UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

PAPEL DO ÓXIDO NÍTRICO NO REPARO MUSCULAR

TESE DE DOUTORADO

LIDIANE ISABEL FILIPPIN

PORTO ALEGRE, 2009.

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LIDIANE ISABEL FILIPPIN

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Tese de Doutorado com área de concentração em Reumatologia apresentada ao Programa de Pós-Graduação em Medicina: Ciências Médicas, para obtenção do grau de Doutor em Medicina.

Tese de Doutorado

PORTO ALEGRE, 2009.

AGRADECIMENTOS

Ao Programa de Pós-Graduação em Medicina: Ciências Médicas e a Universidade Federal do Rio Grande do Sul, pela possibilidade de obtenção deste título.

À equipe do Programa de Pós-Graduação em Medicina: Ciências Médicas pela disposição e auxílio.

Ao Professor Ricardo Xavier pela incansável dedicação, pelos valorosos conhecimentos transmitidos, pelas grandes oportunidades oferecidas e por acreditar no meu trabalho e no meu crescimento profissional.

À professora Norma Marroni pelo suporte ao longo de minha vida acadêmica, oportunidades e incentivo oferecidos.

À professora María José Cuevas pela oportunidade e dedicação.

Ao meu marido pelo amor, paciência dedicada em todos os momentos e pela compreensão nos momentos que estive ausente.

Aos meus amigos e colegas do Laboratório de Hepatologia Experimental e Fisiologia e do Laboratório de Biologia Molecular em Doenças Autoimunes e Infecciosas pela possibilidade da realização deste trabalho, pela amizade e companheirismo.

Aos meus pais pela confiança depositada em mim e pelo carinho em todos os momentos.

Aos funcionários do Laboratório de Experimentação Animal e Laboratório de Patologia do Hospital de Clínicas de Porto Alegre, pelo suporte para realização desta pesquisa.

À Drª Luise Meurer pela competência e tempo dedicado.

ABREVIATURAS E SIGLAS

- α	- Alfa
- ATP	- Adenosina tri-fosfato
- β	- Beta
- cGMP	- monofosfato gualinato-clicase
- $cPLA_2$	- Fosfolipase A_2 citosólica;
- Ca^{2+}	- Cálcio
- CAT	- Catalase
- CS	- Células satélites
- CK	- Creatina quinase
- COX_2	- Ciclooxiganase 2
- DNA	- Ácido desoxirribonucléico
- EAO	- Espécies ativas ao oxigênio
- EAN	- Espécies ativas de nitrogênio
- EGF	- Fator de crescimento epidérmico
- EDRF	- Fator de relaxamento derivado do endotélio
- Fe^{1+}	- Ferro
- GAPDH	- Gliceraldeído-3-fosfato desidrogenase
- GPx	- Enzima endógena glutationa
- GSSH/GHS	- Relação glutationa oxidada/reduzida
- HGF	- Fator de crescimento do hepatócito
- H_2O_2	- Peróxido de hidrogênio
- $HO^{\bullet}$	- Radical hidroxil

- IGF-1 - Fator de crescimento da insulina-1
- IκB - Inibidor kappa-B
- IKK - Inibidor kappa quinase
- INF-γ - Interferon gama
- IL - Interleucinas
- JMT - Junções miotendinosas
- L-arginina - Enzima precursora do óxido nítrico
- $_{L}$-NMMA - NG-monometil-L-arginina
- $_{L}$-NNA - NG-nitro-L-arginina
- $_{L}$-NAME - NG-nitro-L-arginina-metil-éster
- LAP - Proteína associada à latência
- LPS - Lipopolissacarídeos;
- LPO - Lipoperoxidação
- mRNA - Ácido Ribonucléico mensageiro
- MMP - Matrix da metaloproteinase
- MPO - Mieloperoxidase
- NF-κB - Fator de transcrição nuclear kappa-B
- NADPH - Nicotinamida-adenina-dinucleotídeo-fostato-hidrogênio
- NO - Óxido Nítrico
- NOS - Óxido Nítrico Sintase
- cNOS - Óxido Nítrico Sintase constitutiva
- eNOS - Óxido Nítrico Sintase endotelial
- iNOS - Óxido Nítrico Sintase induzível
- nNOS - Óxido Nítrico Sintase neuronal
- $O_2^{\bullet-}$ - Ânion superóxido

- O_2	- Oxigênio
- $ONOO^-$	- Peroxinitrito
- P	- Fosforilação
- PCNA	- Antígeno nuclear de proliferação celular
- $p<0,05$	- Nível de significância de 5%
- PAS	- *Periodic Acid Schift*
- Pax-7	- *Paired box protein*
- -SH	- grupamento sulfidrila
- SOD	- Superóxido dismutase
- TNF-α	- Fator de necrose tumoral alfa
- TGF-α/ TGF-β	- Fator de transformação do crescimento alfa/ beta
- Zn^{2+}	- Zinco

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RESUMO

Óxido nítrico (NO) é uma molécula que exerce uma multiplicidade de funções fisiológicas importantes que vêm sendo estudadas em diversos tecidos. No entanto, o papel do NO em processos fisiológicos e patológicos no músculo estriado é pouco conhecido, apesar de algumas evidências apontarem para uma função de regulação redox e interação com células satélites progenitoras.

Neste estudo avaliamos a participação do NO na regeneração muscular em um modelo de inflamação aguda *in vivo*. O trauma muscular foi induzido por um aparelho similar a uma prensa com impacto direto sobre o gastrocnêmio. Foram utilizados 40 ratos, Wistar, divididos em quatro grupos: *(i)* controle (CO); *(ii)* falso *(sham)* trauma; *(iii)* trauma; *(iv)* trauma com exposição ao nitro-$_L$-arginina metil éster ($_L$-NAME), um inibidor da sintase do óxido nítrico, em dois tempos experimentais: 24 horas e 7 dias após lesão. Vinte e quatro horas após o trauma, o músculo lesionado apresentava intensa vasodilatação e reação inflamatória na análise histológica, lipoperoxidação tecidual e estresse nitrosativo, aumento do mRNA das citocinas pró-inflamatórias (IL-1β, IL-6, iNOS), metaloproteinase-2, expressão proteica de iNOS e MMP-2, ativação do NF-κB e intensa atividade proliferativa. O tratamento com $_L$-NAME diminuiu significativamente os achados das alterações histológicas e moleculares em 24 horas após a lesão e, sete dias após o trauma, houve um aumento na expressão do TGF-β, na deposição de colágeno e proliferação celular. Entretanto, o grupo tratado com $_L$-NAME apresentou uma maior expressão de TGF-β e de deposição de colágeno quando comparado ao grupo trauma e proliferação

celular semelhante. Estes resultados indicam que a lesão muscular é associada com a ativação do sistema NO, o qual parece estar envolvido no equilíbrio entre os processos de regeneração e fibrose durante o reparo.

Palavras chaves: óxido nítrico, regeneração e reparo muscular

ABSTRACT

Nitric oxide (NO) is a molecule that carries a variety of important physiological functions that have been studied in various tissues. However, the role of NO in physiological and pathological processes in striated muscle is poorly known, although some evidence suggests a role of redox regulation and interaction with progenitor satellite cells. We evaluated the role of NO in muscle regeneration in a model of acute inflammation *in vivo*. The muscle trauma was induced by a device similar to a press with a direct impact on the gastrocnemius. A total of 40 rats were divided into four groups: *(i)* control (CO), *(ii)* sham trauma, *(iii)* trauma, *(iv)* trauma + $_{L}$-NAME, an inhibitor of nitric oxide synthase in two experimental phases: 24 hours and 7 days after injury. Twenty-four hours after the trauma, the injured muscle showed intense vasodilatation and inflammation in the histological analysis, tissue lipid peroxidation and nitrosative stress, activation of NF-κB, increased mRNA of pro-inflammatory, (IL-1β, IL-6, iNOS), metalloproteinase-2 and HGF, and increased total cell proliferation. The administration of the $_{L}$-NAME significantly reduced iNOS, MMP-2 and activation of NF-κB reduced the histological and molecular findings 24 hours after injury, and 7 days after injury, there was an increase in the expression of TGF-β, collagen deposition, and total cell proliferation. However, the $_{L}$-NAME group showed increased expression and collagen deposition when compared to the trauma and similar total cell proliferation. These results indicate that muscle injury is associated with activation of the NO, which seems to be involved in the balance between the processes of regeneration and fibrosis during the repair.

Key words: nitric oxide, satellite cells, muscle repair.

SUMÁRIO

1 INTRODUÇÃO

Lesões musculares traumáticas, incluindo lacerações, contusões e estiramentos são relativamente frequentes. Quando ocorrem, podem ter uma implicação determinante na capacidade funcional, frequentemente apresentam recuperação incompleta, demorada e/ou inapropriada com tendência a formação de tecido cicatricial e possibilidade de novas lesões [1, 2].

A cicatrização do músculo ocorre em três fases sobrepostas: *(i)* degeneração e inflamação, *(ii)* regeneração muscular, e *(iii)* fibrose [3]. Simultaneamente à lesão ocorre infiltração do músculo por neutrófilos e macrófagos e, na sequência, ativação da nicotinamida-adenina-dinucleotídeo-fostato-hidrogênio (NADPH) oxidase, uma importante enzima do estresse oxidativo [4, 5]. O processo de regeneração muscular compreende a ativação das células satélites (CS), que têm características progenitoras miofibrilares e se encontram em estado quiescente no músculo esquelético adulto entre a lâmina basal e o sarcolema [6]. Quando o músculo esquelético é estirado ou contundido, essas células entram em ciclo celular, com subsequente divisão, diferenciação e fusão com as fibras musculares para reparar e promover a hipertrofia das fibras musculares [7, 8]. Esse processo de ativação e diferenciação depende da presença de óxido nítrico (NO), metaloproteinases (MMP) ativadas e da liberação do fator de crescimento de hepatócito (HGF) da matriz extracelular [8, 9]. A formação de um tecido cicatricial fibroso, não funcional, também pode ocorrer, e parece ser dependente, pelo menos em parte, da expressão do fator de transformação do crescimento (TGF-β). Essa expressão também pode ser regulada pelos níveis de NO [10].

Assim, a regeneração muscular depende do tipo e da gravidade da lesão, além de adequada resposta inflamatória e equilíbrio nos processos de regeneração e fibrose. Evidências de estudos *in vitro* indicam que em todas estas fases o NO exerce um papel significativo [8, 9, 11-15]. A melhor compreensão do seu papel, bem como da participação de outros mediadores importantes, podem levar ao desenvolvimento de novas estratégias de tratamento. Estudos *in vivo* envolvendo a manipulação da produção de NO em modelos animais de trauma muscular nos permitiria, de maneira original, analisar melhor o papel desse mediador na resposta inflamatória inicial, no equilíbrio entre os processos de regeneração e fibrose e na ativação das CS.

A tese é apresentada em seções. Após a seção de introdução e busca da informação, um e dois, respectivamente, o leitor terá acesso à revisão da literatura, na terceira seção. Neste será abordado à estrutura do tecido muscular esquelético, estruturas microscópicas e função. Em seguida, será descrito alguns mecanismos de lesão muscular mais frequentes e as fases do reparo muscular (fase de inflamação, regeneração e fibrose).

Na quarta seção é abordada a justificativa do trabalho. Na seção seguinte, são apresentados os objetivos do estudo, geral e específico. Logo após, as referências bibliográficas utilizadas para a revisão da literatura são disponibilizadas.

A partir da sétima seção são apresentados os artigos científicos, esses foram divididos em três seguimentos. O primeiro artigo intitulado *Nitric Oxide and Repair of Skeletal Muscle Injury,* apresentada uma revisão da literatura sobre o assunto. O segundo, intitulado *The role of nitric oxide during the healing of trauma to the skeletal*

muscle, apresenta dados originais onde é discutido o impacto da inibição da óxido nítrico sintase pela administração do $_{L}$-NAME (inibidor não específico da NOS) sobre o processo inflamatório. Por fim, no terceiro artigo, é discutido o impacto da inibição da óxido nítrico sintase pela administração do $_{L}$-NAME sobre a regeneraçao muscular e a ativação das células satélites.

2 BUSCA DA INFORMAÇÃO

A revisão bibliográfica foi realizada nas bases de dados da web PUBMED, MEDLINE, SCIENCE DIRECT, SCIELO bem como livros-textos, dissertações, teses e artigos contidos em suas referências.

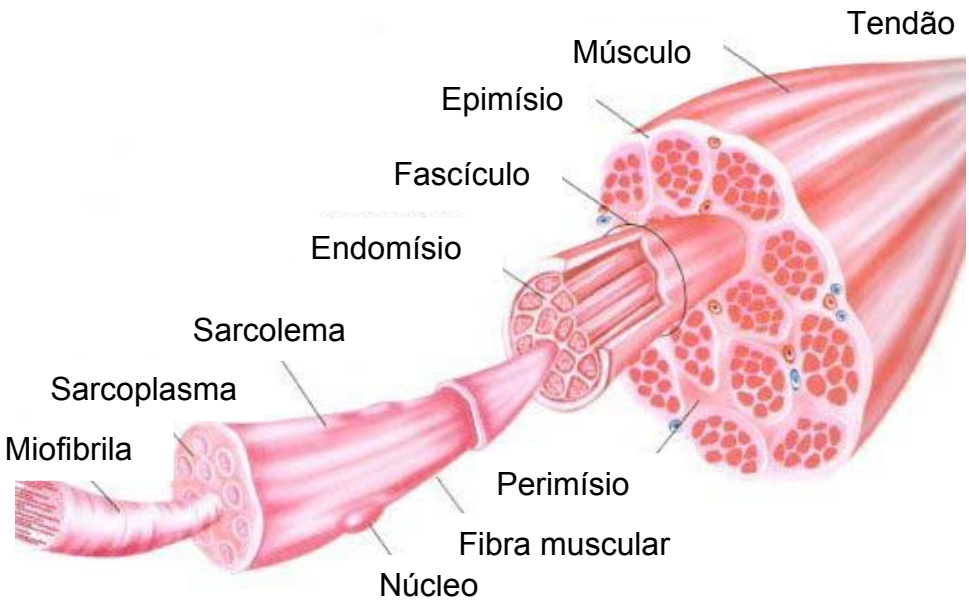
Usando o termo “nitric oxide and skeletal muscle” foram encontrados 1999 artigos na internet. Usando “nitric oxide and muscle repair” foram encontrados 142 artigos. Restringindo para “nitric oxide and skeletal muscle repair and satellite cell” foram encontrados 8 artigos.

3 REVISAO DA LITERATURA

3.1 Tecido Muscular Esquelético

O músculo estriado esquelético é responsável pela autonomia motora do indivíduo, ou seja, fornece sustentação e movimentação corporal. É essencialmente composto de miofibras, responsáveis pela função contrátil do músculo, e tecido conjuntivo, que fornece a sustentação. As células musculares são longas, sinciciais e multinucleadas. Os núcleos são localizados na periferia das células. Alguns núcleos que parecem pertencer à célula muscular podem ser, na verdade, CS. Essas se localizam entre a lâmina basal e o sarcolema e são as células-fonte latentes que podem proliferar e regenerar o músculo após dano tecidual, formando novos mioblastos [16-18].

As miofibras são circundadas e ligadas entre si por um tecido conjuntivo frouxo que contém vasos capilares chamado endomísio. Os feixes de miofibras, ou fascículos, são circundados pelo perimísio, o qual possui vasos sanguíneo maiores. Por fim, um conjunto de fascículos, ou músculo, é circundado por um tecido conjuntivo mais denso chamado epimísio [19]. As extremidades das miofibras são ligadas por um tecido conjuntivo tendinoso, chamado junções miotendinosas (JMT), cuja função é fixar o músculo ao osso (figura 1) [18, 20].



Fonte: www.sport-fitness-advisor.com/muscle-anatomy.html Acesso em: 01 novembro de 2009.

Figura 1- Estrutura do músculo esquelético.

Os músculos esqueléticos são constituídos por uma população heterogênea de fibras com propriedades variáveis de resistência à fadiga e velocidade de contração, podendo ajustar-se eficazmente a diferentes demandas [21].

As fibras musculares são classificadas por diferentes métodos: análise histoquímica (hematoxilina-eosina [HE], *Periodic Acid Schift* [PAS], identificação da isoforma de miosina) e identificação bioquímica de enzimas metabólicas [22] (figura 2).

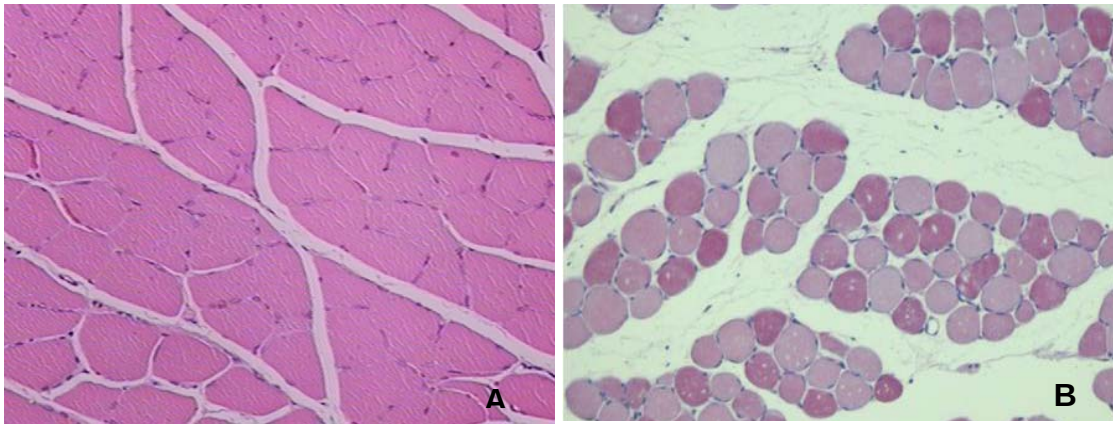


Figura 2 - Distribuição das fibras musculares esqueléticas.

