UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM REABILITAÇÃO FUNCIONAL

Bárbara Santos Luccas Duarte

Efeitos da Crioterapia em Marcadores Bioquímicos, Histológicos e Funcionais após a compressão do nervo isquiático em ratos.

Santa Maria, RS
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Duarte, Bárbara Santos Luccas
Efeitos da Crioterapia em Marcadores Bioquímicos, Histológicos e Funcionais após a compressão do nervo isquiático em ratos. / Bárbara Santos Luccas Duarte.- 2018.
47 p.; 30 cm

Orientador: Gustavo Orione Puntel
Coorientador: Felix Alexandre Antunes Soares
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós Graduação em Reabilitação Funcional, RS, 2018

1. Traumatismos dos Nervos Periféricos 2. Crioterapia 3. Nociceptividade 4. Funcionalidade 5. Estresse Oxidativo I. Puntel, Gustavo Orione II. Soares, Felix Alexandre Antunes III. Título.
Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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Aprovado em 27 de julho de 2018:

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

_____

Luis Ulisses Signoi, Dr. (UFSM)

_____

Cristiane Lenz Dalla Corte, Dra. (UNIPAMPA)

Santa Maria, RS
2018

AGRADECIMENTOS

Agradeço, primeiramente, a Deus por estar sempre guiando meu caminho, abençoando meus passos, me dando força, coragem e colocando pessoas especiais ao meu lado, sem as quais eu não chegaria até aqui.

Ao meu esposo Felipe, meu infinito agradecimento, por sempre estar ao meu lado me dando o suporte necessário para que eu siga sempre em frente mesmo com as adversidades da vida, por compartilhar momentos, por sempre acreditar no meu potencial e por sempre ser essa pessoa incrível da qual eu aprendo todos os dias e que só me faz crescer.

À minha família: principalmente a minha Avó, por todos valores a mim transmitidos, por me ensinar a sempre a ter fé, por ser meu exemplo de mulher e por me ensinar o que é o amor. Ao meu avô, minha mãe e minhas tias pelo amor, carinho, dedicação, compreensão e pelos valores a mim transmitidos. Ao meu pai, irmã e Dani, por mesmo longe me incentivarem a dar o melhor de mim e sempre acreditarem na minha capacidade.

À Liese e Luise, que estiveram ao meu lado durante esses dois anos e me apoiaram até aqui, com vocês aprendi o valor de uma amizade verdadeira.

Ao meu orientador, Professor Gustavo, pela oportunidade, acolhimento, disponibilidade de seu tempo, orientação, atenção e confiança.

Ao meu Co-orientador, Professor Felix, por me aceitar em seu laboratório, pelas orientações, por compartilhar seu conhecimento e oferecer todas as condições para que este trabalho se concretizasse.

Aos colegas de laboratório, em especial a Andrezza, Rodrigo e Diane pela parceria, amizade, risadas e principalmente pela paciência e ajuda em todos os momentos que precisei.

Aos colegas do PPGRF especialmente ao Gustavo, Edneia, Murilo e Tainara pela amizade que formamos, com certeza vocês tornaram este processo mais leve.

Aos demais professores, colegas e funcionários deste Programa de Pós-Graduação, agradeço a disposição para me ajudar e a contribuição, de alguma forma, para a realização do meu trabalho e para a minha formação.

Enfim, agradeço à Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Reabilitação Funcional, a possibilidade de realização deste curso.

RESUMO

EFEITOS DA CRIOTERAPIA EM MARCADORES BIOQUÍMICOS, HISTOLÓGICOS E FUNCIONAIS APÓS A COMPRESSÃO DO NERVO ISQUIÁTICO EM RATOS.

AUTORA: Bárbara Santos Luccas Duarte
ORIENTADOR: Gustavo Orione Puntel

Os nervos periféricos são responsáveis por levar informações sensoriais da pele, músculos e outros órgãos ao sistema nervoso central (SNC) e informações motoras do SNC para músculos somáticos e órgãos efetores controlados pelo sistema autônomo. A ocorrência de lesões neurais periféricas pode acarretar comprometimentos funcionais de caráter motor e sensitivo nos territórios neurais envolvidos. A crioterapia constitui uma técnica terapêutica extensivamente utilizada como forma de analgesia, no entanto seus efeitos sobre modelos animais experimentais de compressão neural periférica são pouco estudados. O presente estudo objetivou avaliar os efeitos da crioterapia sobre as alterações, bioquímicas (indicativas de estresse oxidativo) e comportamentais (indicativas de alterações comportamentais sensoriais álgicas ou motoras) após desenvolvimento de uma lesão por compressão do nervo isquiático em ratos. Os animais foram inicialmente submetidos a avaliação basal do teste de von Frey, 72 horas após o início, os grupos Lesão do Nervo Periférico (PNL), PNL + Crioterapia (Cryo) e Sham foram submetidos ao procedimento cirúrgico de compressão do nervo ciático ou cirurgia simulada. Após 24 horas, o tratamento com 20 minutos de crioterapia foi iniciado uma vez ao dia por 15 dias em todos os grupos, com exceção do Grupo Controle e Sham. Após início do tratamento os testes comportamentais foram repetidos em 7 e 15 dias. No 15°dia de tratamento os animais foram eutanasiados e amostras de tecido sanguíneo e muscular coletado para análise, estresse oxidativo e marcadores de dano. Os resultados deste estudo demonstram que a crioterapia foi capaz melhorar a função ciática de ratos submetidos a lesão do nervo em 7 dias. Além disso através de testes bioquímicos demonstramos que a lesão aumentou o dano oxidativo e a crioterapia foi capaz de atenuar estes níveis.

Palavras Chaves: Traumatismos dos Nervos Periféricos, Crioterapia, Nociceptividade, Funcionalidade, Estresse Oxidativo

ABSTRACT

EFFECTS OF CRYOTHERAPY ON BIOCHEMICAL, HISTOLOGICAL AND FUNCTIONAL MARKERS AFTER SCIATIC NERVE COMPRESSION IN RATS.

Author: Bárbara Santos Luccas Duarte
Adviser: Gustavo Orione Puntel

Peripheral nerves are responsible for conducting sensory information from skin, muscles and other organs to the central nervous system (CNS) and motor information from the CNS to somatic muscles and effector organs controlled by the autonomic system. The occurrence of peripheral neural lesions can lead to functional and motor impairment in the neural territories involved. Cryotherapy is a therapeutic technique extensively used as a form of analgesia. However its effects on experimental animal models of peripheral neural compression are poorly studied. The present study aimed to evaluate the effects of cryotherapy on oxidative stress and behavioral sensorial or painful motor changesafter a sciatic nerve compression injury in rats. The animals were firstly submitted to the von Frey test. After 72 hours the groups Peripheral Nerve Lesion (PNL), PNL + Cryo (Cryo) and Sham were submitted to surgical sciatic nerve compression or surgery simulated. After 24 hours, the 20-minute cryotherapy treatment was started once a day for 15 days in all groups, with the exception of the Control and Sham Group. The tests were repeated in 7 and 15 days after treatment. On the 15^{th} day the the animals were euthanized and samples of blood and muscle tissue collected for analysis, oxidative stress and damage markers. The results demonstrate that cryotherapy was able to improve the sciatic function of rats subjected to nerve damage at the day7. In addition, through biochemical tests, we demonstrated that the lesion increased oxidative damage and cryotherapy was able to attenuate these levels.

Keywords: Peripheral Nerve Injuries, Cryotherapy, Nociception, Functionality, Oxidative Stress.

