Guide. For detailed information pertaining to copyright and permissions requirements, view the Video Permission and Fair Use Quick Guide. For videos with identifiable subjects, subjects will need to sign the Audio-Visual Likeness Release form. It is the author's responsibility to submit signed release forms, if necessary, for each video.

5. Accepted Manuscripts

Once an article is accepted and typeset, authors will be required to carefully read and correct their manuscript proofs that have been copyedited by the publisher. Any extensive changes made by authors on the proofs will be charged to authors at the rate of $2 a line. Authors are responsible for ordering reprints of their articles. Completed articles will be published on our website before print publication.

6. NIH-Supported Studies

Authors of studies funded by grants from the National Institutes of Health can deposit a copy of their accepted final peer-reviewed manuscript and associated figure/table files (pre-typeset versions) to the NIH database after a 12-month embargo period from the time their article is published in *AJSM*.

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