Na figura 2A - fotomicrografia na colocação de HE com preservação dos contornos celulares e núcleos na periferia. A figura 2B – fotomicrografia na colocação PAS onde se observa a diferenciação dos tipos de fibras musculares (tipo I corado em rosa claro; tipo II: corado em rosa escuro). Aumento de 200X. (Filippin, 2009).

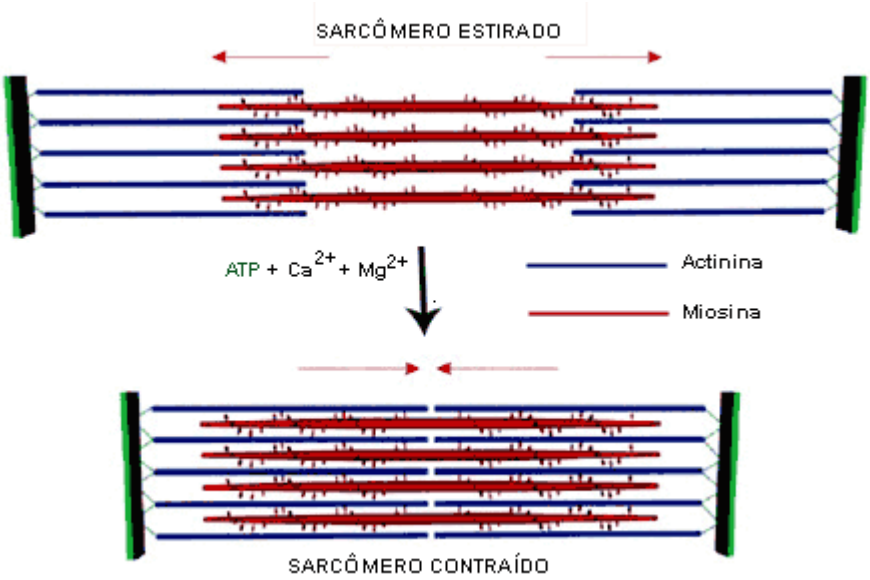
Existem descritos dois tipos de fibras: tipo I e tipo II. As fibras do tipo I, também chamadas fibras vermelhas, de contração lenta, possuem maior resistência à fadiga, maior número de mitocôndrias e maior número de capilares, produzindo energia de forma aeróbica. As fibras do tipo II se subdividem em IIa e IIb. As fibras do tipo IIa, ou fibras brancas, contraem-se rapidamente, possuem menor resistência, menor número de mitocôndrias e vasos sanguíneos e maior armazenamento de glicogênio, produzindo energia de forma anaeróbica. As fibras do tipo IIb, possuem características intermediárias entre as fibras do tipo I e IIa, com baixa capacidade glicolítica e uma grande capacidade oxidativa [17, 18]. Além dessas, existe a fibra do tipo IIc, que são consideradas um tipo de fibra não diferenciada ou fetal (imatura), que está presente em processos de regeneração ou degeneração muscular [23, 24].

3.2 Estruturas microscópicas e funcionais do Músculo Esquelético

O músculo esquelético é um tecido contrátil e está sob controle voluntário. Cada célula muscular esquelética é inervada por um motoneurônio. Os potenciais de ação se propagam ao longo do axônio deste motoneurônio, levando à liberação de acetilcolina na fenda sináptica, despolarização da placa motora, geração de potenciais de ação e subsequente contração desta fibra muscular [25].

Observado por microscopia óptica, o músculo esquelético possui miofibrilas que contêm bandas claras e escuras que se repetem regularmente ao longo de toda fibra. O sarcômero é a unidade contrátil, e são essas unidades repetitivas que fornecem o aspecto estriado ao tecido. Cada miofibrila contêm filamentos grossos (miosina) e finos (actina, tropomiosina e troponina) [26].

Durante a contração os filamentos de actina deslizam sobre os filamentos de miosina a partir da formação das pontes cruzadas dos filamentos de miosina com os de actina em um movimento dependente de Ca^{2+} e ATP, o que provoca o encurtamento da fibra [27] (figura 3).



Fonte:http://www.passeiweb.com/na_ponta_lingua/sala_de_aula/biologia/imagens/sarcomero_est_contr.gif Acesso em: 01 de novembro de 2009.

Figura 3 - Microestrutura do músculo esquelético.

3.3 Mecanismos de lesão muscular

Lesões musculares são frequentes, tanto em atividades desportivas como laborais. Mais de 30% das consultas médicas em emergências podem estar relacionadas à musculatura esquelética [13]. Lesões musculares podem ser causadas por contusão, estiramento, laceração ou por esforços repetitivos. Se a unidade musculotendinosa for mecanicamente alterada em decorrência de lesões parciais ou completas, a unidade pode não mais gerar as forças necessárias para a realização de movimentos simples [28]. A contusão ocorre quando o músculo é

exposto a uma súbita força de compressão. Este tipo de trauma muscular é frequente em esportes de contato, enquanto atividades de corrida e saltos são comumente associadas à lesão por estiramento [14]. As lesões por esforços repetitivos são comuns em atividades laborais específicas. Os músculos biarticulares, como reto femoral, semitendinoso e os músculos gastrocnêmios, são mais suscetíveis às lesões bruscas [15, 16].

3.4 Reparo Muscular

3.4.1 *Destruição e Inflamação Muscular*

Para facilitar o estudo, a cicatrização da lesão muscular pode ser dividida em três fases distintas que se sobrepõem. A fase de destruição é caracterizada pela ruptura e subsequente necrose de fibras musculares, com formação de hematoma, e reação de células inflamatórias. A fase da regeneração consiste na fagocitose do tecido necrosado, ativação das células satélites, com reparo e remodelamento das fibras musculares lesadas. Por fim, ocorre a fase de fibrose, período no qual ocorre a reorganização do tecido cicatricial em áreas onde a regeneração muscular não efetiva [2, 3].

Quando o músculo esquelético sofre uma lesão ocorre ruptura local ou adjacente ao local do impacto, ou ainda próximo às JMT [29]. Esse evento é iniciado geralmente pela ruptura do sarcolema, resultando em aumento da permeabilidade da miofibra. A ruptura da miofibra se reflete pelo aumento dos níveis séricos da proteína muscular creatina quinase (CK). Estas miofibrilas sofrem necrose por

autodigestão mediada por proteases intrínsecas [30]. Edema local e formação de hematoma ocorrem rapidamente após a lesão muscular [31]. Posteriormente, a área de necrose é invadida por pequenos vasos sanguíneos e células mononucleares, macrófagos ativados e linfócitos T, que produzem várias citocinas e fatores de crescimento. A expressão de substâncias como moléculas de adesão (P-selectina, L-selectina e E-selectina) e citocinas (interleucinas [IL]-8, IL-6, IL-1] e fator de necrose tumoral-α [TNF-α]) influenciam o fluxo sanguíneo local e permeabilidade vascular, acelerando a resposta inflamatória [3].

Adicionalmente, outros mediadores são produzidos e liberados, como fator de crescimento da insulina-1 (IGF-1), fator de crescimento do hepatócito (HGF), fator de crescimento epidérmico (EGF) e fatores de transformação do crescimento (TGF-α e TGF-β), os quais regulam a proliferação e diferenciação de mioblastos e irão promover a regeneração muscular [3, 32-35].

A fase inflamatória ainda está associada ao *burst* respiratório via ativação da NADPH oxidase pelos macrófagos, com excessiva liberação de radicais livres e espécies ativas de oxigênio (EAO) [4, 5, 36].

Em geral, a excessiva formação endógena de radicais livres pode ser oriunda da *(i)* ativação aumentada de fagócitos; *(ii)* interrupção dos processos normais de transferência de elétrons na cadeia respiratória mitocondrial; *(iii)* aumento da concentração de íons metálicos de transição por escape do grupamento heme de proteínas em locais de lesão ou doenças metabólicas, e *(iv)* por níveis diminuídos das defesas antioxidantes [4, 37, 38]. As principais EAO conhecidas são: ânion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2) e radical hidroxil ($HO^{\bullet}$).

$O_2^{\bullet-}$ é convertido em H_2O_2 espontaneamente e essa reação pode ser catalisada pela enzima superóxido dismutase (SOD) [36]. Quando formado o $HO^{\bullet}$ rapidamente reage com a molécula mais próxima, que pode ser com lipídeos, proteínas ou bases de DNA. Esse radical livre não possui nenhuma enzima capaz de fazer sua dismutação e é considerado, portanto, um radical altamente reativo [39].

O estresse oxidativo é definido como um desequilíbrio entre pró-oxidantes e antioxidantes em favor do pró-oxidante, levando ao dano potencial [37]. O mecanismo redox tem mostrado influência na sinalização intracelular, e as células parecem ser muito sensíveis à perda destes sistemas de regulação e controle. Estes dois conceitos têm sido recentemente incorporados a uma nova definição do estresse oxidativo como "um desequilíbrio entre oxidantes e antioxidantes em favor dos oxidantes, levando a uma ruptura de redox de sinalização e controle e/ou danos moleculares" [40-42].

Adicionalmente, o $O_2^{\bullet-}$ pode reagir com NO formando o peroxinitrito ($ONOO^-$), uma espécie reativa de nitrogênio (EAN). A adição de $ONOO^-$ às células, tecidos e fluidos corporais, leva à rápida protonação, podendo resultar em: depleção de grupos -SH e outros antioxidantes [4, 39]. Neste sentido, $ONOO^-$ tem sido considerado um importante marcador do estresse nitrosativo em doenças humanas [39].

$ONOO^-$ é capaz de alterar o estado redox da célula levando à ativação do fator de transcrição nuclear kappa-B (NF-κB) [43], através da fosforilação do inibidor kappa quinase (IKK) e subsequente fosforilação do inibidor kappa-B (IκB),

possibilitando a translocação do NF-κB para dentro do núcleo, levando à transcrição de diversos mediadores inflamatórios (figura 4) [44].

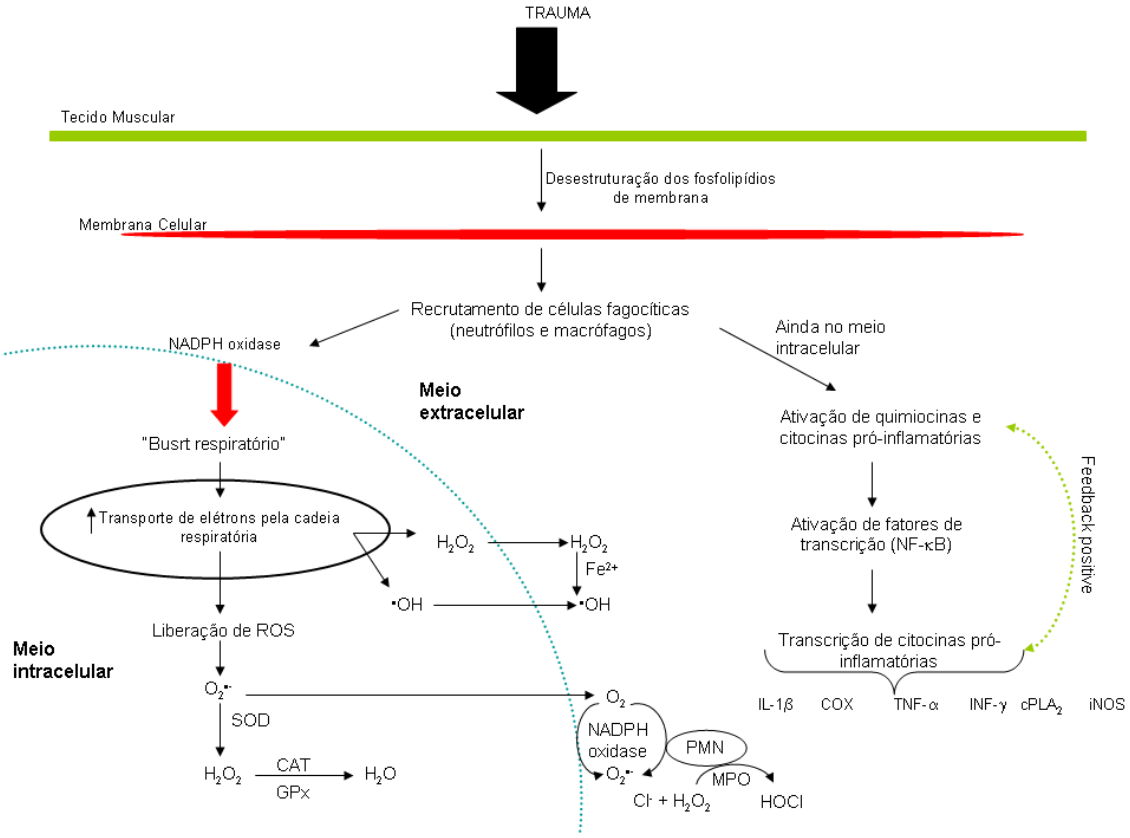


Figura 4 - Formação das espécies ativas de oxigênio e nitrogênio e ativação do NF-κB (Filippin, 2009).

Se por um lado altas concentrações de EAO estão envolvidas na ativação do NF-κB, com a manutenção do processo inflamatório [45], atuando como agente causal de dano a macromoléculas, por outro se sugere que baixas concentrações de EAO participam na regulação de diferentes funções nas células eucarióticas, tais como na proliferação, biosíntese de hormônios, quimiotaxia, apoptose e outras [38].

O desequilíbrio no estado redox tem efeitos potencialmente deletérios sobre a biologia celular. Por isso, existem vários mecanismos antioxidantes, endógenos e exógenos, envolvidos na proteção de células [46, 47].

Pierce *et al*. [48] demonstraram em experimentos *in vitro* que a CK e gliceraldeído-3-fosfato desidrogenase (GAPDH), importantes enzimas no metabolismo energético, podem ter sua conformação alterada pelo estresse oxidativo, pois ambas as proteínas contêm cisteínas altamente reativas em seus sítios ativos e são sensíveis ao estresse oxidativo. Em 2007, este mesmo grupo, estudou essas proteínas em modelo experimental de inflamação por injeção intramuscular de cardiotoxina. Os autores concluíram que as proteínas musculares estavam funcional e estruturalmente alteradas via geração de EAO produzidas durante o processo inflamatório após a lesão muscular, e essas alterações foram irreversíveis [49].

Lesões por isquemia e reperfusão no músculo esquelético ocorrem frequentemente em consequência de eventos vasculares, devido ao excesso na produção de EAO, por diminuição nas defesas antioxidantes, ou ambos. No entanto, se as EAO são geradas durante a hipóxia ou na fase de reoxigenação ainda é bastante controverso na literatura [50].

Também existem relatos demonstrando que as EAN têm papel importante na manutenção da reação inflamatória persistente e destruição óssea e articular em pacientes com doenças inflamatórias crônicas com características autoimunes [51]. A formação de 3-nitrotirosina é um marcador relativamente específico de dano nitrativo mediado pelo NO. Khan *et al*. [51] avaliaram o líquido sinovial de 60

pacientes com diagnóstico de artrite reumatóide, osteoartrite ou lúpus eritematoso sistêmico, e concluíram que os radicais livres de nitrogênio podem produzir inflamação e que os níveis de 3-nitrotirosina são correlacionados com a atividade da doença.

3.4.2 *Regeneração e Reparo Muscular*

Na fase de remodelamento ocorre a contração e reorganização do tecido cicatricial e a recuperação da capacidade funcional do músculo. A recuperação da função contrátil depende do equilíbrio entre estes dois processos: regeneração e fibrose [3] (figura 5).

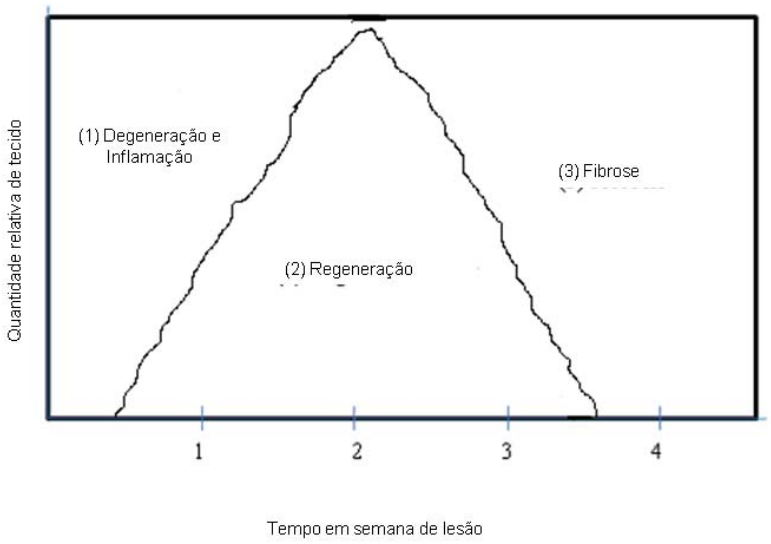


Figura 5 - Estágios de regeneração muscular após-lesão (Filippin *et al.*[52])

A principal célula envolvida no processo de regeneração do músculo esquelético é a célula satélite.

3.4.2.1 Células Satélites

Foram primeiramente descritas em 1961 em fibras musculares de rã por Alexandre Mauro como mioblastos “adormecidos”, que não sofreram total desenvolvimento embrionário, mas que são capazes de retomar a miogênese em resposta a uma lesão muscular [53].