LISTA DE SIGLAS E/OU ABREVIATURAS

ERO: Espécies Reativas de Oxigênio
CK: Creatina Quinase
DCF-RS: diclorofluoresceína oxidada
LDH: Lactato Desidrogenase
LNP: Lesão do Nevo Periférico
MTT: Metil Tetrazólio
SNC: Sistema Nervoso Periférico
SNP: Sistema Nervoso Central

SUMÁRIO

1 INTRODUÇÃO

1.1 ASPÉCTOS MORFOLÓGICOS E FUNCIONAIS DO NERVO PERIFÉRICO APÓS LESÃO.

O Sistema Nervoso Periférico (SNP) é o tecido nervoso situado fora do Sistema Nervoso Central (SNC) e inclui receptores sensitivos, nervos e seus gânglios associados e plexos nervosos (De Graaff, 2003; JUNQUEIRA; CARNEIRO, 1999). Tem a função de estabelecer comunicação e transmitir informações entre o SNC e órgãos da sensibilidade e gânglios e músculos, esta comunicação é executada pela condução nervosa estabelecida por meio de fibras eferentes e aferentes. (JUNQUEIRA; CARNEIRO, 1999). A garantia de uma alta velocidade de condução se dá pela presença de uma bainha de mielina em torno das fibras nervosas e o calibre axônico (ROSSO; YOUNG; SHAHIN, 2017).

Deste modo fibras eferentes são aquelas que saem do SNC conduzindo a informação e estimulando ou ativando a musculatura, ditas assim fibras motoras; já aquelas que conduzem o estímulo ao SNC, são fibras aferentes chamadas de fibras sensitivas (DANGELO; FANTTINI, 2000). No entanto, a maioria dos Nervos levam fibras dos dois tipos, sendo chamados de nervos mistos (JUNQUEIRA; CARNEIRO, 1999). A contração muscular acontece através de nervos motores, cada nervo origina numerosos ramos que se ramificam no tecido conjuntivo.

Lesões nos nervos periféricos (LNP) resultam em perda parcial ou total de funções motoras, sensoriais e autonômicas do membro envolvido, e diminuem substancialmente a velocidade de condução nervosa (RODRÍGUEZ; VALERO-CABRÉ; NAVARRO, 2004; ROSSO; YOUNG; SHAHIN, 2017). Isso ocorre, pois uma das reações mais elementares do SNP é a degeneração Walleriana, que se da quando a continuidade da fibra nervosa é interrompida através de eventos traumáticos, tóxicos, isquêmicos ou metabólicos (DUBOVÝ, 2011). Além disso, LNP promove a degeneração dos axônios e bainhas de mielina distalmente à lesão, refletindo na diminuição de estímulos nervosos que irá repercutir diretamente na perda funcional/motora e/ou sensitiva do membro dependendo do nível e local da lesão (GORDON; TYREMAN; RAJI, 2011; RODRÍGUEZ; VALERO-CABRÉ; NAVARRO, 2004; FERRIGNO, 2005)

Após a LNP, a porção distal do membro inervado pelo nervo ferido sofrerá gradual atrofia e fibrose podendo evoluir de forma irreversível se este não reparado o mais precocemente possível, por este motivo atualmente muitas pesquisas buscam estratégias para promover a regeneração de fibras proximais; minimizando a atrofia da função efetora e de manutenção enquanto aguarda o crescimento (ZHANG et al., 2017). Além das alterações funcionais, lesões no SNP podem vir a contribuir no desenvolvimento de dor neuropática, podendo assim ser associada a disfunção sensorial e alterações na comunicação do SNP e SNC.(GIARDINI et al., 2017) Mais de 70% da população brasileira que procuram um consultório médico por diferentes motivos tem a dor como um deles (ROCHA et al., 2007). A Associação Internacional para o Estudo da Dor (IASP) (MERSKEY; BOGDUK (EDS), 1994) "*define a dor como uma experiência sensorial e emocional desagradável associada a dano real ou potencial de tecidos ou descrita em termos de tal dano*". A dor pode vir a ser definida como dois tipos, "Nociceptiva" ou "Neuropática" (SCHESTATSKY, 2009).

A Dor Neuropática é definida por um estado de má adaptação provocada por alterações funcionais e estruturais das vias sensitivas centrais e periféricas que produzem modificações no processamento das informações nociceptivas (TEIXEIRA, 2003), pessoas com dor neuropática podem apresentar características clínicas comuns, como dores espontâneas e dores evocadas, alodinia mecânica e ainda, hiperalgesia secundária que se dá pelo comprometimento dos tecidos neurais e não neurais, podendo estar associada à sensibilização central (BENNETT et al., 2006; DETLOFF et al., 2014; KRAYCHETE; GOZZANI; KRAYCHETE, 2008). Além dos fatores fisiológicos, pacientes com neuropatia podem apresentar também irritabilidade, alteração do padrão do sono, alteração do humor e isolamento social, o que reflete diretamente no cotidiano e qualidade de vida (VALL; BRAGA, 2005). Sabe-se também que citocinas pró-inflamatórias estão diretamente associadas a modulação da dor, e sua expressão está ligada a dor neuropática (CHEN, 2012; DETLOFF et al., 2014; KRAYCHETE; GOZZANI; KRAYCHETE, 2008).

Algumas modalidades de tratamento conservador vem sendo estudada para modulação da dor, utilizando técnicas como de mobilização, exercícios físicos e crioterapia (DETLOFF et al., 2014; GIARDINI et al., 2017; KARVAT et al., 2016).

1.2 ALTERAÇÕES BIOQUIMICAS APÓS LESÃO DO NERVO PERIFÉRICO

Quando o SNP é exposto a uma lesão, o potencial de regeneração deste é muito superior ao do SNC, isso é devido as diferentes respostas das células da glia, alterações moleculares e celulares que são ativadas no local da lesão (FARONI et al., 2014; IDE, 1996). Diversos mediadores inflamatórios que desempenham funções essenciais na degeneração waleriana são regulados através da expressão de citocinas/quimiocinas no coto distal após lesões nervosas (DUBOVÝ, 2011). Citocinas são proteínas moduladoras da resposta inflamatória que promovem interações entre células em pequenas distâncias (Kraychete et al., 2008) as citoquinas inflamatórias como (factor de necrose tumoral-α (TNF-α), IL-1, IL-6 e muitos outros) são produzidas principalmente por macrófagos e mastócitos e têm um importantes papel na resposta inflamatória, incluindo ativação do endotélio e leucócitos na indução da resposta de fase aguda (MEDZHITOV, 2008). A produção de citoquinas pró-inflamatórias, tais como IL-1, IL-6 e TNF-α liberam uma série de espécies reativas no local da inflamação, levando ao estresse oxidativo exagerado (BISWAS, 2016; SONG et al., 2014). No entanto, a inflamação e o estresse oxidativo são eventos fisiopatológicos que estão inter-relacionados (BISWAS, 2016; LEE; SONG; YEUM, 2015).

Komirishetty et al (2017) mostraram que o estresse oxidativo / nitrosativo após compressão do nervo ciático também desempenhou efeitos importantes na patogênese da neuropatia periférica. O estresse oxidativo é um desequilíbrio das reações pró-oxidantes/antioxidantes resultantes das reações metabólicas que utilizam oxigênio, os mecanismos antioxidantes são contornados ocorrendo dano oxidativo. (VALKO et al., 2007; MOTTA, 2011) O excesso de espécie reativas de oxigênio (ERO) pode reagir com os lipídios celulares, proteínas e/ou DNA, danificando ou inibindo sua função normal, a sua formação pode causar danos significativos nas células. Sua formação é mantida em quantidades mínimas por mecanismos antioxidantes visando o equilíbrio redox.(VALKO et al., 2007; MOTTA, 2011) Alguns estudos implicam o estresse oxidativo em várias doenças humanas, bem como no processo de envelhecimento, e indicam também que o estresse oxidativo é uma das principais causas de dano neural, uma vez que esse dano presente irá modular moléculas antioxidantes, que desempenham um papel importante na regeneração da lesão do nervo periférico. (LANZA et al., 2012; QIU et al., 2014; VALKO et al., 2007). Além de marcadores bioquímicos, alguns estudos também associam a alguns achados histológicos e discutem esta relação, Qiu et

al. (2014), registrou a presença de organelas inchadas comprovando a lesão do nervo periférico, e o aumento da expressão das principais enzimas antioxidantes, indicando assim a ocorrência do estresse oxidativo.