São células progenitoras mononucleares encontradas no músculo maduro entre a lâmina basal e o sarcolema. Normalmente quiescentes, podem ser ativadas em resposta a estímulos de crescimento, remodelamento ou lesão muscular [6, 7]. Na ativação, elas entram no ciclo celular, se dividem, se diferenciam e se fundem com as fibras musculares para reparar as regiões danificadas e/ou para aumentar a hipertrofia das fibras musculares [8].

Há evidências de que as CS são uma população bastante heterogênea, visto que algumas podem sofrer diferenciação imediata, enquanto outras primeiramente proliferam, gerando uma célula filha para diferenciação e outra para futura proliferação [54].

Existem alguns mecanismos tentando explicar a ativação de CS musculares após um trauma. Alguns pesquisadores têm proposto como um desses mecanismos a interrupção da integridade do sarcolema e a lâmina basal, levando a estresse mecânico das CS e sua ativação [55]. Outros sustentam que citocinas liberadas pelas células inflamatórias induziriam a ativação das CS [2]. O mais provável é que exista uma complexa combinação desses e de outros eventos, onde o NO parece ter um papel importante [12].

O padrão de expressão gênica das CS ainda é pouco conhecido, tanto no estado de quiescência, como no estado ativado. Os marcadores destas células são distintos na sua expressão sob dois aspectos: *(i)* condição da CS (quiescente ou ativada); *(ii)* fases do ciclo celular que se encontra após sua ativação. São marcadores conhecidos das CS: Pax-7, antígeno nuclear de proliferação celular (PCNA), Ki-67, MyoD, miogenina, M-caderina e CD-34 [56].

Pax-7 é o marcador que vem sendo empregado mais comumente. Trata-se de um membro da família de fatores de transcrição "*paired box*" que desenvolve papel regulatório do desenvolvimento fetal e no crescimento do câncer. A função específica do gene pax-7 é desconhecida, mas especula-se seu envolvimento na supressão de tumores. Modelos experimentais de lesões musculares têm estudado, ainda muito limitadamente, o papel da proteína pax-7. Seale, *et al.*[57] demonstraram que a proteína pax-7 é expressa exclusivamente em mioblastos primários em proliferação. Além disso, a pax-7 não foi expressa em linhagens não-musculares. Portanto, em camundongos adultos, a expressão de pax-7 parece ser específica para linhagem de CS miogênicas.

O PCNA, marcador também empregado com freqüência, é uma proteína cuja síntese ocorre no início das fases G1 e S do ciclo celular. Embora inespecífico, é um excelente marcador para células em proliferação, inclusive as CS [58].

A ativação das CS e regeneração do tecido muscular são influenciadas por interações intercelulares, com a matriz extracelular e fatores de crescimento secretados. Nos últimos anos, alguns trabalhos têm demonstrado a importância das metaloproteinases (MMP) e do fator crescimento do hepatócito.

3.4.2.2 Metaloproteinases

Orquestram o remodelamento da matriz extracelular do músculo esquelético pós-lesão. São endopeptidases dependentes de íons Zn^{+2} e/ou Ca^{+2}, com estrutura molecular composta de pelo menos três domínios: um pro-peptídeo que mantém a latência da proteína por possuir uma conformação específica entre cisteínas e um íon Zn^{+2}, um sítio catalítico e um domínio catalítico que está ligado a um domínio C-terminal [59]. A expressão e atividade das MMPs são reguladas no nível de transcrição por citocinas e fatores de crescimento e, no nível de pós-tradução, pela secreção de enzimas latentes (pré-pró-MMP) e ativação de zimogênios (pró-MMP) por integrinas e proteases presentes tanto no meio extracelular como as associadas à membrana celular [60]. Existe um balanço delicado entre a produção endógena de inibidores teciduais TIMPs (inibidores de MMP no tecido) e a produção de MMP no microambiente, determinando o remodelamento fisiológico ou destruição patológica do tecido [61].

As MMP-9 (gelatinase A) e MMP-2 (gelatinase B) constituem as principais metaloproteinases envolvidas no reparo do tecido muscular [62]. A MMP-9 está associada com a migração de células inflamatórias e possivelmente à ativação de CS, enquanto a MMP-2 é constitutiva do tecido muscular, mas está aumentada durante o processo de regeneração muscular [63]. A expressão de MMP-2 regula a integridade e composição da matriz extracelular, e também a proliferação e diferenciação de mioblastos [62]. A participação de MMP-2 durante a fusão das CS com as fibras musculares lesadas deve-se em parte à degradação de colágeno tipo IV, como também outros componentes da membrana basal, como a entactina [63].

3.4.3.3 Fator Crescimento do Hepatócito

É uma glicoproteína com múltiplas funções, inicialmente descrita como um potente mitógeno para hepatócitos maduros [64]. Atualmente sabe-se que este fator de crescimento regula a mitogênese, motilidade celular, além da morfogênese em distintas células, ativando uma cascata de sinalização por tirosina quinase após ligar-se ao receptor proto-oncogênico c-Met [65]. HGF é secretado como um polipeptídeo inativo que é clivado por serina-proteases em uma cadeia alfa (α) e beta (β). Uma ligação dissulfeto entre as cadeias α e β produz a molécula heterodimérica ativa [66, 67].

Allen *et al.* [66], Tatsumi *et al*. [68], Sheehan *et al*. [69], através de estudos *in vitro,* demonstraram que o HGF pode ser liberado a partir da matriz do músculo lesado e tem a capacidade de ativar a divisão precoce de CS adultas em cultura e no tecido muscular. Estes autores também demonstraram que mRNA do HGF é expresso em CS maduras e podem atuar de forma autócrina (figura 6). Assim, a regulação da função do HGF no músculo pode ocorrer tanto em um nível de transcrição, quanto pelo seu sequestro na matriz tecidual devido a sua alta afinidade de ligação aos proteoglicanos heparan sulfato [66].

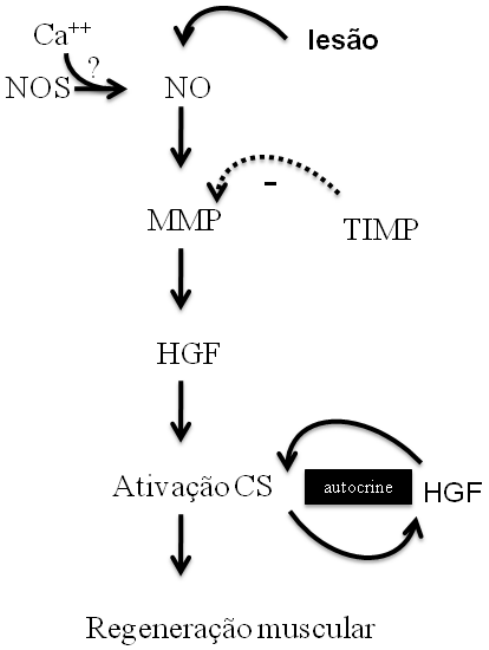


Figura 6 - Ativação das células satélites no músculo esquelético dependente de óxido nítrico (Filippin *et al.* [52]).

3.4.3 *Fibrose Muscular*

A formação de tecido cicatricial pode levar à substituição das fibras musculares por fibrose, com prejuízo da função contrátil. A fibrose muscular é uma superprodução de componentes da matriz extracelular que inicia aproximadamente duas semanas após a lesão e progride até a 4° semana. Fatores que influenciam o equilíbrio entre fibrose e regeneração não são bem compreendidos, e podem incluir a intensidade da resposta inflamatória aguda, expressão dos diversos fatores de

crescimento e citocinas, em especial o TGF-β, presentes no local da lesão, e ativação das CS (figura 7) [10].

TGF-β é uma citocina produzida principalmente por fibroblastos, algumas células epiteliais e macrófagos. Acredita-se que desempenhe um papel crucial em doenças fibróticas [10].

TGF-β1 foi identificado pela primeira vez em plaquetas humanas como uma proteína com massa molecular de 25 kDa. Existem três isoformas de TGF-β em mamíferos – TGF- β1, - β2 e β3, contudo a fibrose tecidual é atribuída a isoforma β1 [70]. É armazenado na forma inativa, como um homodímero, não covalentemente acoplado a proteína associada à latência (LAP). A ligação da citocina aos seus receptores requer a dissociação do LAP, um processo catalisado por vários agentes, entre eles plasminogênio, trombospondina e MMPs [71].

In vitro, o TGF-β é um potente quimioatraente para fibroblastos que estimulam a síntese de colágeno e fibronectina [3]. No músculo, a cascata fibrogênica pode ser iniciada por TGF-β1. Li *et al*. [71] demonstraram em estudo *in vitro* (células C2C12) que a estimulação de TGF-β1 pode induzir mioblastos a expressar TGF-β1 de forma autócrina e suprimir a expressão de proteínas musculares, com o respectivo aumento na produção de proteínas relacionadas à fibrose.

Também se acredita que o TGF-β inibe a diferenciação das células miogênicas e impede a expressão do MyoD, fator de transcrição que controla a diferenciação de células da linhagem miogênica [72]. Portanto, este fator de crescimento pode ativar cascatas fibróticas e desencadear a diferenciação de

mioblastos em miofibroblastos. Esses são responsáveis pela retração das células musculares lesionadas, e esse efeito pode ser inibido via administração de decorina, um inibidor do TGF-β1 [71].

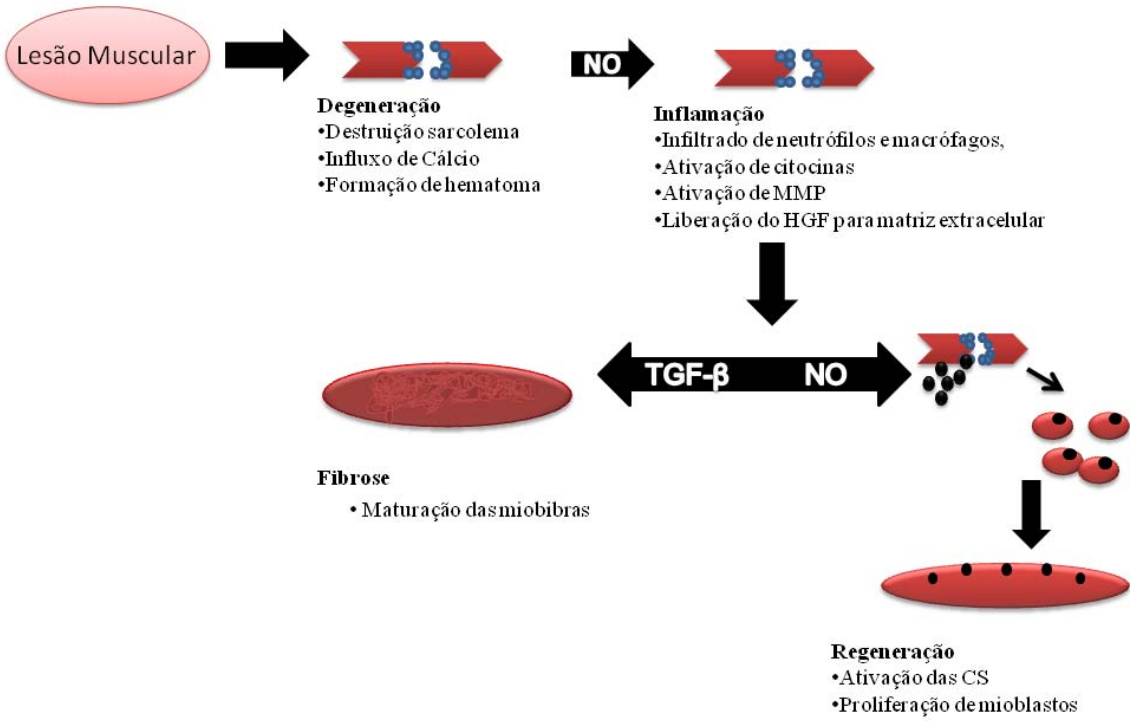


Figura 7 – Fase de regeneração muscular (Filippin *et al.* [52]).

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3.5 Óxido Nítrico na Cicatrização Muscular

Em 1980, Furchogott *et al*. [73] demonstraram que o relaxamento vascular induzido por acetilcolina era dependente da presença do endotélio e evidenciaram que este efeito era mediado por um fator humoral lábil, mais tarde conhecido como fator de relaxamento dependente do endotélio (EDRF). Em 1987 foi demonstrado que esse fator de relaxamento derivado do endotélio era um radical livre, o NO.

NO pode ser um oxidante ou um redutor dependendo do meio em que ele está e é rapidamente oxidado pelo O_2, produzindo nitrito e nitrato. Possui o menor peso molecular de qualquer produto de secreção celular de mamíferos; sua meia-vida é curta e a especificidade de suas reações é mínima. O NO é citotóxico e vasodilatador e modula reações inflamatórias ou antiinflamatórias, dependendo do tipo celular e do estímulo [74, 75].

A molécula do NO tem um elétron não pareado e reage facilmente com oxigênio, radical superóxido, ou metais de transição, como ferro. O NO tem alta afinidade com o grupamento heme, encontrado em proteínas intracelulares (óxido nítricosintase, cicloxigenase e guanilato ciclase) e também se liga a grupos - SH, formando tiol [75-77].

NO é produzido a partir da L-arginina, que inicialmente é transformada em um intermediário, a NG-hidroxi-L-arginina com a presença de NADPH e Ca^{2+}. Por fim, em presença de mais NADPH e O_2 há formação de L-citrulina e NO [78]. A L-arginina é um aminoácido semi-essencial produzido no organismo, porém em quantidade insuficiente para todas as necessidades. Além do ciclo da uréia, a

arginina é utilizada na síntese de creatinina e fornece ornitina para a síntese de poliaminas [79]. A síntese de NO pode ser inibida por análogos inibidores da L-arginina, tais como NG-monometil-L-arginina (L-NMMA), NG-nitro-L-arginina (L-NNA) e NG-nitro-L-arginina-metil-éster (L-NAME). Estes inibidores têm grande importância na pesquisa dos prováveis efeitos do NO nos tecidos [80].

Diversas células produzem NO, dentre elas, as células do músculo esquelético e cardíaco, células epiteliais, células endoteliais, macrófagos, fibroblastos e hepatócitos [81], através de uma reação catalisada pela enzima óxido nítrico sintase (NOS) [82, 83]. Três isoformas de NOS foram caracterizadas, cada uma codificada por diferentes cromossomos. Duas isoformas da enzima são expressas constitutivamente (eNOS-endotelial e neuronal, nNOS), enquanto que uma isoforma é induzível (iNOS), inicialmente descrita nos macrófagos [82].

O NO é sintetizado pela ativação da cNOS basal (em células endoteliais vasculares e neurônios) segundos a minutos após o aumento na concentração de Ca^{2+} em resposta à ativação de receptores da superfície celular e mecanismos de transdução de sinal. A cNOS apresenta forma monomérica, é dependente do sistema cálcio-calmodulina, mas é expressa continuamente na ausência de agentes indutores, com síntese basal de concentração picomolar [78].

Ao contrário, a iNOS não depende de cálcio para ativação, mas a síntese *de novo* de mRNA da iNOS é necessária para sua atividade, não sendo detectável em condições basais. LPS ou endotoxinas bacterianas, junto com citocinas, como TNF-α, IL-1β ou interferon gama (IFN-γ), induzem a síntese de iNOS, de 2 a 4 horas após

a exposição ao agente. É encontrada sob forma de monômero e tetrâmero com peso molecular de 130 kd [78].

NO tem demonstrado ser um modulador da função do músculo esquelético, e um provável mediador de lesões e doença [84]. Atividade NOS em músculo esquelético de ratos tem sido correlacionada com a densidade da fibra muscular. Kobzik *et al.* [85], observaram que a função contrátil do músculo esquelético foi prejudicada por bloqueadores de NOS e inibiu a atividade da guanilato ciclase. Esses eventos foram diminuídos por doadores de NO e com aumento dos níveis de GMP cíclico. Esses resultados suportam duas funções fisiológicas do NO no músculo esquelético: 1°) promover o relaxamento através da cGMP; 2°) modular o aumento da contração.

NO também está relacionado com o desenvolvimento da força [86]. NO é sintetizado no músculo esquelético pela nNOS, que está localizada ao sarcolema das fibras de contração rápida, ligado a α1-syntrophin, proteína associada a distrofina [30]. A ausência de distrofina, devido a uma mutação no gene para a proteína, causa um secundária perda de nNOS no sarcolema, levando à inflamação do músculo, lise da membrana muscular, perda de massa muscular e morte. Isso ocorre em pacientes com distrofia muscular de Duchenne e em modelo animal da doença (ratos mdx) onde o NO demonstra um importante efeito protetor [78].

Além disso, o NO tem papel na regulação do diâmetro dos vasos sanguíneos e a circulação sanguínea [79], modulação do tônus vascular, bem como propriedades contráteis musculares. A produção de NO pelas células endoteliais vasculares mantém um tônus vascular; as plaquetas também produzem NO, o qual

modula sua agregabilidade; e, no sistema nervoso central o NO medeia os efeitos dos aminoácidos excitatórios [79].

Em todos estes tecidos o NO atua como um mecanismo de transdução de guanilato ciclase solúvel, na qual não é o estimulador endógeno. NO também é liberado após a estimulação imunológica pelos macrófagos, neutrófilos e outras células. NO age assim, lançado como parte do mecanismo de defesa do hospedeiro, pois é citotóxico para as células tumorais e organismos invasores e podem mediar outros aspectos da resposta imunológica [79].

Assim, NO é uma molécula sinalizadora no músculo esquelético. No entanto, existem poucos estudos investigando o papel do NO na inflamação pós-lesão muscular e de reparação.

4 JUSTIFICATIVA DA PESQUISA

Óxido nítrico desempenha um papel importante durante a resposta inflamatória, contribuindo tanto com ações pró-inflamatórias, quanto com ações estimuladoras de resolução do processo. Os eventos moleculares que ocorrem na lesão e regeneração do músculo esquelético ainda são pobremente entendidos. Para isso o uso de modelos experimentais de inflamação no sistema osteomuscular, comumente empregados para avançar nosso conhecimento sobre a sinalização intracelular e sobre a resposta de reparo, apresentam-se como uma ferramenta interessante para uma melhor compreensão destes eventos. Estudar o efeito da administração de agentes inibitórios da produção de óxido nítrico, especificamente o $_{L}$-NAME, sobre o balanço oxidativo tecidual, com atenção especial na influência dessa inibição na resolução ou cronificação da resposta inflamatória e proliferação celular é fundamental. Uma melhor compreensão desse processo seria importante para desenvolvimento futuro de terapias moduladoras da resposta inflamatória aguda, como em casos de trauma muscular, objeto desse projeto, visando otimizar a recuperação e evitar cicatrização com predomínio de fibrose, com comprometimento funcional.

5 OBJETIVOS DO ESTUDO

5.1 Objetivo geral

- Avaliar o impacto da inibição da produção de óxido nítrico com administração do $_{L}$-NAME no reparo do músculo estriado em modelo murino de trauma muscular.

5.2 Objetivos específicos

- Avaliar o efeito da administração de $_{L}$-NAME sobre:

  - o infiltrado inflamatório do músculo traumatizado, através de exame histopatológico semiquantitativo, em diferentes períodos após o trauma;

  - a fibrose através de exame histopatológico semiquantitativo e por exame de imagem com auxílio de software (Adobe Photoshop CS3 Extended versão 10.0 [Adobe Systems Inc, San Jose, CA]);

  - a lipoperoxidação tecidual, a atividade da enzima antioxidante superóxido dismutase, através de testes bioquímicos;
  - a ativação do NF-κB, por técnica de EMSA;
  - a expressão do mRNA da iNOS, MMP-2, HGF, IL-1β, IL-6 por RT-PCR;
  - a expressão da proteína 3-nitrotirosina, medido por Western Blotting;
  - a expressão das proteínas iNOS, TGF-β, MMP-2, MPO por imunohistoquímica;
  - a proliferação celular pela expressão da proteína PCNA, por imunohistoquímica;

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7 ARTIGOS EM INGLÊS

7.1 Artigo 1

Nitric oxide and repair of skeletal muscle injury

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Publicado em: **Nitric Oxide: Biology and Chemistry** v.21 (2009) p.157-163.

Este trabalho foi realizado com financiamento da Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) e do Fundo de Incentivo a Pesquisa e Eventos (FIPE).

ABSTRACT

The muscle wound healing occurs in three overlapping phases: (1) degeneration and inflammation, (2) muscle regeneration, and (3) fibrosis. Simultaneously to injury cellular infiltration by neutrophils and macrophages occur, as well as cellular 'respiratory burst' via activation of the enzyme NADPH oxidase. When skeletal muscle is stretched or injured, myogenic satellite cells are activated to enter the cell cycle, divide, differentiate and fuse with muscle fibers to repair damaged regions and to enhance hypertrophy of muscle fibers. This process depends on nitric oxide (NO) production, metalloproteinase (MMP) activation and release of hepatocyte growth factor (HGF) from the extracellular matrix. Generation of a fibrotic scar tissue, with partial loss of function, can also occur, and seems to be dependent, at least in part, on local TGFβ expression, which can be downregulated by NO. Hence, regeneration the muscle depends on the type and severity of the injury, the appropriate inflammatory response and on the balance of the processes of remodeling and fibrosis. It appears that in all these phases NO exerts a significant role. Better comprehension of this role, as well as of the participation of other important mediators, may lead to development of new treatment strategies trying to tip the balance in favor of greater regeneration over fibrosis, resulting in better functional recovery.

Key words: nitric oxide, skeletal muscle, satellite cells, repair

NORMAL STRUCTURE OF MUSCLE

Traumatic muscle injuries, including crush, contusion, laceration or freezing is relatively frequent. However, when they do occur, can have dramatic and prolonged effects on muscle functional capacity [1]. Often, the injured muscle heals slowly and improperly, leading to an incomplete functional recovery, a tendency for recurrent injuries and/or scar tissue formation [2]. Hence, the use of therapeutic interventions aiming at enhancement of muscle regeneration and prevention of muscle fibrosis would be very useful in order to preserve function. However, the development of these strategies will require a better understanding of how these processes occur and what are the major factors involved.

Skeletal muscle is essentially composed of two main components: the *myofibers* and their innervating nerves, which are responsible for the contractile function of the muscle, and the *connective tissue*, which provides the framework to transform contraction force into movement. Myofibers and connective tissue have an intimate relation, which is essential for effective, coordinated function. The individual myofibers are bound together by a connective tissue structure composed of 3 levels of sheaths called the endomysium, perimysium, and epimysium [3]. Moreover, each myofiber is attached at both ends to the connective tissue of tendon or tendon-like fascia at the so-called myotendinous junctions (MTJs) [4].

To facilitate the study, healing of muscle injury can be divided into three distinct, but overlapping phases. The *destruction phase* is characterized by the rupture and ensuing necrosis of the myofibers, the formation of a hematoma, and the inflammatory cell reaction. The *repair phase,* consisting of the phagocytosis of the

necrotized tissue, the regeneration of the myofibers, production of a connective tissue scar; and the *remodeling phase*, a period throughout which the maturation of the regenerated myofibers, the contraction and reorganization of the scar tissue and the recovery of the functional capacity of the muscle occur. Remodeling phase is constituted of two processes, remodeling and fibrosis, the balance of which defines de degree of function recovery and scar tissue amount (figure 1) [2; 5]. Therefore, understanding the mechanisms and factors involved in this balance could have a significant impact on therapeutic strategies for muscle injuries.

The nitric oxide (NO) is a very small molecule, freely diffusible through cell membranes, which regulates an ample variety of cellular functions [6; 7]. It is produced by several cells, such as skeletal and cardiac muscle cells, epithelial cells, endothelial cells, macrophages, fibroblasts and hepatocytes, [8] via a reaction catalyzed by the nitric oxide synthase (NOS) [9; 10]. Three NOS isoforms have been characterized, each encoded by different chromosomes. Two enzyme isoforms are constitutively expressed (endothelial –eNOS- and neuronal -nNOS), whereas one isoform is an inducible enzyme (iNOS), initially found in macrophages [9].

NO can act as a signal molecule that activates guanylate cyclase and as a cytostatic/cytotoxic molecule that inhibits mitochondrial iron containing cytochromes and aconitase, as well as inhibiting ribonucleotide reductase. NO may also regulate gene expression by activating or inhibiting transcription factor binding, by reacting with the thiol binding site of the transcription factor NF-κB [11; 12].

NO has been demonstrated to be a modulator of skeletal muscle function, and a likely mediator of injury and disease [13]. NOS activity in rat skeletal muscles has

been correlated with muscle fiber density [14], force development [15], regulation of blood vessel diameter and blood flow [16], modulation of the vascular tone, as well as muscle contractile properties. However, there are very few published studies investigating the role of NO in post-injury muscle inflammation and repair.

We will subsequently summarize the biological and pathological processes that occur in skeletal muscle post-injury and the possible roles of NO in these processes. Finally, we will discuss current trends in therapy research, trying to enhance regeneration and the inhibit fibrosis in injured skeletal muscle.

DESCTRUCTION PHASE OF MUSCLE INJURY

When muscle is injured, overused, or mechanically stretched, tissue necrosis occurs, followed by cellular infiltration through several stages, generally characterized by early neutrophils invasion and sequential increase of macrophages [17]. Macrophages can lyse target muscle cells by a NO-dependent, superoxide independent mechanism and their cytolytic capacity is increased by the presence of neutrophils. [18] Additionally, the presence of muscle cells increases NO production by macrophages, suggesting that there may be a positive-feedback mechanism promoting lysis, in which initial muscle damage promotes increased NO-mediated toxicity by macrophages [19]. Therefore, the macrophages play a decisive role in the removal of necrotic tissue, and together with the fibroblasts, produce complementary chemotactic signals (cytokines, growth factors, and chemokines) for attraction of circulating inflammatory cells [5; 17; 19]. Proinflammatory cytokines, such as

interleukin-1beta (IL-1β), IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), are important to modulate chemotaxis to injured muscle [17].

These cytokines can also stimulate pathways that contribute to activation of the enzyme NADPH oxidase, which generates a 'respiratory burst' and the subsequent release of reactive oxygen species (ROS) [19; 20; 21; 22]. ROS are produced during normal aerobic cell metabolism, have important physiological roles in maintaining cell redox status and are required for normal cellular functions, including cell proliferation, aggregation, chemotaxis and apoptosis, as well as regulation of intracellular signaling pathways and the activity of transcription factors, such as nuclear factor(NF)-κB, activator protein 1 (AP-1) and hypoxia-inducible factor-1alpha (HIF-1α) [23; 24; 25].

It seems likely that ROS generated by either PMN or macrophages during muscle injury cause oxidative modification of muscle proteins, such as creatine kinase (CK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Nevertheless, the extent of these potentially significant biomolecular transformations of specific proteins is largely unknown [26; 27].

On the other hand, ROS may also have a positive role in the wound healing. They can enhance the affinity of the growth factors (FGF-2) to its receptor and also induce its expression. Hydrogen peroxide (H_2O_2), a specific ROS, induces collagen type I, III and IV synthesis and their subsequent cross linking. ROS also mediate conversion of fibroblasts to myofibroblasts, thus aiding in wound contraction [28; 29].

Different cells have the capability to produce NO in the skeletal muscle. In human muscle nNOS is constitutively expressed in both type I and II fibers [14; 30].

nNOS is located in specialized structures at the surface membrane, by binding to α1-syntrophin, a dystrophin-associated protein [30]. The absence of dystrophin, due to a mutation in the gene for the protein, causes a secondary loss of nNOS from the sarcolemma, leading to muscle inflammation, muscle membrane lysis, muscle wasting, and death. This occurs in patients with Duchenne muscular dystrophy and an animal model of this disease (mdx mice), and demonstrates an important protective effect of NO.

The isoform eNOS is expressed in endothelial cells [8]. Although immunoblot analysis shows that rat extensor digital longus and soleus muscles express eNOS, its low level of expression has made it more difficult to localize and study [30]. Finally, iNOS activity in skeletal muscle depends on the disease state and species investigated. iNOS mRNA is absent or present at very low levels in normal skeletal muscles of rats [31] and human [32], however its levels are markedly increased in inflammatory conditions, such as autoimmune inflammatory myopathies [33].

During the inflammatory phase there is a great increase in local NO production. Several studies confirmed this data. Rubinstein *et al*,[31] in an experimental model of muscle crush injury observed the expression of all three NOS isoforms both in normal and crushed untreated limbs. However, induction of iNOS was observed only in the crushed limb, which was maximal at 6h after the injury. Darmani, *et. al*.[34] investigated iNOS activity in crush-injured digital flexor tendon and observed a greatly increased expression of iNOS and TGF-β at 3 days subsequent to injury, which gradually returned to normal. Zhang *et.al*,[35] investigated the change of local NO level in a rat experimental model of muscle crushing. In this study, the expressions of eNOS and iNOS were upregulated, and

NOS activity and NO level in local muscles and serum were significantly increased in crush group compared with the sham group. The likely source of NO in this process is primarily iNOS expressed by macrophages and/or muscle fibers [13].

Using an experimental model of muscle crush injury, we have also observed an increase of the iNOS mRNA at 6 hours after trauma. However, when the animals were exposed to $_{L}$-NAME, they showed a great increase in the iNOS mRNA, probably attempting to compensate the inhibited NO synthesis. This demonstrates that NO increased levels post-injury can be strongly regulated at the transcriptional level. We also observed increased transcription of the pro-inflammatory cytokines, imbalance of redox status and increased local MPO expression.

Muscle-derived NO appears to be a particularly important regulator of muscle inflammation and muscle damage by invading inflammatory cells [18]. *In vitro* and *in vivo* studies have shown that muscle-derived NO reduces of damage by inflammatory cells by increasing their apoptosis and inhibiting the expression of adhesion molecules, such as intracellular adhesion molecules (ICAM), E-selectin and P-selectin. Muscle-derived NO can reduce neutrophil-mediated lysis of muscle cells and decrease superoxide concentration, forming less reactive intermediates, such as $ONOO^-$ [30].

In skin, it is well known that the NO is implicated as a regulator of all phases of wound healing [36]. The beneficial effects of NO on wound repair may be attributed to its functional influences on angiogenesis, inflammation, cell proliferation, matrix deposition and remodeling [37]. Experimental studies demonstrated that inhibitors of NOS have been reported to delay wound healing [38; 39] and the administration of

NO donors has beneficial effects on wound repair, on both inflammatory and proliferative phases, improves wound contraction and, principally, accelerate reepithelialization [36; 40]. Whether similar effects of NO occurs during muscle injury and repair processes is not known.

An essential process in the regeneration of an injured muscle is the vascularization. Angiogenesis is a prerequisite for the subsequent morphological and functional healing of the injured muscle. It leads to rebuilding of the damaged vessels, restoration of the blood flow and restoration of the oxygen supply to the tissue. NO plays a key role because it can act as a vasodilator and can promote activation of several growth factors involved, such as vascular endothelial growth factor and fibroblast growth factor [41].

Rubinstein, *et. al.,*[31], in an experimental model, observed that muscle crush injury caused a threefold increase in the capillary mean cross-sectional area and a three- to fourfold increase in the femoral blood flow and capillary blood flow. Administration of Nω-nitro-L-arginine methyl ester ($_{L}$-NAME), a NOS inhibitor, largely decreased the hyperperfusion that follows the muscle injury. Therefore, this data clearly suggest that the vasodilatation in the injured skeletal muscle is promoted by local NO production.

REPAIR AND REMODELING PHASE OF MUSCLE INJURY

Compared with inflammatory phase, the repair process is somewhat delayed, beginning about 7 days after injury.

A likely role of neutrophils in muscle repair or remodeling is the oxidative or proteolytic modification of injured tissue, to allow phagocytosis of debris by neutrophils or macrophages [18]. Although the mechanisms responsible for attracting macrophages remain unknown, some the pro-inflammatory cytokines be involved in repair process, such as IL-6. These cytokine may be involved in protein degradation and muscle damage [42] or it is induce proliferation of satellite cells, raising the possibility at least for a potential role for IL-6 in muscle regeneration [43]. Therefore, it appears that IL-6 plays a role in muscle inflammation and repair with the exact details yet to be determined.

The key cell involved in regeneration of skeletal muscle is the **satellite cell** (SC). These cells are mononuclear progenitor cells found in mature muscle between the basal lamina and sarcolemma. In mature skeletal muscles, they are normally quiescent, but can be activated in response muscle injury [44; 45] (see figure 2). On activation, they enter the cell cycle, divide, differentiate and fuse with muscle fibers to repair damaged regions and to enhance hypertrophy of muscle fibers [46].

Satellite cell activation is limited to areas where there is necrosis of myofibers and can continue for nine to ten days, depending on the severity of the injury [2; 47]. Apparently mechanical changes in muscle can lead to SC activation, although the mechanisms involved have not been clarified. There are several postulated mechanisms trying to explain the activation of muscle satellite cells after trauma. Some researchers have postulated that disruption of the integrity of the sarcolemma and basal lamina activates satellite cells [48]. Others sustain that cytokines released by infiltrating inflammatory cells result in satellite cell activation [2] Most likely there

are a complex combination of these and other events, and NO seems to have a critical role.

During a crushing injury of the muscle fibers, macrophages invade the area of injury, phagocytose the necrotic tissue and produce several growth factors that are mitogenic for muscle precursor cells, such as FGF, insulin-like growth factor (IGF), TGF-β, hepatocyte growth factor (HGF), and IL-6 [5; 29; 44; 49] (see table 1).

Hepatocyte growth factor/scatter factor (HGF) has been recently well studied as an activator of satellite cells [50]. HGF is an α-β heterodimer produced by proteolytic cleavage of a single-chain inactive precursor of 728 amino acids. It is localized in the extracellular domain of uninjured skeletal muscle fibers [51; 52; 53]. Allen, *et. al.,*[51], Tatsumi, *et. al.,*[50], Sheehan, *et. al.,*[54] have demonstrated that HGF can be released from muscle matrix upon injury, and has the ability to activate early division of adult satellite cells in culture and in muscle tissue. These authors have also demonstrated that HGF mRNA is expressed in adult SC and can act in autocrine fashion. Thus, regulation of HGF function in muscle can occur both in a transcriptional level and by its high-affinity binding to heparan sulfate proteoglycans of the tissue matrix [55].

HGF role in muscle regeneration is essential during the early phase of the repair process as demonstrated by the decrease in HGF immunostaining with time after injury and the inability of exogenous HGF injection to affect muscle regeneration when performed at later stages of muscle regeneration [56]**.**

Tatsumi, *et. al.,*[57], based in an *in vitro* experiments, proposed that mechanical stretch stimuli triggers an intracellular cascade of events in the muscle

fibers, which is pH-dependent and involves NOS activation, eventually leading to HGF release from the extracellular compartment and subsequent SC activation. These authors studied whether the mechanism of release involves a proteolytic activation of pro-HGF, and observed that the active form of HGF is present in the extracellular compartment of uninjured skeletal muscle. Therefore, the mechanism of HGF release in response to stretch does not require proteolytic activation of pro-HGF [58].