Sendo assim muitas modalidades de tratamento tem sido investigada para inibição dos efeitos do estresse oxidativo e investigação de demais possíveis envolvimentos para diminuição de dano neural após lesões do nervo periférico.

1.3 CRIOTERAPIA

A técnica de crioterapia é bastante utilizada por profissionais da área da saúde. Um dos motivos são o baixo custo e fácil aplicação, podendo ser utilizada para analgesia, manutenção do edema e na recuperação pós-trauma (GUIRRO; ABIB; MÁXIMO, 1999). Segundo Uchôa; Freitas, (2006) A característica do tratamento através da aplicação do frio se dá pela diminuição de movimento molecular, e é utilizado com o objetivo de diminuir a temperatura local fazendo com que a área de tratamento reaja com uma série de respostas locais e sistêmicas. Após 15 a 20 minutos de aplicação do gelo ocorre uma diminuição do metabolismo levando a limitação da formação de edema, da liberação da histamina, da ativação de neutrófilos, da ação colagenase, de leucócitos sinoviais e maior drenagem linfática (BRANCACCIO et al., 2005).

Muitas publicações discutem os efeitos da crioterapia, porém (GUTIÉRREZ ESPINOZA; LAVADO BUSTAMANTE; MÉNDEZ PÉREZ, 2010) em um estudo de revisão sistemática trazem que existe uma quantidade escassa de publicações de alta qualidade metodológica que a abordam com objetivo de utiliza-la como ferramenta terapêutica no gerenciamento da dor. A variação da temperatura tem grandes efeitos no sistema nervoso periférico, entre os eles, sabe-se que causa um retardo na condução das fibras nervosas contribuindo com o processo de analgesia (FARIAS et al., 2010; RUTKOVE, 2001). Entretanto, dois fatores podem influenciar nos efeitos desta terapia na recuperação do nervo e modulação da dor, que são, a temperatura e o tempo de exposição ao método (HOOSHMAND; HASHMI; EM, 2004). E ainda, relaciona o grau de hipotermia ao dano nervoso, pois acredita-se que a esta diminui a velocidade do impulso quimioelétrico em virtude da solidificação mielínica interrompendo assim a condutibilidade dos nervos mielínicos. Porém, quando exposto a temperaturas muito

baixas, pode evoluir com processo de degeneração axonal distal ao nível da lesão, mas a porção proximal do axônio permanece preservada, essa característica mantém a possibilidade de regeneração do nervo (HSU; STEVENSON, 2015)

Com base na pesquisa da literatura é possível observar que o gelo diminui a velocidade de condução nervosa podendo assim contribuir no processo de analgesia. Entretanto é possível observar também que essa alteração da condutibilidade do nervo pode causar ou induzir processos de hiperalgesia ou danos nervosos quando expostos de maneira inadequada. A crioterapia quando utilizada por um longo período de tempo pode trazer outras características de dor, principalmente de dor neuropática (JU et al., 2012).

Sendo assim mais estudos que investiguem os efeitos variados nos diferentes limiares de tempo com esta terapia são necessários. Existe uma variedade de estudos envolvendo o uso da crioterapia como recurso terapêutico, no entanto os resultados são controversos e inconclusivos, isso se dá pelo amplo número de protocolos utilizados com tempo de aplicação variado (GUIRRO; ABIB; MÁXIMO, 1999; GUTIÉRREZ ESPINOZA; LAVADO BUSTAMANTE; MÉNDEZ PÉREZ, 2010). Outro fator determinante para se estabelecer a relevância da técnica se dá por poucos estudos que investiguem os possíveis efeitos negativos desta terapia (RUTKOVE, 2001), tais como as alterações bioquímicas e funcionais, decorrentes do uso incorreto deste recurso, sobre a funcionalidade de nevos periféricos.

1.4 OBJETIVOS

1.4.1 Objetivo Geral

Estudar os efeitos da crioterapia sobre as alterações bioquímicas e comportamentais após a compressão cirúrgica do nervo isquiático em ratos.

1.4.2 Objetivos Específicos

- Analisar os efeitos da compressão cirúrgica do nervo isquiático sobre as alterações bioquímicas indicativas de dano oxidativo no tecido muscular do território de inervação do mesmo, a partir da quantificação da viabilidade

celular, dos níveis de diclorofluoriceina oxidada (DCF-RS), da peroxidação lipídica.

- Analisar os níveis plasmáticos de atividade das enzimas creatina quinase (CK) e lactato desidrogenase (LDH) determinados pela compressão cirúrgica do nervo isquiático;
- Analisar as alterações comportamentais motoras de marcha e de percepção sensorial dos animais submetidos à compressão cirúrgica do nervo isquiático;
- Analisar as alterações histopatológicas relacionadas ao processo de cicatrização do nervo isquiático seguintes à compressão cirúrgica do mesmo;
- Investigar os efeitos da crioterapia sobre os objetivos específicos supracitados;

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*.

2.1 ARTIGO CIENTÍFICO

Full title:

EFFECTS OF LONG-TERM THERAPEUTIC COLD TREATMENT ON BIOCHEMICAL AND FUNCTIONAL MARKERS AFTER SCIATIC NERVE COMPRESSION IN RATS.

EFFECTS OF LONG-TERM THERAPEUTIC COLD TREATMENT ON BIOCHEMICAL AND FUNCTIONAL MARKERS AFTER SCIATIC NERVE COMPRESSION IN RATS.

Bárbara S.L. Duarte[1], Andrezza B.V. Furtado[1], Diane D. Hartmann[2], Rodrigo P. Martins[2], Dèbora F. Gonçalves[2], Pamela C. da Rosa[2], Felix A. Soares[2], Gustavo O. Puntel[1,2].

[1]Centro de Ciências da Saúde, Programa de Pós-graduação em Reabilitação Funcional, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil.

[2]Centro de Ciências Naturais e Exatas, Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

ABSTRACT

Peripheral nerves are responsible for conducting sensory information from skin, muscles and other organs to the central nervous system (CNS) and motor information from the CNS to somatic muscles and effector organs controlled by the autonomic system. The occurrence of peripheral neural lesions can lead to functional and motor impairment in the neural territories involved. Cryotherapy is a therapeutic technique extensively used as a form of analgesia. However its effects on experimental animal models of peripheral neural compression are poorly studied. The present study aimed to evaluate the effects of cryotherapy on oxidative stress and behavioral sensorial or painful motor changes after a sciatic nerve compression injury in rats. The animals were firstly submitted to the von Frey test. After 72 hours the groups Peripheral Nerve Lesion (PNL), PNL + Cryo (Cryo) and Sham were submitted to surgical sciatic nerve compression or surgery simulated. After 24 hours, the 20-minute cryotherapy treatment was started once a day for 15 days in all groups, with the exception of the Control and Sham Group. The tests were repeated in 7 and 15 days after treatment. On the 15th day the animals were euthanized and samples of blood and muscle tissue collected for analysis, oxidative stress and damage markers. The results demonstrated that cryotherapy was able to improve the sciatic function of rats subjected to nerve damage at the day 7. In addition, through biochemical tests, we demonstrated that the lesion increased oxidative damage and cryotherapy was able to attenuate these levels.

Keywords: Peripheral Nerve Injuries, Cryotherapy, Nociception, Functionality, Oxidative Stress.

INTRODUCTION

The integrity and balance between the structures related with movements are responsible for the functional ability of the human's body members. There is a regulatory role of the Central Nervous System (CNS) that obtains sensory information, integrates and processes the signals received, and produces the most adequate response for its preservation and regulating comfort to each position and action. Such information is received and transmitted through the afferent and efferent fibers of the Peripheral Nervous System (PNS)(1). The peripheral nerve is the extension of the CNS, being responsible for the motor and sensory function of the organism. The basic functional unit of the peripheral nerve is the neuron, consisting of a cellular body and a nerve fiber. This functional unit of the PNS demands the intimate association of two different cell types: the Schwann cells (SCs) with the neuronal axons (1,2)

PNS lesions are commonly associated with mechanical trauma, and less frequent after surgical procedures, resulting in acute traumatic compression or crushing (3,4). However, the PNS has an intrinsic capacity for regeneration and repair that is related to factors such as age, mechanism of injury and location of the lesion(3). The functional loss resulting from the trauma is influenced by several mechanisms, which may be reversible. As a result of peripheral lesions, an inflammatory response occurs, leading to changes in vascular permeability, increased blood flow, swelling and hyperalgesia(5). However, existing treatments for accelerated recovery and nerve regeneration are not fully available (4). Currently, there are many difficulties both in diagnosis and in treatment, with little capacity to predict lesions in which recovery is even possible(4). For better recovery and rehabilitation of these lesions, many conservative modalities are being studied (6).

Cryotherapy is one of the conservative modalities of treatment commonly used in clinical practice for inflammatory response control (pain relief, edema and hyperthermia reduction, prevention of functionality lost). Commonly used by athletes, and population in general, the mostly employed methods of cryotherapy consist in application of wet ice or submersion of body treated areas in cold water. The differences of treatment protocols consists basically on the duration of each application or the global period in days after the lesion onset (7). It is known that a 30 minutes treatment produces twice the physiological effect of a 15 minute treatment, and can produce a different result under the tissue (7). In this sense, it is perceived that the action of the treatment by cryotherapy is beneficial. However, negative effects such as the solidification and hardening of the myelinic lipid, leading to an impairment of the afferent and efferent nerve stimulus conduction, were already observed depending on the time of application of the tissue (8),

The sciatic nerve is the most important motor nerve, and one important sensorial nerve, of animal's lower/hind limb. Experimental models of chronic constriction injury (CCI) already demonstrated impairs in motor skill abilities and sensorial behavior. Moreover, morphological changes indicative of the sciatic nerve degeneration were already observed. (5,9) However, biochemical changes suggestive of an oxidative stress of the skeletal muscle innervated by them is stills not well described.

Although the beneficial effects of cryotherapy on reducing the oxidative damage of skeletal muscle after injuries such as contusion and distension were already described (10,11) , there is no consensus about cryotherapy effects on experimental models of nerve injury. Therefore, the present work aimed to investigate for the first time the effects of cryotherapy on oxidative damage in the skeletal muscle represented by an

experimental model of CCI in rats. In addition, also in motor changes and sensory behavior resulting from CCI.

Materials and methods

Animals and reagents

Wistar male rats, three to four months old weighing between 200 and 250g, were used for this study, from the central laboratory of the Federal University of Santa Maria (UFSM). The animals were kept in boxes with free access to food and water, in a room with controlled temperature (22 ± 2 ° C) and with a light / dark cycle of 12 h. All experimental procedures were conducted according to the ethical principles in animal experimentation elaborated by the Brazilian College of Animal Experimentation (COBEA), and according to principles determined in the procedures law for the scientific use of animals n ° 11,794, of October 08, 2008 . The detailed design of the experimental procedures of the lesion were submitted 63 to the Committee of Ethics and Animal Welfare of the Federal University of Santa 64 Maria (UFSM), All the procedure were in accordance with the guidelines of the 65 Committee on Care and Use of Experimental Animal Resources of the Federal 66 University of Santa Maria, Brazil (UFSM; 4185290915)

Experimental groups

The animals (n=29) were randomized and divided into five main homogeneous groups:

1. Control Group (Ctrl) (n = 5): No surgical intervention or treatment.
2. Sham group (Sham) (n = 6): submitted to the surgical procedure without compression of the Sciatic Nerve.

3. Peripheral nerve injury group (PNI) (n = 6): submitted to surgical procedure and compression of the Sciatic Nerve.
4. Cryotherapy group (Cryo) (n = 6): submitted to 20 minutes of daily cryotherapy session without any surgical procedure involved.
5. Peripheral Nerve Injury and Cryotherapy Group (PNI+Cryo) (n = 6): submitted to the surgical procedure with compression of the Sciatic Nerve and 20 minutes of cryotherapy daily session.

Study design

The animals were initially submitted to a basal evaluation of the von Frey test, 72 hours after control, the PNI, PNI + Cryo and Sham groups underwent the surgical procedure of sciatic nerve compression or sham surgery. After 24 hours the treatment with 20 minutes of cryotherapy was started once a day for 15 days in all the groups except for the Control and Sham Group.

On the seventh day after the start of the experiments, all animals underwent behavioral and functional analyzes (von-frey and IFC) to evaluate the mechanical allodynia test and sciatic function index. On the 15th day of cryotherapy, the animals were again submitted to functional and behavioral tests and were immediately euthanized after the tests. Then the tissues (blood, gastrocnemius muscle and sciatic nerve) were collected for biochemical and histological analyzes.

The animals were euthanized immediately after the cryotherapy application protocol. Blood was collected by cardiac puncture in a pre-heparinized syringe, and both gastrocnemius muscles were quickly removed, weighed, and placed on ice. Samples of skeletal muscle tissue were homogenized in 10 min in 10 volumes of 10mM cold Tris (pH 7.4) and centrifuged at 4000 xg for 10 min to produce the low-speed floating fraction that was used for different biochemical assays at all essay. Whole

blood samples were centrifuged at 1500 xg for 10 min for plasma separation which was used for biochemical analysis.

Induction of sciatic nerve injury by chronic constriction injury (CCI)

The rats were anesthetized (n = 18) with ketamine hydrochloride (i.p. 40mg / kg) associated with xylazine hydrochloride (i.p.10mg / kg). The common sciatic nerve was exposed to the medial thigh level by incision through the femoral biceps, proximally to the trifurcation of the sciatic tissue releasing tissue along approximately 7 mm of the sciatic nerve only, distal to the major trochanter of the femoral bone. The sciatic nerve was exposed through the femoral biceps muscle, and then its 3 terminal branches (the sural, common peroneal and tibial) were identified. Four wired ligatures (4.0 black silk) were tied around the sciatic nerve as described by Bennett GJ, Xie YK (1988) (12) Stricture of the sciatic nerve was controlled by ligation when a small, brief contraction occurred in the muscle surrounding the exposed sciatic nerve. The muscle, adjacent fascia and skin incisions were closed with 4/0 silk thread, and the animal was returned to its recovery cage. Sham surgeries (n =6) involved the exposure of the sciatic nerve and its branches with the same procedures, but without creating any constriction / lesion.

Cryotherapy application protocol

After 24 hours of the PNI, the cryotherapy sections were performed individually for each animal during 20 minutes by using a disposable container with water and ice in order to kept the temperature at 5 °C (the temperature was monitored by a

analogical/digital thermometer submerged in the container along the entire period of treatment). The right hind limb of the animal was submerged until the medial region of the knee joint accordingly for Karvat et al., 2016 (6). This protocol was repeated daily for 15 consecutive days, always between 10 AM and 1PM hours. It is important to emphasize that this treatment protocol was innovator in the treatment of a peripheral nerve injury in rats.