The role of NO in the release of HGF from the extracellular matrix was investigated by administering $_{L}$-NAME, an inhibitor of NOS function, or D-NAME, an inactive stereoisomer, before stretch treatment. *In vivo* activation of SC in stretched muscle was inhibited by $_{L}$-NAME, but not by D-NAME, indicating the stretching of muscle fibers induces liberation of HGF in a NO-dependent manner [59].

The NO-dependent HGF-matrix release could be mediated by matrix metalloproteinases (MMP), a large family of zinc-dependent endopeptidases that are capable of degrading one or several extracellular matrix proteins, such as collagens, elastin, fibronectin, laminin, and proteoglycans [60]. Numerous MMPs, including MMP2, 3, 7, and 9, are found in skeletal muscle [46; 61], possibly playing fundamental roles in muscle fiber growth and repair by regulating the integrity and composition of extracellular matrix in skeletal muscle [61].

Yamada *et. al.,*[62] demonstrated that MMPs are involved in the NO-dependent release of HGF. When mechanically stretched rat SC were treated with NO donors in the presence of recombinant tissue inhibitor-1 of MMPs (TIMP-1), the activation response was inhibited, providing strong evidence that MMPs mediate

HGF release from the matrix, and this process can be regulated by the presence of TIMPs.

Yamada *et. al.,*[46] demonstrated that MMP-2 mediates stretch-induced activation of skeletal muscle SC in a nitric oxide-dependent manner. In these experiments, the SC muscle isolated from 9-month-old male Sprague–Dawley rats of gastronomies were treated with sodium nitroprusside, a NO donor; mechanical cyclic stretch or $_{L}$-NAME. MMP-2 was identified in both stretch-simulated and NO donor-treated SC culture, but not in $_{L}$-NAME-treated, and HGF expression was detected in MMP-2, stretch and NO donor-treated cultures. Thus, results from this study provide evidence that NO-activated MMP2 may cause release of HGF from the extracellular matrix of SC and contribute to their activation *in vitro*.

More recently, Tatsumi *et. al*.,[63] demonstrated that calcium-calmodulin is also involved in the SC activation cascade *in vitro*. Cultures of SC that were treated with a calcium ionophore for 2h induced production of HGF and activation of these cells, similarly to the effect of mechanical stretch or NO donor treatments. The response was abolished by addition of calmodulin inhibitors. Therefore, results from these experiments provide an additional insight that calcium-calmodulin mediates HGF release from the matrix and that this step in the activation pathway is upstream from NO synthesis.

Based in this series of experiments, a NO-dependent pathway of SC activation has been proposed as summarized in Figure 3. Since HGF plays a central role in SC activation, it is likely that direct administration of HGF into damaged muscle may represent a potentially useful approach for stimulating muscle repair. However,

although this is the best studied, it does not exclude other different pathways where NO could or could no be involved.

FIBROSIS OF MUSCLE INJURY

The fibrotic scar tissue formation may lead to inadequate healing and a deficient muscle function. The muscle fibrosis is an overproliferation of components of the extracellular matrix beginning approximately 2 weeks after injury and accelerating thereafter for as long as 4 weeks.

Skeletal muscle healing following trauma can be understood as a balance between fibrosis and regeneration. Factors influencing this balance consist of inflammation, the growth factors and cytokines present in site of injury, and the interaction between infiltrating inflammatory cells and native myogenic cells [64].

During this process the predominant cell is the fibroblast and proteins, such as collagen (predominantly types I and III), glycoproteins, and proteoglycans. During tissue repair, fibroblasts continue to secrete their own pro-inflammatory cytokines, such as IL-6 and IL-1, as well as to recruit additional neutrophils through the production of IL-8. Ultimately, fibroblasts can perpetuate the chronic inflammatory response through their release of PGE2 in response to cell stress and strain. Although SC proliferation has been shown to be enhanced in the presence of macrophages, the release of excessive quantities of TGF-β1 inhibits SC differentiation, compromising fiber regeneration [19].

Some growth factors, such as EGF, myostatin, and TGF-β1, are released from inflammatory cells in initial phase and have been shown to stimulate proliferation of the ECM and inhibit skeletal muscle regeneration both *in vitro* and *in vivo* as early as 72h after injury [64].

TGF-β is believed to be responsible for the scar formation during the wound/skeletal muscle repair [5]. Two mechanisms through which TGF-β1 promotes fibrosis have been postulated. TGF-β1 stimulates the production of ECM proteins and simultaneously blocks their degradation and also promotes myogenic cells differentiation into myofibroblasts that produce collagen type I [65; 66; 67].

In both skeletal muscle disease and injury, TGF-β appears to be a major determinant for connective tissue proliferation and fibrosis. In muscle diseases characterized by fibrosis, such as muscular dystrophy and inflammatory myopathy, TGF-β has been localized to the extracellular matrix between myofibers and areas of inflammatory cell infiltration. [68].

Smith *et. al.* [68] determined whether TGF-β protein is present and active 48h following skeletal muscle strain injury in rats. In this study, the TGF-β expression and synthesis were evaluated by immunohistochemistry, RT-PCR and immunoblot analysis. TGF-β1 was detected in areas of myofiber injury and, by RT-PCR analysis, there was increased expression of TGF-β1 and TGF-β2 precursors. Although there was no correlation between the extent of cellular injury and TGF-β transcript and protein amounts, elevated levels of TGF-β1 and TGF-β2 precursor proteins were present in strain-injured skeletal muscles 48 h after injury.

NO and TGF- β may have antagonist effects in muscle injury repair. Darmani *et. al.*[34] in their study examined the expression of iNOS and TGF-β in macrophage infiltrates within crush-injured digital flexor tendon and synovium of control and $_{L}$-NAME-treatment rats. The results showed that during normal tendon healing the levels of TGF-β are high at first and gradually decrease after 3 weeks of injury to slightly above control uninjured levels. However, inhibition of NOS by $_{L}$-NAME-treatment at the time of injury leads to a chronic overexpression of TGF-β *in vivo* at 5 weeks after the injury, with no evidence of reduction.

We have also observed an increased TGF-β expression in an experimental model of muscle crush injury caused by a single impact blunt. Administration of $_{L}$-NAME 2 hours after injury leads to an overexpression of TGF-β at 7th day after injury, implicating an inhibitory effect of NO. This expression was large in fibroblasts and cells of skeletal muscle (unpublished data).

It is well established that chronic elevation of TGF-β levels in the healing wound will lead to increased deposition of collagen by stimulated fibroblasts and this chronic accumulation of collagen in the healing tendon and synovium is critical in the progressive loss of function in tendon healing. [34].

Thus, based on these initial studies it can be hypothesized that the regulation of the regeneration/repair and fibrosis is dependent of the balance between NO and TGF-β local levels, with NO stimulating regeneration by induction of SC activation, and TGFβ inducing collagen deposition and fibrosis.

THERAPEUTIC STRATEGIES

Studies designed for therapeutic strategies are extremely limited, especially considering the clinical importance of effective strategies to improve regeneration/repair of muscle after injury. Therapeutic interventions to improve outcomes following muscle injuries revolve around 3 concepts: regulating inflammation, enhancing regeneration and blocking excessive fibrosis.

The knowledge about the role of NO in the inflammatory process has significant potential to provide an understanding of the mechanisms of action of current therapies and, especially, for developing new therapeutic strategies to handle the repair process.

As previously mentioned, the arginine is substrate of NOS resulting in the production of citruline. Arginine is a precursor for three pathways, the products of which are involved in tissue injury and repair: 1) nitric oxide; 2) polyamines, which are required for DNA synthesis and cell growth; and 3) proline, which serve as substrate for collagen synthesis [69; 70].

Additionally, the L-arginine might be metabolized via arginase, which is present in high concentrations in healing wounds due to macrophage production [71]. Through the action of arginase, ornithine is formed which is a precursor for proline and polyamine generation [72]. Therefore, arginine supplementation could have an impact in muscle regenerative processes.

Barbul, *et. al.,*[73] and Williams, *et. al.,*[74] established that dietary L-arginine intake can improve collagen deposition and wound strength in both animals and

humans. Nevertheless, the finding that L-arginine intake does not improve collagen deposition in iNOS-deficient mice to the same extent as in wild-type littermates implicates that part of L-arginine effect involves NO directly [75].

Betters *et. al.,*[76] investigated supplementation of the L-arginine or diethylenetriamine (DETA) to muscle cell cultures from mice in different stages of life. Single intact myofibers were isolated from the gastrocnemius muscles of young (2 mo), adult (10 mo), and aged (22 mo) mice. They reported that L-arginine bioavailability and NO production can enhance SC activity in old myofibers, and the decline in SC activity in early senescence can be partially extended.

Buchman *et. al.* [77] investigated the effect of arginine or glycine supplementation on gastrointestinal function, muscle injury, serum amino acid concentrations and performance during a marathon run in a randomized controlled trial. The extremity pain scores and fluid intake was similar between both groups; CPK increased significantly and similarly in both groups immediately post-race, and even more dramatically after 48 hours. The authors concluded that skeletal muscle injury was unaffected by arginine or glycine supplementation.

Zhang *et. al.*, [78] investigated the effect of L-arginine supplementation on protein metabolism in skin wound and muscle in anesthetized rabbits. L-[ring-(13)C(6)]phenylalanine was infused as a tracer on day 7 after ear injury, and the scalded ear and uninjured hindlimb were used as arteriovenous units to reflect protein kinetics in these two tissues. One group received amino acid mixture (10% Travasol) with supplemental L-arginine, and the other, $_{L}$-NAME. The arginine supplementation increased net protein balance in skin wound and muscle, indicating

an anabolic effect. In group 2, $_{L}$-NAME infusion markedly reduced the blood flow rate in the scalded ear and increased net protein balance in skin wound and in muscle. Thus, arginine supplementation increased net protein balance in skin wound and muscle by a mechanism which was independent of nitric oxide production.

Besides arginine, few studies have proposed the use of exogenous MMP to improve repair. Bedair *et. al.,*[79] have showed that the introduction of exogenous MMP-1 into the zone of injury following skeletal muscle laceration can decrease the amount of residual fibrosis and, in turn, result in more regenerating myofibers in the area of injury. These results suggest that the direct injection of MMP-1 into the zone of injury during fibrosis can enhance muscle regeneration by increasing the number of myofibers and decreasing the amount of fibrous tissue.

Wang *et. al.,*[80] propose that MMP-1 could enhance muscle regeneration by improving myoblast migration and differentiation, which is a critical step in the sequence of muscle regeneration. By biomolecular analyses of *in vitro* wound healing assays (C2C12 cells culture), they demonstrated that MMP-1 enhances myoblast migration but is not chemoattractive. *In vivo*, myoblast transplantation was greatly improved after MMP-1 treatment within the dystrophic skeletal muscles of MDX mice. MMP-1 may therefore be able to improve muscle function recovery after injury or disease by increasing both the number of myofibers that are generated by activated myoblasts and the size of myoblast coverage area by promoting migration.

Besides, pharmacological blockade pro-inflammatory cytokines, such as TNF-α activity, have been proposed to dystrophic skeletal muscles treatment. Hodgetts, S *et. al.*, [81] analyzed dystrophin-deficient mice, that the initial sarcolemmal

breakdown resulting from dystrophin deficiency is exacerbated by inflammatory cells, specifically neutrophils, and that cytokines, specifically TNFα, contribute to myofibre necrosis. Antibody depletion of host neutrophils resulted in a delayed and significantly reduced amount of skeletal muscle breakdown in young dystrophic mdx mice. A more striking and prolonged protective effect was seen after pharmacological blockade of TNFα bioactivity using Etanercept. These data show a clear role for neutrophils and TNFα in necrosis of dystrophic mdx muscle *in vivo*.

Physical therapies, besides immobilization, are commonly used for the treatment of muscle injuries, but their mechanism of action and effects at the molecular level have not been well studied. We have investigated the effect of low-level laser therapy (LLLT) of gallium arsenide (Ga-As) in our experimental model of gastrocnemius muscle crush injury. LLLT application 24 hours post-trauma by 7 days or 14 days markedly inhibited the oxidative stress, followed by inhibition of iNOS expression, NF-κB activation and collagen deposition [82]. None of these effects could be seen when LLLT was applied to uninjured muscle.

An additional therapeutic strategy used for the treatment of muscle injuries is the ultrasound (US), although the scientific evidence on its effectiveness is somewhat unclear. Rantanen, J *et. al.,*[83] investigated the regeneration of contusion injury to the rat gastrocnemius muscle during treatment with pulsed ultrasound. The speed of myoregeneration in ultrasound-treated animals was compared with that in control animals by immunohistochemical, morphometric, and scintigraphic analyses. This study concluded that pulsed ultrasound-treatment does not seem to have significant effects on the overall morphological manifestations of muscle regeneration.

Early mobilization was first recommended for the acute treatment of muscle trauma [84]. This therapeutic strategy has been shown that induces more rapid and intensive capillary in growth into the injured area, better regeneration of muscle fibers, and more parallel orientation of the regenerating myofibers in comparison to immobilization, the earlier preferred treatment for injured muscle [5]. However, the nitric oxide role in this strategy not is clear.

CONCLUSION

In summary, muscle injury healing occurs in overlapping phases and this process is influenced by the type and severity of the injury and involves numerous growth factors and signaling molecules. The repair process depends on a delicate balance between muscle regeneration and fibrosis, which is certainly determined by the effectors of the early inflammatory response. As discussed, NO is intimately involved in all these phases, mediating many aspects of the inflammatory response and SC activation and probably modulating TGF-β mediated fibrosis. However, a better assessment of the importance of the NO role in muscle repair requires further studies.

The best treatment for muscle injuries has not clearly been defined yet. Further research is necessary to improve our attempt to understand the muscle healing process, in order to develop the necessary methodology to promote efficient muscle healing and also to achieve complete functional recovery, and perhaps to contribute for the development of innovative muscle diseases therapies.

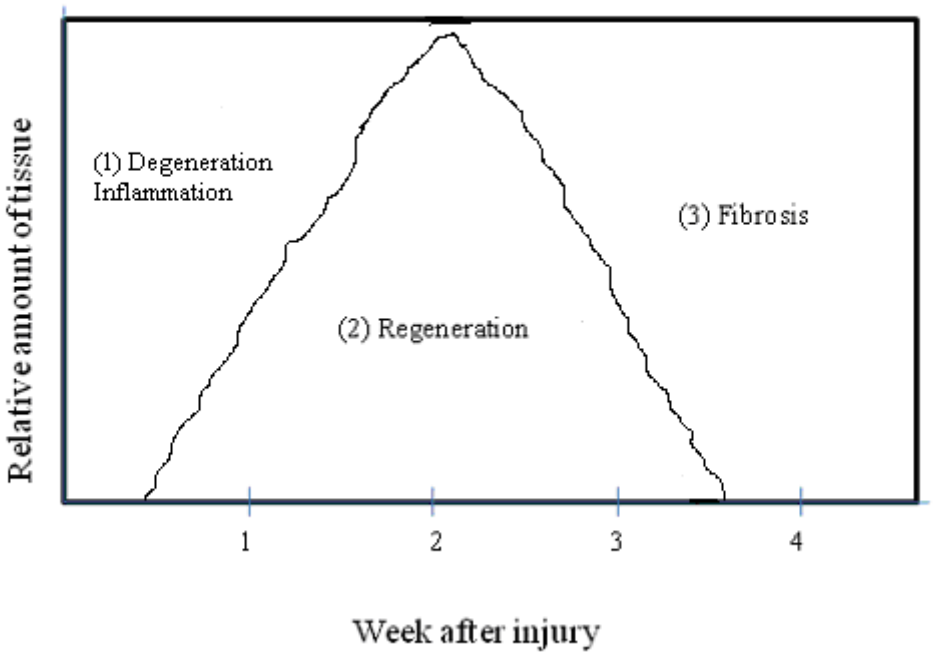

Figure 1 - The different stages of muscle healing after muscle injury. The first event is muscle degeneration and inflammation, which occurs within the first minutes and continues for up to one to two weeks after injury. Muscle regeneration begins in the first week post-injury and peaks at about fourteen days post-injury. Fibrosis usually occurs at two weeks post-injury and increases over time for up to four weeks post-injury.