Sensorial and motor behavioral tests

The evaluation of mechanical allodynia and motor was performed using the Von Frey and Sciatic Function Index (SFI) tests.

Von Frey was used to measure the 50% paw withdrawal threshold, the 50% threshold was determined using the method proposed by Dixon WJ. (1988) (13). To perform the tests the rats were allocated in individual stainless steel chambers with a wire mesh floor. Seven von Frey filaments (Remington's Sensory-Touch Sensors, Remington Medical Equipment Ltd., ON, Canada) were applied to the plantar surfaces of the right hind paw. The stimulation with the filaments was performed 3 consecutive times. Removal of the hind paw was considered a positive response. The results were analyzed with their respective baseline results.

The SFI proposed by De Medinaceli (9) was used to evaluate the motor function in order to evaluate the functional recovery of the sciatic nerve after injury and cryotherapy application. The animals were placed on a wooden path so they could only walk forward, with a dark shelter at the end. The animals were placed to walk on a white paper, the paws will be stamped so that the appropriate measures were taken for analysis. The following calculation was based on the equation proposed by (14): SFI = 283.3 [(EPL-NPL) / NPL] + 109.5 [ETS-NTS] / NTS] +13.3 [-EITS) / NITS] -8.8.

PL = Print length (distance from the heel to the third toe).

TS = Toe extension (distance from first to fifth toe)

ITS = Spread of the middle finger (distance from the second to the fourth toe).

E = Paw of the experiment.

N = Contralateral leg.

Biochemical analisis in skeletal muscle

Oxidized dichlorofluorescein (DCF-RS) levels

DCF-RS levels were measured according to MYHRE (15) 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-RS and the results were corrected by mg of protein.

Measurement of 3-(4,5-Dimethylthiazol – 2 –ye)- 2,5- Diphenyl-tetrazolium Bromide (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 (16).

Thiobarbituric Acid Reactive Substances (TBARS)

TBARS levels were determined according to the method described by Ohkawa et al. Aliquots of 500 μL of supernatant fraction obtained after blood sample precipitation or 200 μL of skeletal muscle S1 were added to color reaction. TBARS levels were measured at 532 nm using a standard curve of MDA and corrected by the protein content.

Catalase (CAT)

Catalase enzyme activity was measured in the skeletal muscle homogenate according to the method described by Aebi. A sample of skeletal muscle was added in a medium containing potassium phosphate buffer (TFK 50 mM, pH 7.4) and H_2O_2 (1 mM). The CAT kinetic analysis was initiated after the hydrogen peroxide H_2O_2 addition; colorimetric reaction was measured at 240 nm.

Protein quantification

The protein content was estimated by the Bradford method (17) using bovine serum albumin (BSA) as the standard.

Biochemical analysis in plasma after 15 days

Activity of the creatine kinase enzyme (CK)

CK activity was determined spectrophotometrically in plasma samples using diagnostic kits (Labtest Diagnóstica S. A. Lagoa Santa, MG).

Activity of the enzyme lactate dehydrogenase (LDH)

LDH activity was determined spectrophotometrically from muscle tissue samples by the use of diagnostic kits (Labtest Diagnóstica S. A. Lagoa Santa, MG).

Statistical analysis

Graphpad Prism 5.0 (San Diego, CA, USA) was used for all analyzes. Data were expressed as mean and standard deviation (SD). Significance was assessed by Two-Way Variance Analysis (ANOVA) followed by Bonferrni's post-hoc test for behavioral analysis of mechanical allodynia. The significance of the SFI was evaluated by analysis of variance MANOVA followed by the Bonferrni post-hoc test. The other results, significance was assessed by analysis of one-way variance followed by the Tukey post-hoc test.

Results

Motor and sensorial behavorial tests

The SFI measurement data are shown in Figure 1A. In this study we observed that 7 days after the injury there was a significant loss of motor function in the PNI group when compared to the Ctrl group ($p > 0.001$), indicating that the surgery was correctly performed. This hypothesis asserts when we compare the PNI group to Sham, we observed that the PNI group had a significant loss of function when compared to Sham ($p > 0.001$), thus indicating success in surgery and sham surgery.

However, 15 days after the injury there was a recovery of function in the PNI group compared to Ctrl ($p > 0.05$). This same pattern was demonstrated after 15 days in the Sham group when compared to PNI after 15 days ($p > 0, 05$). We thus suggest that this fact is reasserted by the intrinsic re-emergence of the nerve.

In 7 days after the injury, PNI + Cryo group presented recovery of nerve function ($p > 0.001$), the result was not statistically significant after 15 days, however, it is still possible to observe improvement of the function compared to the surgery group.

FIGURE 1A: **Walking track analysis to evaluate functional recovery.** Cryotherapy was performed for 15 consecutive days for 20 minutes, gait alteration was evaluated 7 and 15 days of ice application. Data are expressed as ± SEM and two-way

RM ANOVA was performed after the Bonferroni test. N = 6. * p <0.05 and *** p <0.001 vs. ctrl, # p <0.05 and ### p <0.001 vs PNI + Cryo, $ p <0.05 vs Cryotherapy group.

The results of the sensorial analysis through Von Frey were demonstrated in Figure 1B. No statistical difference was observed between the groups for nociception. However, it was possible to observe that the PNI group lowered the threshold throughout the 15 days while the PNL + Cryo group increased its threshold in the first 7 days and maintained in the next 7 days.

FIGURE 2B: Analysis of the paw withdrawal index after 7 and 15 days of injury and cryotherapy treatment. Cryotherapy treatment decrease (g) removal paw after PNI Cryotherapy was performed for 15 consecutive days for 20min, sensitive body change that was investigated by the Frey test. The basal sensorial limit was performed prior to the application of cryotherapy and surgery and the sensory alteration was evaluated after 7 days and 15 days after application. Data are expressed as SEM and two-way ANOVA was performed after the Bonferroni test. N = 6

Oxidative stress markers in skeletal muscle

Figure 2 shows the effect of cryotherapy on skeletal muscle levels of DCFH-RS in rats exposed to sciatic nerve injury. Data are expressed as mean ± SEM (n = 6) and analyzed by One-Way ANOVA, followed by the Tukey test where appropriate. The PNI and Sham groups significantly increased DCF-RS levels when compared to the control group (p> 0.01 and p> 0.05). There was no difference in Cryo and PNI + Cryo groups when compared to control, but compared to PNI and Sham there was a lower level of DCF-RS (p> 0.05 and p> 0.01).

FIGURE 2: Effect of Cryotherapy (5° C) on DCF-RS oxidation in gastrocnemius muscle in rats exposed to injury to the sciatic nerve. The rats were exposed to CCI and were treated with Cryotherapy (5° C) for 20 min for day in 15 days. Data are expressed as mean ± SEM (n = 6) and were analyzed by One-Way ANOVA, followed by Tukey test when appropriated. *p<0.05 ** p < 0,01 compared to Control group. #p<0.05 ## p < 0,01 compared to PNI + Cryo group. $ p<0.05 $$p<0,01 compared to cryotherapy group.

However, oxidative damage measure by Thiobarbituric Acid Reactive Substances (TBARS) did not demonstrate significantly difference in gastrocnemius muscle levels with cryotherapy treatment, the results were demonstrated in figure 3.

FIGURE 3: Effect of Cryotherapy on of Thiobarbituric Acid Reactive Substances (TBARS) levels in gastrocnemius muscle in rats exposed to CCI. The rats were exposed to surgical injury in sciatic nerve and treated with Cryotherapy (5° C) for 20 minutos for day in 15 days. Data are expressed as mean ± SEM (n = 6) and were analyzed by One-Way ANOVA, followed by Tukey test when appropriated.

In addition, MTT levels decrease in PNI group compared to Control (p>0.05) in gastrocnemius muscle after the Sciatic injury (Fig. 4).