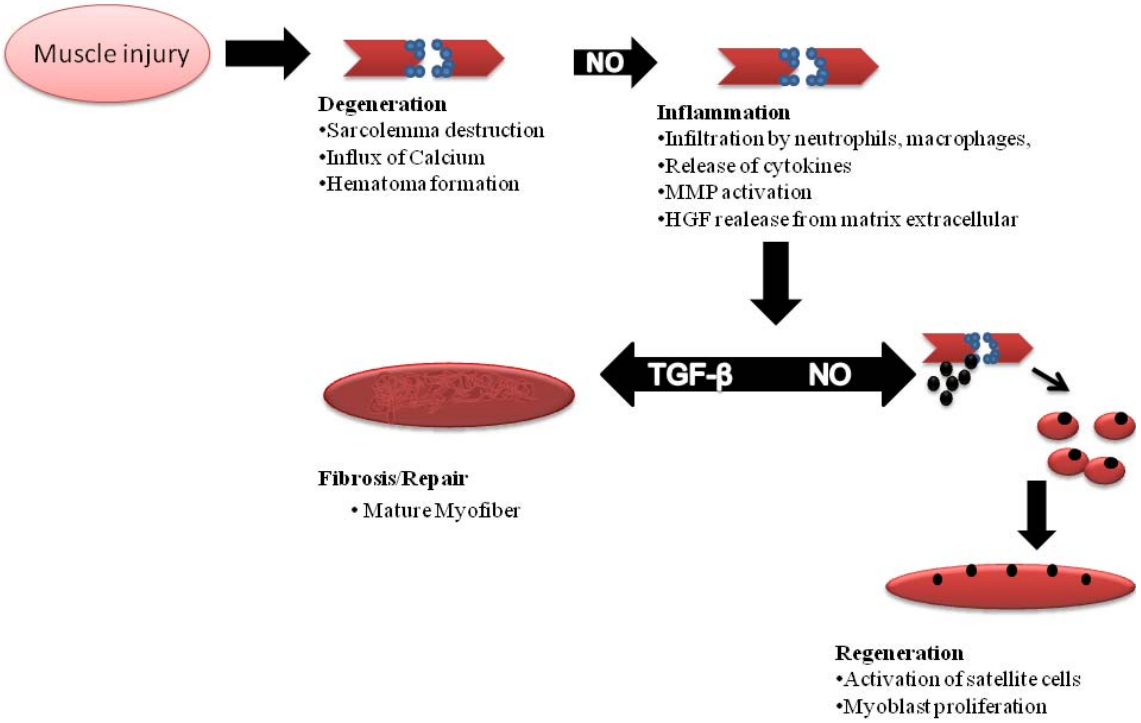

Figure 2 – Regeneration phase of muscle.

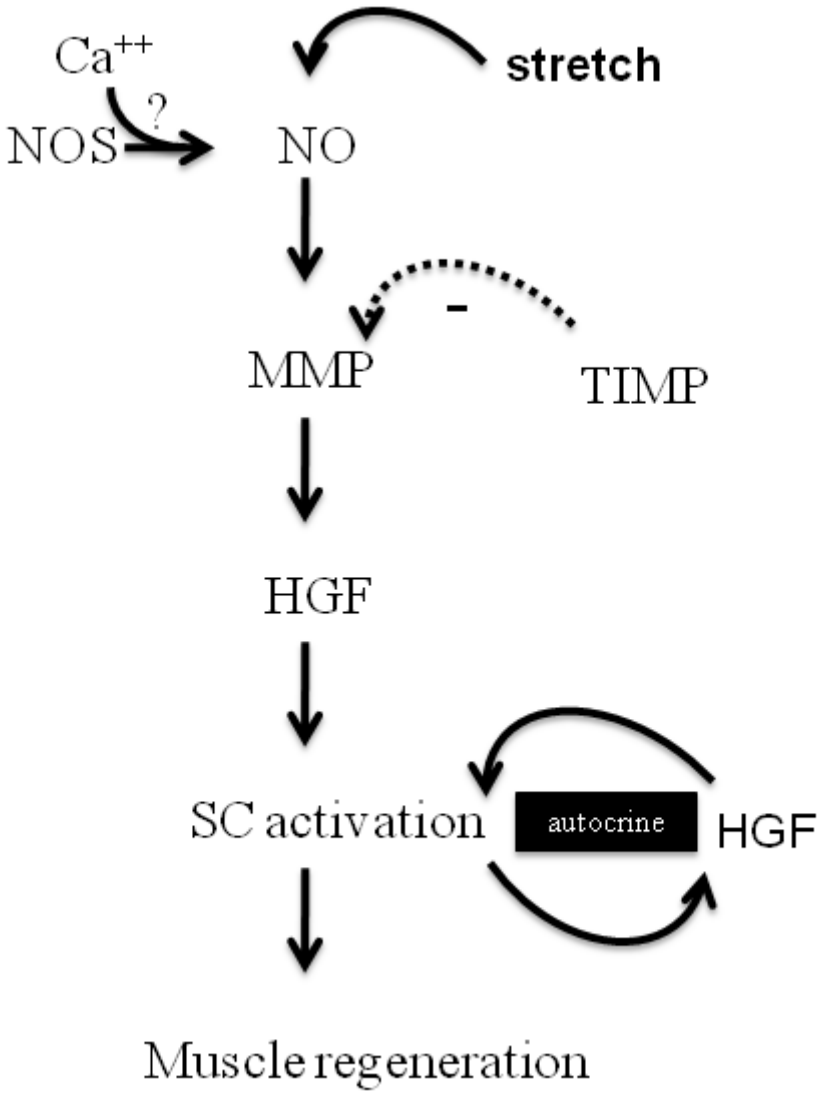

Figure 3 – Activation skeletal muscle SC NO-dependent. See the text.

Table 1 - Effect of Growth Factors on Myoblasts *in Vitro* and *in vivo.*

Growth factor	**Cell Proliferation**	**Cell differentiation**	**Ref.**
Hepatocyte growth factor (HGF)	↑	↑	[85]
Fibroblast growth factor 2 (FGF-2)	↑	↓	[86]
Insulin-like growth factor-1 (IGF-1)	↑	↑	[87]
Interleukin-6 (IL-6)	↑	↑	[88]
Epidermal growth factor (EGF)	↓	↓	[5]
Transforming growth factor-beta (TGF-β1)	↓	↓	[89]

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7.2 Artigo 2

The role of nitric oxide during the healing of trauma to the skeletal muscle

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Submetido em: **Muscle and Nerve** (2009).

Este trabalho foi realizado com financiamento da Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) e do Fundo de Incentivo a Pesquisa e Eventos (FIPE).

The role of nitric oxide during the healing of trauma to the skeletal muscle

Nitric Oxide during the healing muscle

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Article origin: Serviço de Reumatologia do Hospital de Clínicas de Porto Alegre (HCPA) e Universidade Federal do Rio Grande do Sul (UFRGS).

Supporting Agency: Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundo de Incentivo a Pesquisa e Eventos (FIPE).

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ABSTRACT

Introduction: The role of NO in the muscle inflammatory is not clear. **Methods:** We examined the involvement of the NO system in the development of muscle damage in an experimental model of crush injury. The animals were divided into four groups: *(i)* control (CO), *(ii)* sham trauma, *(iii)* trauma, *(iv)* trauma + $_{L}$-NAME in two experimental phases: 24 hours and 7 days after injury. **Results:** Twenty-four hours after injury, the crushed muscle was characterized by intense inflammatory reaction. These changes were accompanied by increased oxidative damage, increased transcription of mRNA cytokines, increased NF-κB binding ability and growth factor expression TGF-β in the gastrocnemius muscle. Treatment with $_{L}$-NAME markedly decreased histological and molecular abnormalities at 24 hours. However, at 7 days post-trauma an increased collagen formation was seen in the $_{L}$-NAME group. **Discussion:** These findings indicate that muscle injury is involved in the balance between healing with regeneration and fibrosis.

Keywords: nitric oxide, oxidative stress, muscle repair, nitrative stress, $_{L}$-NAME

INTRODUCTION

Traumatic muscle injuries, including crush, contusion, laceration or freezing, are common and, when they do occur, can have dramatic and prolonged effects on muscle functional capacity [29]. Often, the injured muscle heals slowly and incompletely, leading to a loss of functional capacity, a tendency for recurrent injuries and scar tissue formation [22]. After injury, muscle undergoes a healing process, which includes a degeneration/inflammatory phase, followed after 7 to 10 days by regeneration and/or fibrosis [24]. The mechanisms of control of this sequential process are not well defined, but involve the participation of numerous inflammatory mediators, including cytokines. It would be clinically important to be able to manipulate these processes in order to minimize the damage and fibrosis and to improve the regeneration and functional recovery.

During the muscle injury process, there is tissue necrosis and cellular infiltration generally characterized by early neutrophil invasion, followed by sequential increase of macrophages and local production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [57,42]. Polymorphonuclear neutrophilic leukocytes (PMN) are the inflammatory cells predominant in the injury site [42], and key role players in the early hours of the inflammatory response. These cells remove necrotic tissue and release cytokines, such as interleukin-1beta (IL-1β), IL-6, IL-8, tumor necrosis factor (TNF-α), to modulate further cell chemotaxis and the subsequent muscle regeneration and fibrosis [8,13].

Other important cytokine during the fibrosis process is transforming growth factor (TGF-β), believed to be responsible for the scar formation during skeletal muscle repair [24]. Two mechanisms through which TGF-β1 promotes fibrosis have been postulated. TGF-β1 can stimulate the production of extracellular matrix (ECM) proteins and simultaneously block their degradation. Additionally, TGF-β1 can induce myogenic cell differentiation into myofibroblasts that produce collagen type I [50,45,35].

Proinflammatory cytokines stimulate pathways that contribute to activate the enzyme NADPH oxidase, which generates a respiratory burst and subsequent release of ROS [8,13,21]. Neutrophils can generate hypochlorous acid (HCPO) via a myeloperoxidase-mediated reaction and superoxide ($O_2^{\bullet-}$) via NADPH oxidase. When there is an imbalance between ROS production and the antioxidant capacity, the oxidative stress occurs, which can be defined as 'an imbalance between oxidants and anti-oxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage' [17,25]. The oxidative and/or nitrosative stress leads to potential damage to lipids, membranes, proteins and nuclear acids [13].

Recently, the nitric oxide (NO) system has also been described as a regulator of several skeletal muscle functions. During muscle injury, NO can act as a pro-inflammatory molecule by activating cyclooxigenases and thereby increasing prostaglandin production [30], which can promote inflammation and muscle proteolysis; or may also function as an anti-inflammatory molecule through its ability to inhibit the synthesis of reactive free oxygen radicals by scavenging superoxide anions [9,10].

The NO production during the inflammatory response occurs mainly by the inducible isoform of NO synthase (iNOS), which plays a crucial role in numerous and

diverse pathophysiological processes, particularly as the principal mediator of the microbicidal and tumoricidal actions of macrophages [41]. However, little is known of NO-mediated participation in redox regulation and muscle repair.

This study was planned to explore the role of NO during the inflammatory and regeneration/fibrosis stages of the muscle injury process using an NO synthase inhibitor (nitro-$_L$-arginine methyl ester: $_L$-NAME), with attention to its effect on the local oxidative balance, iNOS and TGF-β activation, inflammatory cytokine synthesis, and consequent histological changes.

MATERIALS AND METHODS

Animals and experimental groups. Male Wistar rats weighing 250–300 g were used. The animals were caged at 22°C, with 12 hours light-dark cycle and free access to food and water until the time of experiments. All experiments were performed according to the Guiding Principles for Research Involving Animals (NAS), and to the Committee of Research and Ethics in Health of the Research and Postgraduate Group of the Hospital de Clínicas de Porto Alegre.

Experimental animals were first randomly divided into four groups of ten animals each: *(i)* C (control), *(ii)* ST (sham trauma + L-NAME), *(iii)* T (trauma), *(iv)* $_L$-NAME (trauma + $_L$-NAME). Right gastrocnemius injury was induced by a single impact blunt trauma with a press developed by the Centro Industrial de Equipamentos de Ensino e Pesquisa Ltda (CIDEP/RS, Brazil) using the procedure of

Lech *et al* [33]. Briefly, injury was produced by a metal mass (0.459 kg) falling through a metal guide from a height of 18 cm on the middle third of the gastrocnemius muscle belly. The impact kinetic energy delivered was 0.811 J [46].

Experimental procedures. During the procedure, rats were anesthetized with ketamine chlorhidrate (Ketalar, Parke Davis, 100 mg/kg) and xilazine 2% (Rompun, Bayer, 50 mg/kg) cocktail i.p. Sham trauma rats were also anesthetized to ensure standardization, but did not received muscle trauma. The animals of the ST and $_{L}$-NAME groups received a 100mg/kg dosage of $_{L}$-NAME, intraperitoneally, 2 hours after the trauma. Rats were killed 24 hours or 7 days later for biochemical evaluation and muscle histological analysis. The animals were anesthetized with ketamine chlorhidrate and xilazine 2% cocktail i.p. The gastrocnemius muscle was rapidly removed from both legs, snap frozen in liquid nitrogen, and stored at -80°C until analysis. The entire surgical procedure took less than 10 minutes. The gastrocnemius left paw was used as internal control. However, since the results were similar to the sham trauma controls, they will not be presented.

Histology. For histological examination a section of the muscle of all animals was trimmed and fixed by immersion in 10% buffered formalin for 24 hours. The blocks were dehydrated in a graded series of ethanol and embedded in paraffin wax. Serial 4-mm sections were stained with hematoxylin and eosin or picrossirius. Five sections from each sample were analyzed by two independent pathologists who had no prior knowledge of the animal groups.

Oxidative damage determination. Frozen tissue from each rat was homogenized in ice-cold phosphate buffer (KCl 140 mM, phosphate 20 mM, pH 7,4) and centrifuged at 3,000 rpm for 10 minutes. Oxidative stress was determined by measuring the concentration of aldehydic products (MDA) by thiobarbituric acid reactive substances (TBARS)[7]. Spectrophotometric absorbance was determined in the supernatant at 535 nm.

Antioxidant enzyme activity. Cytosolic superoxide dismutase (SOD) (EC 1.5.1.1) was assayed according to Misra and Fridovich at 30°C [40,39]. The rate of autoxidation of epinephrine, which is progressively inhibited by increasing amounts of SOD in the homogenate, was monitored spectrophotpmetrically at 560nm. The amount of enzyme that inhibits 50% of the epinephrine autoxidation is defined as 1 U of SOD activity.

Real-Time RT-PCR. Total RNA was obtained by Promega Kit (Promega, Madison, WI) and quantified by Nanodrop Technologies Spectrophotometer (ND – 1000 UV/VIS). First-standard cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). The negative control (no transcriptase control) was performed in parallel. cDNA was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI 7000 (Applied Biosystems). Commercially available TaqMan-Gene Expression Assays (Applied Biosystems, Weiterstadt, Germany) for Interleukin 1β (GenBank accession no M98820.1 and Rn00580432_m1), and Interleukin 6 (GenBank accession no

M26744.1 and Rn99999011_m1), and the housekeeping gene hypoxanthine phosphoribosyl-transferase (HPRT) (GenBank accession no M63983.1 and Rn01527840_m1), genes were used. Relative changes in gene expression levels were determined using the $^{2-\Delta\Delta CT}$ method as described previously [37]. The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of HPRT detection, referred to as ΔCT.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from gastrocnemius muscle by the method of Dignam *et al* [12] with modifications. Binding activity of NF-κB was determined in nuclear extracts of DVL by means of electrophoretic mobility shift assay (EMSA). Oligonucleotides were end-labeled with (γ-^{32}P) ATP to a specific activity >5 x 10^7 cpm/μg DNA-NF-κB consensus: 5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5'. Nuclear extract (40μg) was incubated for 20 min at room temperature in binding buffer in the presence of ≈ 1 ng labeled oligonucleotide [≈250 μCi (GE Healthcare Bio-sciences AB, Uppsala, Sweden)]. In order to verify that the results from EMSA analysis did not arise from non-specific binding, competition experiments were also carried out using a negative control (Cold probe) and muscle sample + non-specific competitor (NC probe). The non-specific competitor reaction used an oligonucleotide with a different sequence to the specific oligonucleotide listed above. In this instance, SP1 oligonucleotide (Promega Corporation, Madison, USA) was used. If the signal was specific, addition of non-radiolabeled specific competitor would decrease the signal intensity. An additional aliquot was prepared and loaded onto the gel which

contained all reagents with the exception of sample (negative control or cold probe). Protein–DNA complexes were separated from the free DNA probe by electrophoresis through 6% native polyacrylamide gels containing 10% ammonium persulfate and 0.5x Tris-borate-EDTA buffer. Gels were dried under vacuum on Whatmann DE-81 paper and exposed for 48–72 h to Amersham Hyperfilms at -80ºC.

Western Blotting Analysis. For Western blot analysis of nitrotyrosine muscle homogenates were prepared and protein concentration was measured by the Bradford assay. Protein extracts (25-50μg) were separated by 10-12% sodium dodecyl sulphatepolyacrylamide gel electrophoresis and transferred electrically to polyvinyllidene difluoride membranes (Millipore, USA). The membranes were incubated overnight at 4ºC with monoclonal anti-nitrotirosine (SIGMA-ALDRICH, St. Louis, MO). Subsequent, the membranes were incubated with secondary HRP conjugated antibody (Dako, Glostrup, Denmark), and visualized using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden). Polyclonal anti-□actin antibody was used to confirm equal loading of protein (1:1.000, SIGMA). The density of the specific bands was quantified with an imaging densitometer (Scion Image, Maryland, MA, USA).

Nitrite and nitrate quantification. Nitric oxide production in muscle tissue was measured indirectly using a quantitative colorimetric assay based on Greiss reaction as previously described by Granger *et. al* [18]. A method for the determination of NO involves spectrophotometric measurement of its stable decomposition products NO3-

and then NO2- determined by the Griess' reaction. The reaction was measured in a 546 nm absorbance, using a sodium nitrate solution as standard.

Immunohistochemistry. The muscle sections were washed with Tris-buffered saline (TBS), and treated with dilute normal serum for 15 min. The slides were drained and incubated with anti-iNOS (SIGMA-ALDRICH, St. Louis, MO), anti-TGF-β (Santa Cruz Biotechnology, Inc.) and anti-MPO (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. After been washed three times with TBS for 5 min, the slides were treated with biotin-conjugated secondary antibody for 45 min at room temperature. Subsequent to three further 5-min washes with TBS, the sections were incubated with alkaline phosphate-conjugated streptavidin (Sigma) at a dilution of 1:100 for 30 min. The sections were washed three times in TBS and then visualized using fast 5-bromo-4-chloro-3 indolyl phosphate/nitro blue tetrazolium substrate (Sigma), known positive and negative tissue biopsies were used to control the observations.

Statistical Analysis. Results are expressed as mean values with 95% confidence intervals (95%IC) when symmetric variables and as median and percentiles (25% and 75%) when asymmetric variables. The data were compared by analysis of variance (ANOVA); when the analysis indicated the presence of a significant difference, the means were compared with the Tukey test. Significance was accepted at $p<0.05$. Values were analyzed using the statistical package SPSS 13.0 (SPSS, Inc., Chicago, IL).

RESULTS:

Hystological findings

Muscle histological analysis of trauma group after 24 hours showed the typical modifications in normal architecture, with inflammatory reaction, vasodilatation, edema, angiogenesis and extracellular matrix formation (figure 1A). A single administration of the nitric oxide synthase inhibitor $_{L}$-NAME (100mg/kg i.p. 2 hour after the trauma) markedly attenuated all the inflammatory histological abnormalities at 24 hours, with less cellular infiltrate and edema than in the trauma group (figure 1B). On the 7th day post trauma there was distinct diffuse and poorly organized fibrosis (figure 2C e figure 2D) and increased collagen concentration (figure 1E) in the trauma group. The administration of $_{L}$-NAME was associated with more intense and focal formation of fibrotic tissue (figure 1F). Results from the sham trauma group and left gastrocnemius muscle (internal control) were similar to the untraumatized control group not shown.

Oxidative stress

Since the $_{L}$-NAME administration decreased acute inflammatory changes, we next explored whether it could influence the local production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Trauma induced a dramatic increase in tissue lipoperoxidation, measured by TBARS, by about 561% at 24 hours (Table1), indicating a strong local ROS production and lipid damage. $_{L}$-NAME administration

reduced this effect by about 51% ($p<0.05$). Moreover, myeloperoxidase (MPO) expression, commonly used as a marker of the oxidative stress and PMN inflammatory infiltration, was decreased in the $_L$-NAME group after 24 hours in comparison with the trauma group by about 34% ($p<0.05$) (Figure 2A and 2B). The nitrated proteins, measured by nitrotirosine expression, increased about 129% at 24 hours (Table 1), and the $_L$-NAME administration reduced this effect by about 71%. On the 7th day after trauma the lipoperoxidation, nitrotyrosine (table 2) and MPO (data not shown) expression were no longer observed in either group, not even where injured muscle cells were noted. NO quantification evaluated by Greiss reaction did not show any significant differences between groups in both times studied (24 hours and 7 days) (data not shown). Since generation of antioxidant enzymes is an important mechanism for the maintenance of low levels of free radicals and non-radical reactive species, we measured SOD activity, a major component of the antioxidant system and the first line of defense against cellular damage, and observed that it is increased after $_L$-NAME exposure in comparison to trauma and control groups (table 1). On 7th day after lesion the SOD activity was not significantly different between groups (table 2).

Cytokine expression and NF-κB activation

As the histological data and oxidative stress markers demonstrated that $_L$-NAME dampens muscle acute inflammatory changes after trauma, we decided to evaluate local pro inflammatory cytokine expression by RT-PCR analysis. At 24 hours, the trauma group demonstrated significantly higher mRNA levels of IL-1β and IL-6 when

compared with control and L-NAME groups ($p<0.05$) (table 1). This analysis was also done at 7 days of muscle injury and both cytokines still demonstrated significantly increased levels in trauma group compared to control group ($p<0.05$) (table 2). These cytokines are expressed in response to the activation of the transcription factor nuclear factor-κB (NF-κB). Therefore, we studied the effects on NF-κB-binding activity. The control group demonstrated no activation of NF-κB, as expected, while the trauma group showed increased activation at 24h by about 77%. Exposure to L-NAME suppressed this activation, bringing NF-κB-binding activity to control levels. On the 7th day both trauma and L-NAME treated groups did not present activation of NF-κB (figure 3).

We also studied the influence of L-NAME exposure in TGF-β expression. This cytokine is involved in muscle repair and remodeling. There was no expression in the control (untraumatized) animals. The trauma groups not did presented expression at 24h and 7 days (figure 4A and 4C). However, L-NAME treated group showed moderate expression at 24 hours with positive cells as compared to untreated mice and with stronger staining in interstitial areas at 7 days after lesion (figure 4B and 4D).

iNOS expression

Since L-NAME exposure appears to decrease the intensity of acute inflammation, we evaluated its impact on iNOS tissue expression. The control group showed no

expression of iNOS, as expected. In the trauma group there was increased iNOS expression in the cytoplasm of the muscle fiber at 24 hours by about 67% as compared to $_{L}$-NAME group (figure 5A and 5B). At 7 days both trauma and $_{L}$-NAME treated groups did not present any iNOS expression (data no shown).

Discussion

Inflammation of injured muscle is characterized by infiltrating inflammatory cells, especially neutrophil that contribute in the removal of necrotic tissue and in cytokine release [51]. A potential mechanism by which inflammatory or other cells could induce muscle injury after acute trauma is through the generation of reactive metabolites of nitrogen and oxygen, which can cause additional damage to protein, lipids and nucleic acids [43]. On the other hand, there are evidences indicating that NO can play a significant role in normal wound repair [52], although the role in collagen synthesis is not clear.

In our experimental work rats were treated with $_{L}$-NAME, a competitive inhibitor of NOS, to study the effects of decreased NO production in the acute inflammatory reaction and repair process of traumatized muscle. Acute muscle trauma was accompanied by the presence of edema, vasodilatation and inflammatory infiltration with a large number of neutrophils and macrophages (figure 1A), in line with previous findings that trauma to the muscle-tendon structure produces important inflammatory infiltrates [16,46,47]. $_{L}$-NAME administration significantly decreased this acute inflammatory reaction, with reduction of the edema and cell infiltrate. Therefore, NO production seems to have an important role in this

initial phase of the inflammation-regeneration process. Rubenstein *et al* [47] have demonstrated that L-NAME treatment in similar conditions largely decreased the tissue hyperperfusion, with decreased femoral artery blood flow and decreased capillary cross section area and blood flow, confirming the important role of NO in vasodilatation during acute muscle inflammation.

Oxidative and/or nitrative stress might be important mechanisms implicated during the destruction of the muscles fibers [56]. The inflammatory process is associated with the generation of ROS, which are produced principally during oxidative phosphorylation, while there are several sources of ROS [34,13,8], and in excess may lead to lipid, protein, and nucleic acids damage [26,13]. Although there is an extensive literature describing oxidative stress in pathophysiological situations where ROS generation are reported to be increased, or antioxidant defenses are compromised, data demonstrating that this contributes to aberrant redox signaling in skeletal muscle is scarce [23].

Our findings demonstrate an excessive degree of oxidative in traumatized muscle, as evidenced by the greater than 5-fold increase in the concentration of lipid peroxidation marker, TBARS (+561%), increase in the NO-dependent protein nitration (nitrotyrosine expression +129%), as well as increase in MPO, without alteration of the antioxidant status (SOD). L-NAME exposure diminished lipid peroxidation (-51%), tyrosine nitration (71%), as well as increased SOD antioxidant status (+189%). This was expected since NO can act as a pro-oxidant agent by reacting with $O_2^{\bullet-}$ and generating $ONOO^-$ [48,32]. The difference in enzyme activities between the two experimental groups might arise from the different levels of superoxide anion, because, in the L-NAME group, NO levels were decreased due to

$_L$-NAME administration. Therefore, the decreased interaction of superoxide anion with NO caused the increased amount of $O_2^{\bullet-}$, leading to greater Cu-Zn/SOD activity. Therefore, inhibiting NO production acutely appears to suppress both, the acute inflammatory reaction (edema, cell infiltration) and the oxidative stress response after muscle trauma. The nitrite/nitrate levels did not differ in the groups at both experimental times (24h and 7 days). Kerkweg *et al* [28] evaluated the local and systemic formation of ROS and nitric oxide 5, 45 and 180 min after induction of blunt trauma to the mouse gastrocnemius muscle and demonstrated that the local formation of ROS in the injured muscle started immediately upon induction of the mechanical trauma as indicated by changes in the glutathione redox balance, but the nitrite levels were not found to be increased at these times.

There is some evidence that the oxidative stress can elicit varying effects on the activity of antioxidant enzymes. The three primary scavenger enzymes involved in detoxifying ROS in mammalian systems are Cu-Zn/SOD, catalase and glutathione peroxidase [19]. Kocaturk *et al* [31] investigated the effect of $_L$-NAME on Cu-Zn/SOD concentration in diabetes-induced rats. The results showed that Cu-Zn/SOD activity was significantly increased in diabetic and diabetic + $_L$-NAME groups compared to control, and the increase in the second group was higher than in the first group. This study suggests that $_L$-NAME administration has ensured an additive effect on the antioxidant defense system, which was proved by the increase in Cu-Zn/SOD activity. This increase might have a protective effect against tissue damage in the acute period, with corresponding changes in zinc and copper concentrations.

Therefore, the inhibition of NO seems to have an additive effect on the endogenous antioxidant defenses. However, this increase in antioxidant effect seems

to impair the normal process of muscle repair, as studies have suggested that inhibition of NO may tip the regeneration/fibrosis balance toward fibrosis [15]. There are evidences indicating that adequate rates of NO production are essential for normal wound healing and that iNOS has been shown to be expressed during wound repair [59]. It has been shown by Schaffer *et al* [49] that NO synthesis is critical to wound collagen accumulation and acquisition of mechanical strength. Accordingly, Tews *et al* [55] have demonstrated that there is iNOS upregulation in skeletal muscle fibers during inflammatory myopathies.

In the present study, trauma was able to induce an activation of transcription factor (NF)-κB that was accompanied by increased iNOS expression and pro-inflammatory cytokines (IL-1β and IL-6), and $_{L}$-NAME exposure decreased these effects. These data point to NO-dependent activation of the NF-κB, which is a pleiotropic transcription factor activated by a number of immunological and pathological stimuli, including cytokines, oxidative stress, toll-like receptors, and bacterial and viral products [20,27]. NF-κB enhances the expression of different genes, including iNOS, further increasing NO production in a positive feedback loop [1]. Such correlation between NF-κB activation and iNOS expression has been observed in skeletal muscle of patients with chronic heart failure [2], flexor tendon repair [54], and in the model of ischemia/reperfusion of skeletal muscle [36].

After muscle injury, the first events are muscle degeneration and inflammation. Subsequently, muscle regeneration (complete repair of the muscle fibers) and fibrosis (scar tissue formation) occur concomitantly [15]. The mechanisms involved in the balance between regeneration and fibrosis are poorly understood. We observed

significantly higher levels of collagen deposition 7 days after trauma, and L-NAME treatment induced more intense formation of fibrotic tissue (figure 1F), indicating that inhibition of NO production in the early phase of muscle healing could shift the balance toward scar formation instead of muscle repair. There are a number of studies indicating that NO stimulates collagen synthesis in various cell types, like afferent arterioles, vascular smooth muscle cells, pulmonary tissue and cutaneous wound, Peyronie's disease tissue from both human specimens and from a rat model of PD [14,3,6,38,4,5], but the role of NO in regulation of collagen level in crush muscle injury model has not been previously studied.

NO and ROS seem to influence in the balance of collagen production and muscle regeneration could also involve the activation of TGF-β, which appears to be a major determinant for connective tissue proliferation and fibrosis [53]. In our study, the TGF-β1 was increased in L-NAME group at 24 hours as compared to the trauma group. This data can be correlated with the increased of the collagen that occur at the 7th day after the trauma in L-NAME group. In skeletal muscle strain injury in rats, Smith *et al* [53] determined whether TGF-β protein is present and active 48h following injury. In this study, the TGF-β expression and synthesis were evaluated by immunohistochemistry, RT-PCR and immunoblot analysis. It revealed the presence of TGF-β1 in areas of myofiber injury and increased TGF-β1 and TGF-β2 precursors. Darmani *et al* [11] examined the expression of iNOS and TGF-β in macrophage infiltrates within crush-injured digital flexor tendon and synovium of control and L-NAME-treatment rats. The results showed that during normal tendon healing the levels of TGF-β are high at first and gradually decrease after 3 weeks of injury to slightly above control uninjured levels. However, inhibition of NOS by L-NAME-

treatment at the time of injury leads to a chronic overexpression of TGF-β *in vivo* at 5 weeks after the injury, with no evidence of reduction. This observation supports our model of low NO, leading to high TGF-β and a shift to increased fibrosis.

ROS have also been linked in the pathogenesis of fibrosis. Qi *et al* [44] reported that superoxide toxicity can activate human lung fibroblast, which plays a key role in the development of pulmonary fibrosis. In this study, $O_2^{\bullet-}$ (generated from xanthine and xanthine oxidase) activated lung fibroblasts by increasing the release of TGF-β1 and collagen. Furthermore, Treiber *et al* [58] investigated the relationship between ROS and collagen. In this study they evaluated the MnSOD expression in response of the contraction of free floating collagen lattice in human dermal fibroblasts. The enhanced collagen lattice contraction was in part due to an increase in the activation of TGF-β1 and in H_2O_2 levels in collagen lattices populated with MnSOD overexpressing fibroblasts. These data suggest that ROS or $ONOO^-$ can function as key second messengers for collagen lattice contraction and perhaps act on the TGF-β1 activation pathway.

In summary, our results point to an important role of NO in the healing processes after acute muscle trauma. After a single early exposure to the NOS inhibitor $_L$-NAME there was a reduction of oxidative and nitrative stress markers and inflammatory reaction, a marked decrease in pro-inflammatory cytokines and iNOS and increase in TGF-□ expression at 24 hours. After 7 days, there was a well defined increased deposition of collagen, pointing to a shift toward healing with fibrosis under the conditions of low early concentrations of NO. The exact molecular mechanisms of this obviously important role of NO in the repair of muscle injury still need to be

further studied. It could involve an early vasodilatation effect, intracellular signaling with regulation of matrix protein synthesis and satellite cells activation. Better understanding of the molecular regulation of muscle repair will certainly have significant therapeutic implications.

Abbreviations

α	Alpha
β	Beta
C	Control group
ECM	Extracellular matrix
EMSA	Electrophoretic mobility shift assays
HTRP	Housekeeping gene hypoxanthine phosphoribosyl-transferase
HCPO	Hypochlorous acid
95%IC	Confidence intervals
IL	Iinterleukin
$_L$-NAME	nitro-$_L$-arginine methyl ester
$_L$-NAME	Trauma + $_L$-NAME group
LPO	Lipoperoxidation
mRNA	Messenger ribonucleic acid
25% and 75%	Median and percentiles
MMP	Matrix da metaloproteinase
MPO	Myeloperoxidase
NF-κB	Factor nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthesis
iNOS	Nitric oxide synthesis inducible
$O_2^{\bullet-}$	Anion superoxide
O_2	Oxygen
$ONOO^-$	Peroxinitrite
P<0.05	Significant level

Pax-7	*Paired box protein*
PMN	Polymorphonuclear neutrophilic leukocytes
Real-Time RT-PCR	Real-Time reverse transcriptase - Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
ST	Sham trauma + $_{L}$-NAME group
SOD	Superoxide dismutase
T	Trauma group
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor alpha
TGF-β	Transforming growth factor beta

Table 1 - Values of tissue lipid peroxidation and antioxidant status markers in muscle injury rats and control group, mRNA cytokines and nitrotyrosine expression and statistical significance in 24 hours after lesion

	C (n=10)	T (n=10)	$_{L}$-NAME (n=10)
TBARS (nmol/mg prot)	0.50 (0.45 – 0.55)	2.3 (2.08 – 2.52)*	1.17 (1.09 – 1.25)
SOD (USOD/mg prot)	7.8 (7.6 – 8.1)	4.70 (4.14 – 5.26)†	8.9 (6.6 -11.2)
IL-1β (Relative IL-1β RNA level)	100 (98 – 101)	478,05 (434 - 522)*	325,3 (294 – 356)‡
IL-6 (Relative IL-6 RNA level)	100 (98 – 101)	1199,7 (1180,1 - 1219,4)*	706,65 (654,6 - 758,6)‡
Nitrotyrosine (Nitrotyrosine [arbitrary units])	100 (97.3 – 102.7)	129 (125 – 133)*	92 (89.7 – 94.3)

Data were shown by mean ± 95%IC values

* p<0.05 higher versus control and $_{L}$-NAME groups

† p<0.05 lesser versus control and trauma groups

‡p<0.05 higher versus control group

Table 2 – Values of tissue lipid peroxidation and antioxidant status markers in muscle injury rats and control group, mRNA cytokines and nitrotyrosine expression and statistical significance in 7 days after lesion

	C (n=10)	T (n=10)	$_{L}$-NAME (n=10)	Significant
TBARS (nmol/mg prot)	1,59 (1,43 - 1,75)	1.92 (1.75 – 2.09)	1.67 (1.38 – 1.96)	ns
SOD (USOD/mg prot)	8,47 (7,03 – 9,91)	8.28 (7.13 – 9.43)	9.56 (8.91 – 10.21)	ns
IL-1β (Relative IL-1β RNA level)	100 (98 – 101)	113.09 (111 - 115)*	103.4 (80 - 126)	*p<0,05
IL-6 (Relative IL-6 RNA level)	100 (98 – 101)	173.3 (172.5 – 174.2)*	138.1 (113 – 163)*	*p<0,05
Nitrotyrosine (Nitrotyrosine [arbitrary units])	100 (93.7 – 106.3)	109 (106.7 – 111.3)	109 (107 – 110,9)	ns