FIGURE 4: Effect of Cryotherapy in MTT activity, in gastrocnemius muscle in rats exposed to sciatic nerve injury. The rats were exposed to PNI in sciatic nerve and treated with Cryotherapy (5° C) for 20 minutes a day during 15 days. Data are expressed as mean ± SEM (n = 6) and were analyzed by One-Way ANOVA, followed by Tukey test when appropriated. *p<0.05 compared to Control group

Enzymatic antioxidant mechanism (CAT)

Effect of cryotherapy on catalase enzyme activity (CAT) was demonstrated in figure 5. Fifteen days after the injury, the CAT enzyme activity of the treated group was higher compared to the control ($p < 0.05$) and surgery ($p < 0.05$).

FIGURE 5: **Effect of Cryotherapy in catalase enzymeactivity (CAT) in gastrocnemius muscle exposed to sciatic nerve injury**. The rats were exposed to surgical injury in sciatic nerve and treated with Cryotherapy (5° C) for 20 minutes a day during 15 days. Data are expressed as mean ± SEM (n = 6) and were analyzed by One-Way ANOVA, followed by Tukey test when appropriated. *$p<0.05$ compared to Control group. #$p<0.05$ compared to surgery group. $ $p<0.05$ compared to cryotherapy group.

Skeletal muscle injury markers in plasma

Cryotherapy treatment during 15 days after CCI did not alter plasmatic creatine kinase levels (Fig. 6), this result explained because this enzyme returns basal levels at 7 days after damage. Nevertheless, cryotherapy applicantion increased LDH activity in treatment group without injury (Fig. 7).

FIGURE 6: Effect of cryotherapy (5 ° C) on creatine kinase (CK) activity in plasma of rats exposed to sciatic nerve injury. Rats were exposed to CCI in sciatic nerve and treated with cryotherapy (5 ºC) for 20 minutes for 15 days. Data are expressed as mean ± SEM (n = 6) and analyzed by One-Way ANOVA, followed by Tukey's test when appropriate.

FIGURE 7: **Effect of cryotherapy (5 ° C) on lactate dehydrogenase (LDH) enzyme activity in plasma of rats exposed to sciatic CCI.** Rats were exposed to surgical injury in sciatic nerve and treated with cryotherapy (5 ºC) for 20 minutes for 15 days. Data are expressed as mean ± SEM (n = 6) and analyzed by One-Way ANOVA, followed by Tukey's test when appropriate. * $p < 0.001$ to the control group.

Discussion

In the present study, we evaluated the cryotherapy (once daily for 20 minutes for 15 days) effects on biochemical and functional alterations in rats submitted to peripheral nerve injury (PNI) of the sciatic nerve. To the best of our knowledge, there is no other studies that seek to assess the long-term effects of cryotherapy on the treatment of a peripheral nerve injury. Through this study, we demonstrated that the application of cryotherapy for 15 days, determined an improvement of the motor function of the sciatic nerve associated with significant biochemical but not significant sensorial changes.

The SFI evaluates the improvement of motor function, with values varying from 0 (normal nerve function) to 100 (complete loss of nerve function) and is a reliable measure to evaluate the condition of the sciatic nerve in rats (9).

In this study, we observed that rats that underwent peripheral nerve compression had their motor function impaired (Fig. 1A). This is because with nerve compression the axonal nervous stimulus conduction and also the blood supply of the nervous fiber are impaired (5). In addition, the myelin sheath thickness around the fibers could be damaged after a constriction injury of the nerve leading to an impaired speed of nervous stimulus conduction. In fact, the impact of traumatic events on nervous fibers functionality depends on the size and thickness of the myelin sheath, since that its greater size means a greater neuronal conduction velocity (5,18). For this reason, compression lesions lead to a reduction in conduction velocity, resulting in severe physiological consequences, Furthermore, a traumatic nerve damage could result in the neuronal cell body ruptures with the consequent degeneration of axons distal to the lesion, determining denervation of target organs (19,20).

We observed that the application of cryotherapy PNI rats improved sciatic function in the first seven days compared to the group that had undergone compression

and had not been treated (Fig 1A). We suggest that this phenomenon can happen because the peripheral nervous system is highly temperature sensitive (18). When a cooling process occurs, there is an increase in the threshold of excitation of the nerve cells as a function of time, which is inversely proportional to the decrease of the impulse. Therefore, the sensory nerves react with an increase in the duration of the action potential leading to the depolarization of the fibers and consequently the decrease of pain (21). In another study investigating the effects of temperature on peripheral nerves, the authors observed that, when temperature increased, the duration, distal latency, area and amplitude of the sensory nerve action potential decreased, increasing the neuronal stimulus conduction velocity (22).

In contrast, when we analyzed the gait functionality after 15 days, we observed that the PNI + Cryo was not able to bring about significant improvement of the sciatic function, however, there was an improvement of the PNI group function. We report that this improvement was probably due to the fact that the time threshold was higher resulting in intrinsic nerve regeneration, which may have influenced the result when compared to the treated group. This finding corroborates with other studies that report that from the 3rd week after nerve compression the regeneration process begins and consequently the neuronal function improves (8).

In this study, we tried to verify a link between the mechanisms of functional impairment and biochemical changes indicative of oxidative stress in the skeletal muscle innervated by the sciatic nerve that underwent the compression injury. Some studies have reported that the excessive production of reactive oxygen species (ROS), which characterize an oxidative stress status, is involved in the induction of neuropathic pain (23,24). It is known that after compression of the sciatic nerve occurs an oxidative / nitrosative stress, which contributes to the genesis of peripheral neuropathy. Moreover,

339 oxidative stress is recognized as one of the main causes of neural damage, not only due
340 to increased ROS levels but also due to an impairment of antioxidant systems, which
341 play an important role in the regeneration of peripheral nerve injury (25–27). The
342 cryotherapy protocol tested in this study determined a reduction of the oxidative
343 damage in skeletal muscle as were indicated by a decreased DCF-RS levels production
344 (Fig. 2).

345 According to Siqueira et al (2016), the application of cryotherapy during the
346 phase of destruction after a skeletal muscle lesion can reverse the increase of DCF-RS
347 and TBARS levels (28). In another study involving cryotherapy after a skeletal muscle
348 damage, the authors suggest that excessive ROS levels could not only induce DCF-RS
349 formation, but also trigger a complex cascade of reactions leading to lipid peroxidation
350 (increased levels of TBARS) (10).

351 In agreement with this, we have demonstrated here that cryotherapy attenuated
352 the increase of DCF-RS levels after PNI in rats (restoring control levels), reinforcing its
353 antioxidant effects. However, we did not observe a significant lipid peroxidation in
354 skeletal muscle in response to the PNI.

355 It is known that excessive levels of ROS production can cause changes in the
356 structure and function of enzymes, and as a consequence, defects in some important
357 enzymatic antioxidant systems (such as catalase) (10). LDH is a cytosolic key enzyme
358 involved in the energetic metabolism of several cells, such as the myocite. This enzyme
359 has the role to catalyze the reversible dehydrogenation of lactate, converting it into
360 pyruvate (29). Our results demonstrated that there was an increase in plasma LDH
361 activity in the group that only performed cryotherapy (Fig. 7), indicating an increase in
362 rupture of myocite membranes and the leakage of this enzyme from cytosol to
363 circulatory system. We understand that this unexpected result means that cryotherapy,

when applied for prolonged periods (20 minutes, daily for 15 days) in situations where there is no skeletal muscle lesion, can be harmful to myocites membranes integrity.

Mitochondria play an essential role for survival and cellular function, including energy production, redox control, calcium homeostasis, metabolic and biosynthetic factors(30). Them are also the main source of ROS, which have a fundamental role in normal cellular functioning and also in cell death mechanisms (30). In this study we evaluated the skeletal muscle mitochondria functionality via measuring the methyltetrazolium salt (MTT) reduction to formazan (Fig. 4). This chemical reaction is associated with dehydrogenase enzyme activities, which are mainly located in mitochondria and reflects indirectly its viability. Therefore, a decreased MTT reduction activity means a reduced cellular viability (31), We observed that rats underwent to the nerve compression had lower levels of MTT reduction when compared to the control group animals (Fig 4). The other groups maintained the levels of cellular viability without statistical difference to control, depicting that cryotherapy could contribute to maintain the myocite viability of PNI injured rats.

The increase in the antioxidant catalase activity of the PNI + Cryo treated rats suggests that cryotherapy determined an increase the antioxidant status of these animals. (Fig 5). This result corroborates with the literature that shows different protocols of cryotherapy are able to increase this enzyme activity (32).

The results of this study indicate that the experimental model of PNI in rats determined a significant impairment of motor behavior that was associated with significant changes in biochemical markers of oxidative stress. We note that cryotherapy has shown that it may be a potential treatment resource for pain management. However, most of the studies interrupt the treatment in the acute phase after injury, and we do not have clarity of what happens functionally and

physiologically after this period. For this reason, we sought to advance the treatment period to 15 days in order to study and deepen the effects that cryotherapy may have on the peripheral and muscular nervous system and to understand its mechanism of action in the treatment of pain, functional aspects and repercussions in the biochemical mechanisms.

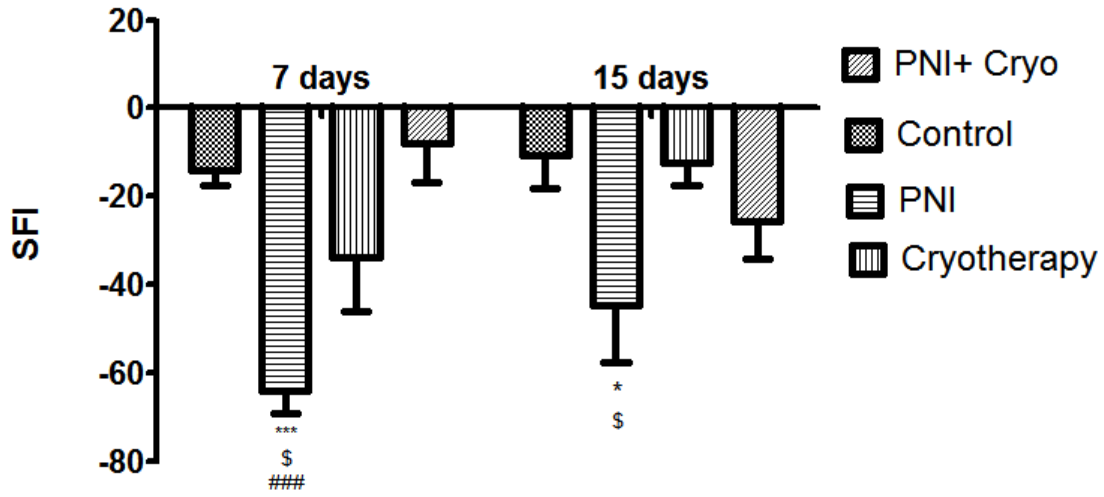
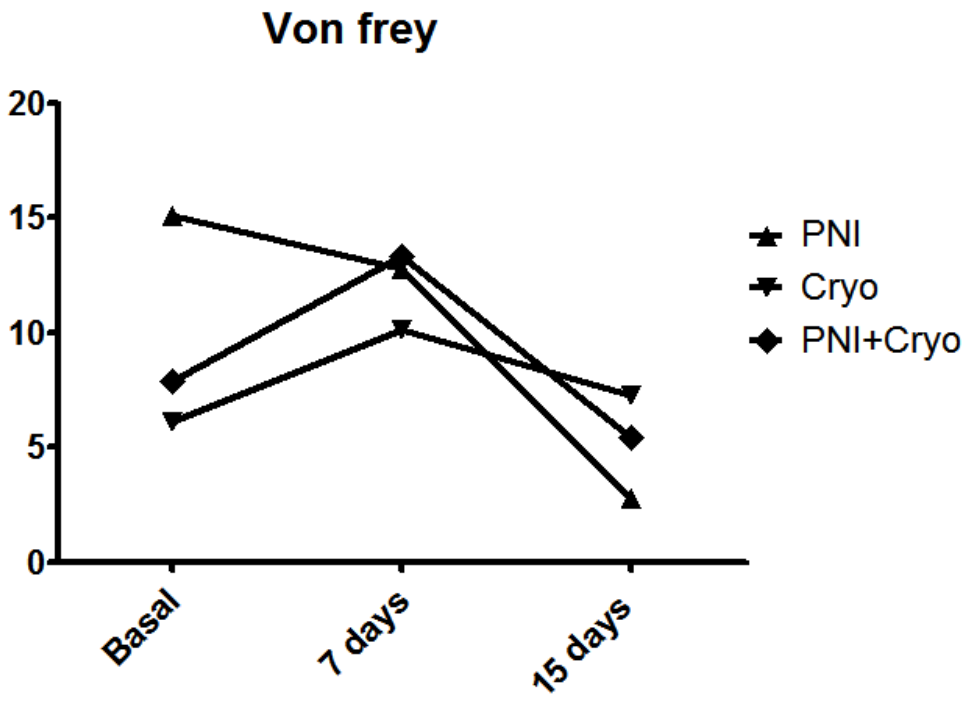


Fig 1A



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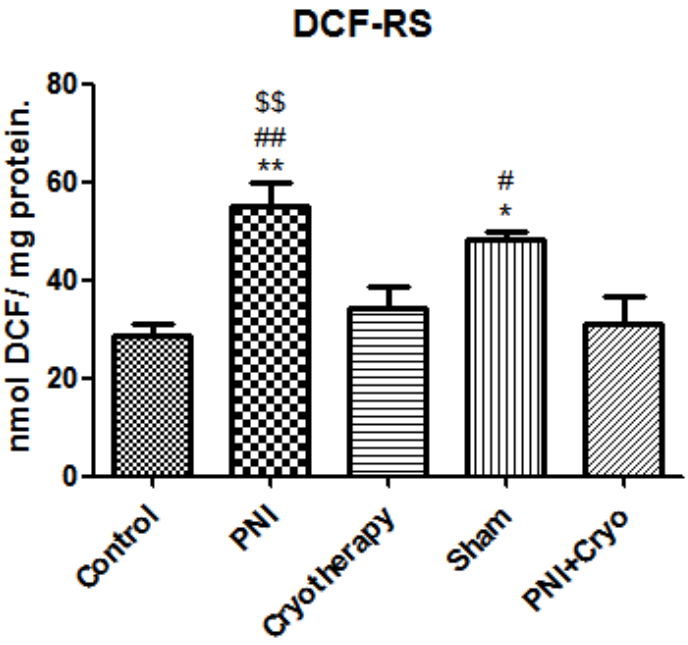

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402 Fig 2

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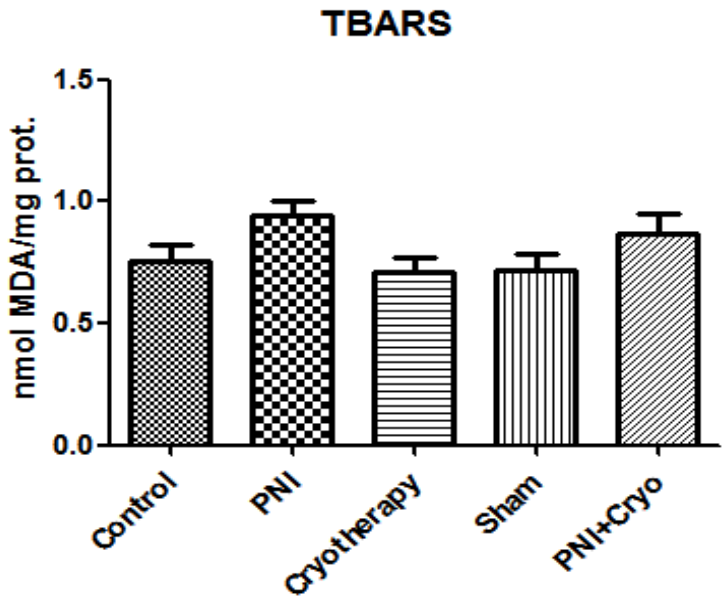

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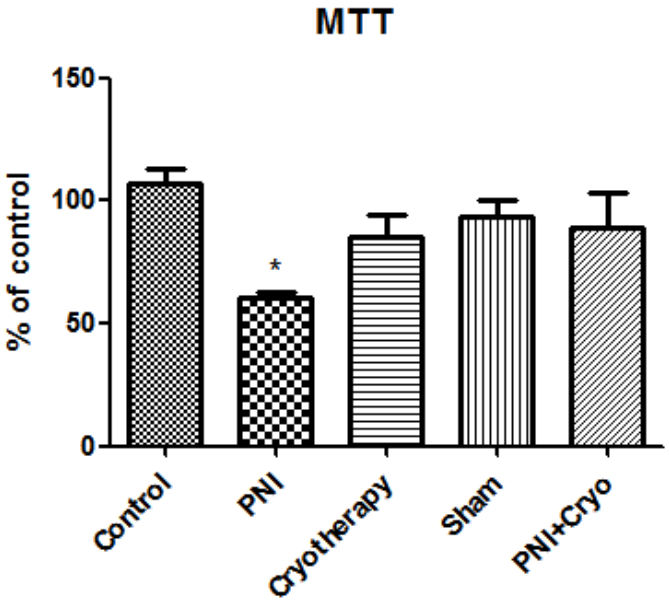

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407 Fig 4

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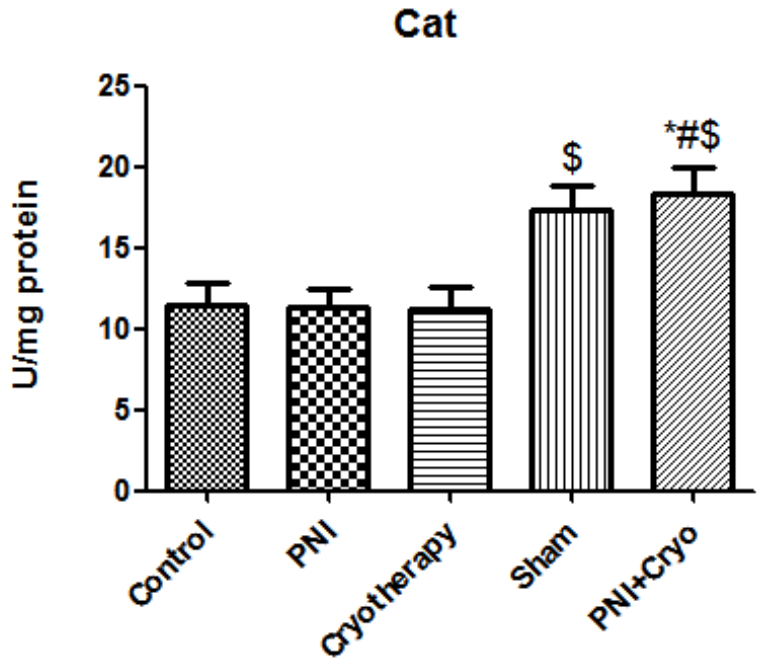

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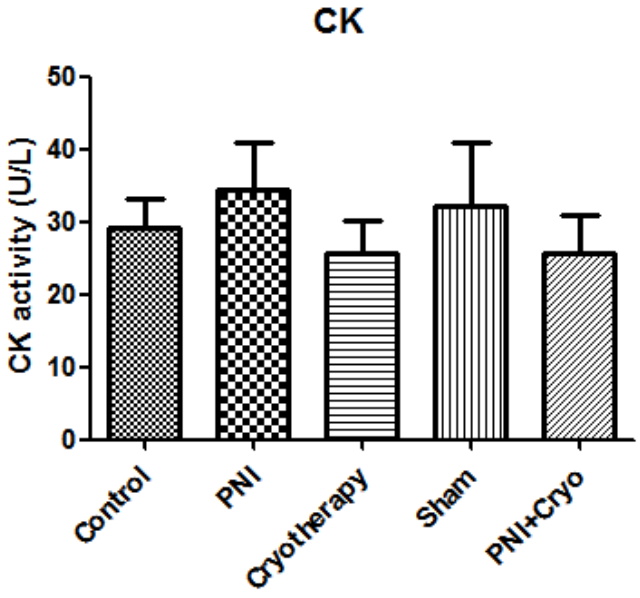

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Fig 6

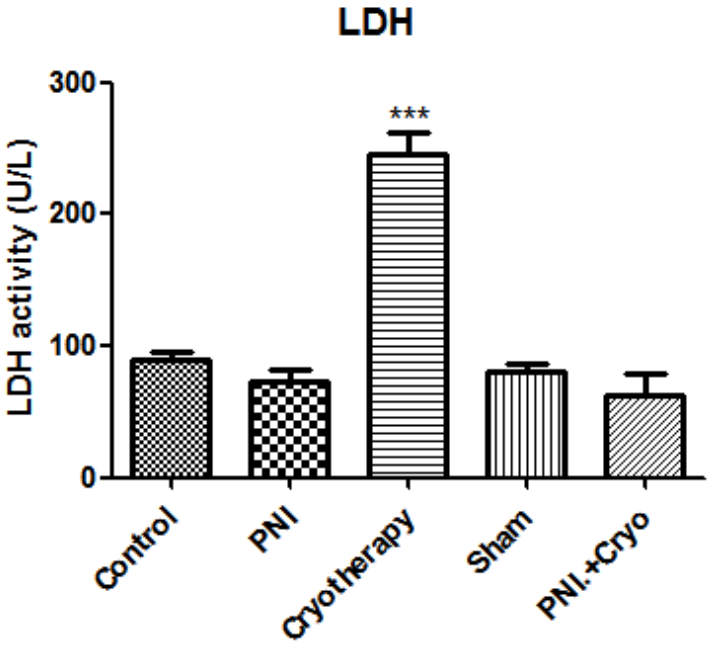

Fig 7

Acknowledgments

The authors express their gratitude to CAPES, CNPq, PPGRF-UFSM and PPGBTOX – UFSM.

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3. CONCLUSÃO

Ao planejar este estudo, objetivou-se preencher lacunas deixadas por pesquisas previamente realizadas. A maioria dos estudos que envolvem a crioterapia interrompem o tratamento durante a fase aguda após a lesão, e não temos clareza do que acontece funcionalmente e fisiologicamente após esse período. Os resultados deste estudo indicam que o modelo experimental de compressão nervosa em ratos determinou um comprometimento significativo do comportamento motor associado a mudanças significativas nos marcadores bioquímicos do estresse oxidativo. Notamos que a crioterapia durante um limiar de tempo maior pode ser um recurso de tratamento potencial para o tratamento da dor. Por essa razão, procuramos avançar o período de tratamento para 15 dias, a fim de estudar e aprofundar os efeitos que a crioterapia pode ter sobre o sistema nervoso periférico e muscular e compreender seu mecanismo de ação no tratamento da dor, aspectos funcionais e repercussões nos mecanismos bioquímicos.

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