Data were shown by mean ± 95%IC values

* p<0.05 higher versus control group

ns – not significance

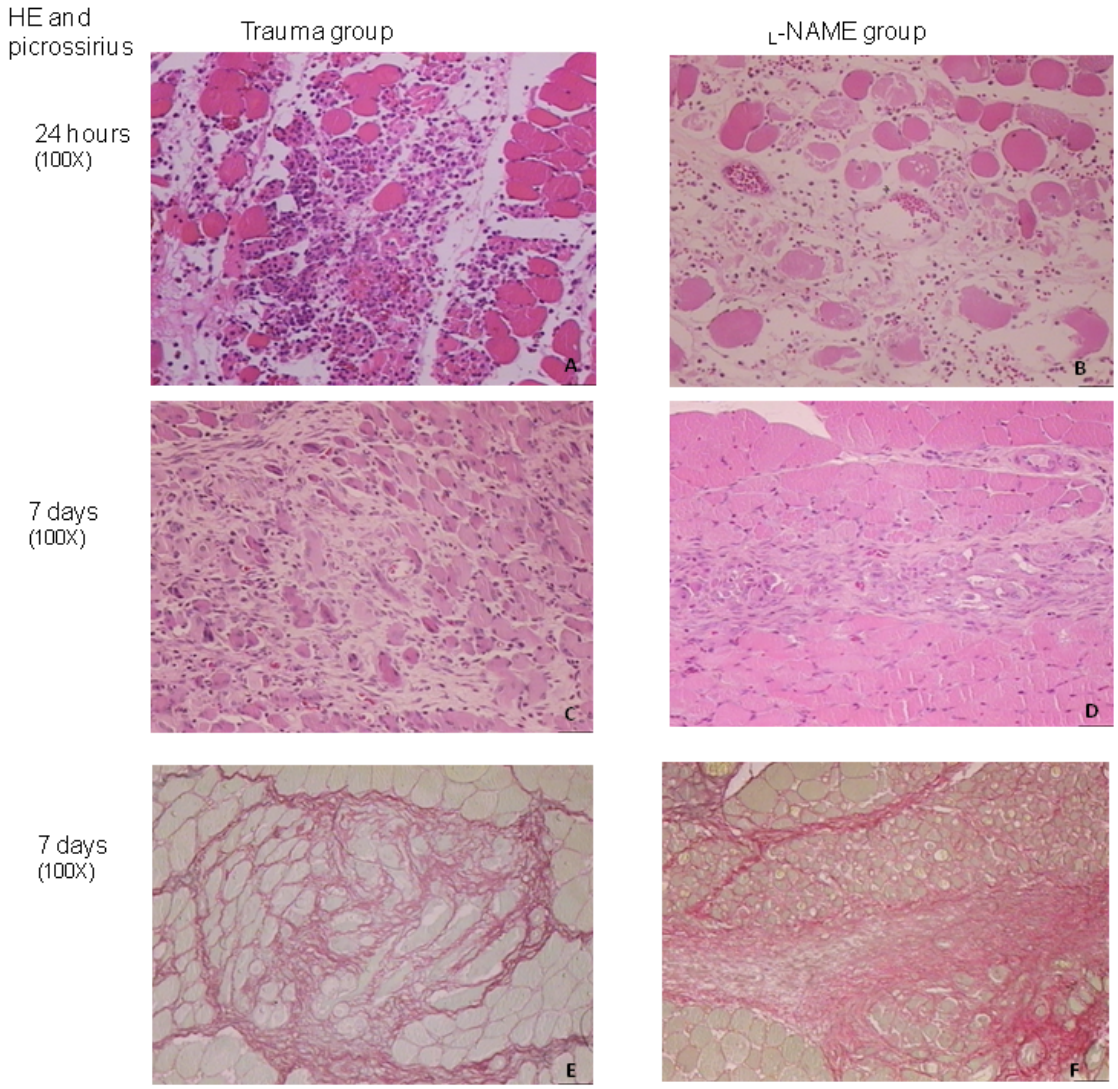

Figure 1 – Histological analysis in muscle section in coloration of the hematoxilin-eosin and picrossirius.

In A: the trauma group (HE – 100X) showed modifications of normal architecture, with angiogenesis, vasodilatation, edema and inflammatory reaction with important infiltrate of neutrophils. B :(HE – 100X) the treatment with L-NAME decreased the inflammatory infiltrate. C: trauma group (HE – 100X) in 7 days after muscle injury with increase of the fibrosis confirmed in E in picrossiruis stainning (100X). D: the L-NAME group (HE- 100X) demonstrated fewer area of repair. F: important increase of the deposition of collagen (picrossirius – 100X).

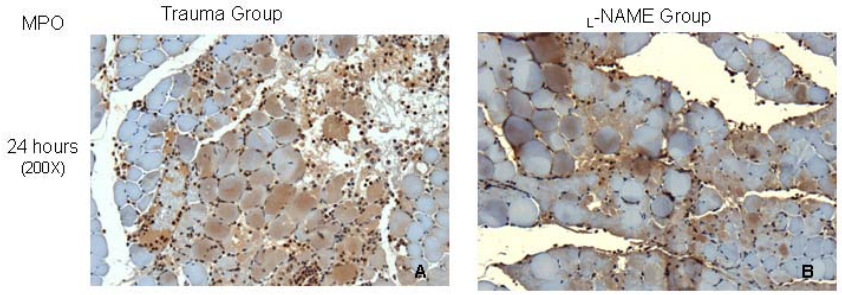

Figure 2 – Immunohistochemistry of MPO at 24 hours after trauma.

In photomicrography A the trauma group (immunohistochemistry – 200X) showed increase of the infiltrate of neutrophils in trauma group. In photomicrography B (immunohistochemistry – 200X) the treatment with $_{L}$-NAME decreased inflammatory infiltrate and MPO expression. At 7 days both trauma and $_{L}$-NAME treated group did not present any MPO expression.

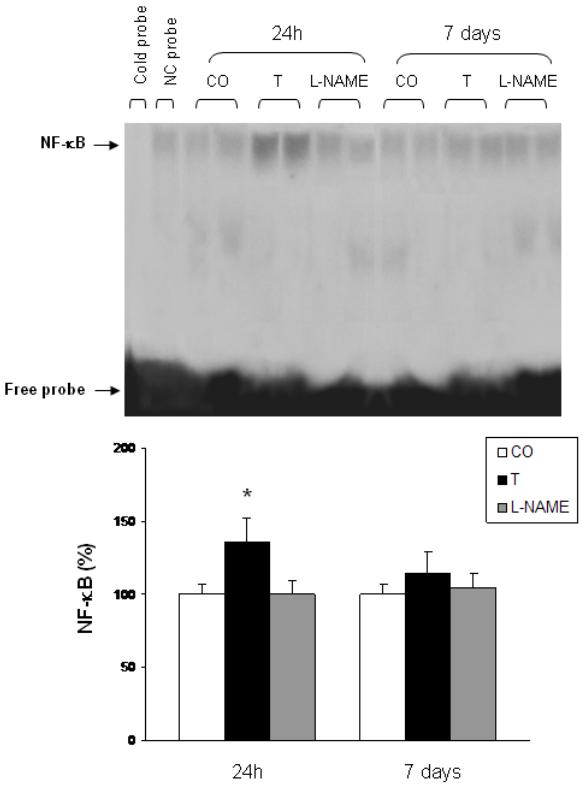

Figure 3 – NF-κB-binding activity (see text)**.**

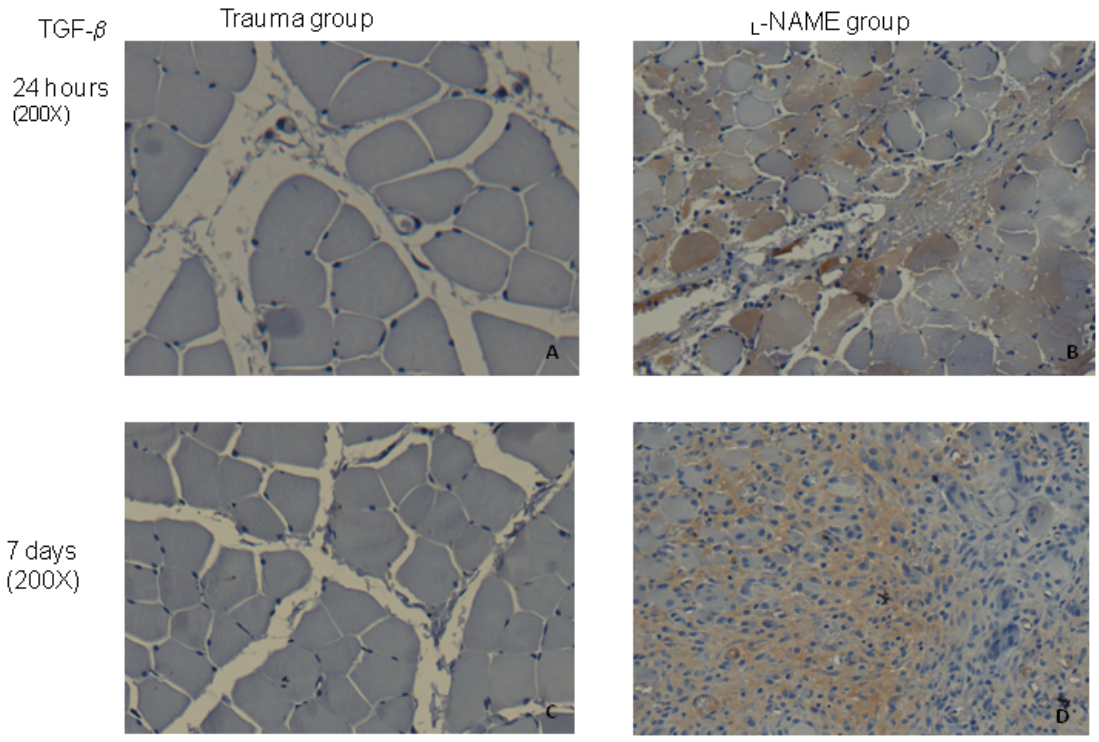

Figure 4 – Immunohistochemistry of TGF-□ at 24 hours and 7 days after trauma.

In photomicrography A the trauma group at 24 hours and the photomicrography C the trauma group at 7 days (immunohistochemistry – 200X) not did present any TGF-β expression. In photomicrography B (immunohistochemistry – 200X) the treatment with L-NAME at 24 hours showed moderate TGF-β expression. At 7 days, L-NAME treated group presented stronger staining in interstitial area.

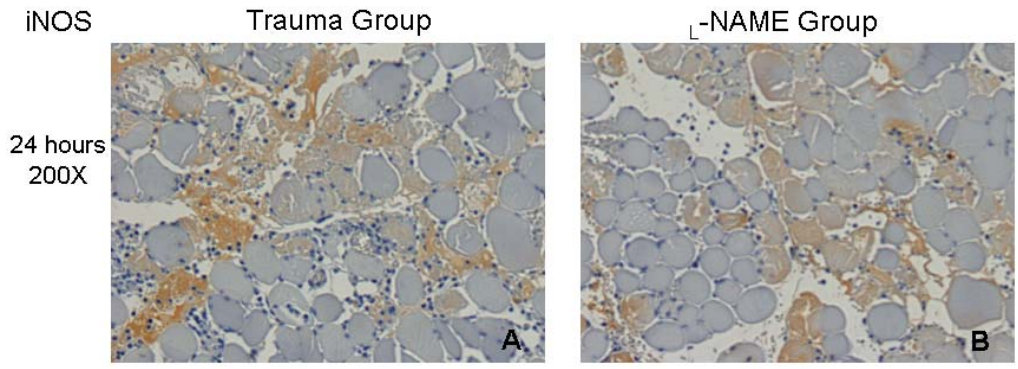

Figure 5 – Immunohistochemistry of iNOS at 24 hours after trauma.

In photomicrography A the trauma group (immunohistochemistry – 200X) showed increase of iNOS expression in the cytoplasm of the muscle fiber. In photomicrography B (immunohistochemistry – 200X) the treatment with $_{L}$-NAME decrease iNOS expression. At 7 days both trauma and $_{L}$-NAME treated group did not present any iNOS expression.

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7.3 Artigo 3

NO is involved in the regulation of regeneration and tissue fibrosis in injured skeletal muscle

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Este trabalho foi realizado com financiamento da Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) e do Fundo de Incentivo a Pesquisa e Eventos (FIPE).

NO is involved in the regulation of regeneration and tissue fibrosis in injured skeletal muscle

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Article origin: Serviço de Reumatologia do Hospital de Clínicas de Porto Alegre – (HCPA) e Universidade Federal do Rio Grande do Sul (UFRGS).

Supporting Agency: Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundo de Incentivo a Pesquisa e Eventos (FIPE).

ABSTRACT

Skeletal muscle repair can be understood as a balance between fibrosis and regeneration, the result of which may lead to complete recovery or loss of muscle function. To study the involvement of nitric oxide in post-trauma muscle repair, we used an experimental murine model of crush injury muscle. A total of 40 rats were divided into four groups: *(i)* control (CO), *(ii)* sham trauma, *(iii)* trauma, *(iv)* trauma+$_{L}$-NAME, an inhibitor of nitric oxide synthase, and the muscle tissue was analyzed in two time-points: 24 hours and 7 days. Twenty-four hours after injury, the crushed muscle was characterized by intense vasodilatation and inflammatory cell infiltrate demonstrated by histological analysis. These changes were accompanied by increased mRNA transcription for iNOS, MMP-2 and HGF and increased protein expression of the iNOS and MMP-2. Crushing injury also promoted cell proliferation and increased activation of muscle satellite cell, responsible for the regeneration of the muscle fiber. Treatment with $_{L}$-NAME blocking local NO production, greatly attenuated these histological and molecular findings at 24 hours. On the 7th day the molecular findings of both groups were comparable to the control (sham trauma) group. However, the $_{L}$-NAME group showed increase deposition of collagen and decrease of SC activation. These findings demonstrate that activation of NO during muscle crush is critical in the early phases of the skeletal muscle repair process and indicate its possible role as a regulator of the balance between muscle regeneration and fibrosis.

Key words: satellite cell, $_{L}$-NAME, muscle fibrosis, regeneration

INTRODUCTION

Skeletal muscle satellite cells (SC) are resident myogenic stem cells normally found in a quiescent state in adult skeletal muscles. These cells are mononuclear progenitor cells found in mature muscle between the basal lamina and sarcolemma (1-3). On activation, they enter the cell cycle, divide, differentiate and fuse with muscle fibers to repair damaged regions after injuries of muscle fibers (4). The mechanisms of control of this sequential process are not well defined, although it has been reported that mechanical changes in muscle can initiate events that lead to SC activation, such as a disruption of the integrity of the sarcolemma and basal lamina activates SCs (5) and/or the cytokines released by infiltrating inflammatory cells (6) Probably there are a complex combination of these multiple events, and NO seems to play a critical role. It would be clinically important to be able to manipulate these processes in order to improve muscle regeneration and minimize fibrotic scar tissue formation, resulting in better muscle functional recovery after injury.

During a crushing injury of the muscle fibers, macrophages invade the area of injury, phagocytose the necrotic tissue and produce some growth factors that are mitogenic for muscle precursor cells, such as fibroblast growth factor (FGF), transforming growth factor (TGF-β, interleukin-6), and hepatocyte growth factor (HGF) (2, 7, 8). HGF has been recently well studied as an activator of SC. It is a α–β heterodimer produced by proteolytic cleavage of a single-chain inactive precursor of 728 amino acids, localized in the extracellular domain of uninjured skeletal muscle fibers, attached to the extracellular matrix (9-11).

The role of NO in the release of HGF from the extracellular matrix has been investigated. Tatsumi *et al* (12) studied the effects of administration of $_{L}$-NAME, an inhibitor of NOS function, or $_{D}$-NAME, an inactive stereoisomer, before a stretching stimulus to the muscle. *In vivo* activation of SC and HGF release in stretched muscle was inhibited by $_{L}$-NAME, but not by $_{D}$-NAME, indicating that these processes are dependent of local NO production.

The NO-dependent HGF-matrix release could be mediated by matrix metalloproteinases (MMP), a large family of zinc-dependent endopeptidases that are capable of degrading one or several extracellular matrix proteins, such as collagens, elastin, fibronectin, laminin, and proteoglycans (13). Numerous MMPs, including MMP-2, -3, -7, and -9, are found in skeletal muscle (4, 14), possibly playing fundamental roles in muscle fiber growth and repair by regulating the integrity and composition of extracellular matrix (15). Yamada *et al* (4) demonstrated that MMP-2 mediates stretch-induced activation of skeletal muscle SC in a NO-dependent manner. Results from this study also provide evidence that NO-activated MMP2 may cause release of HGF from the extracellular matrix and contribute to SC activation *in vitro*. However, there are no studies *in vivo* confirming these observations.

This study explored the role of NO during repair process in a experimental murine model of skeletal muscle injury using an NO synthase inhibitor (nitro-$_{L}$-arginine methyl ester: $_{L}$-NAME), with attention to MMP, HGF, total cell proliferation and SC activation.

MATERIALS AND METHODS

Animals and experimental groups. Male Wistar rats weighing 250–300g were used. The animals were caged at 22°C, with 12-hour light-dark cycles and free access to food and water until the time of experiments. All experiments were performed according to the Guiding Principles for Research Involving Animals (NAS), and to the Committee of Research and Ethics in Health of the Research and Postgraduate Group of the Hospital de Clínicas de Porto Alegre.

Experimental animals were first randomly divided into four groups of ten animals each: *(i)* C (control), *(ii)* ST (sham trauma + $_{L}$-NAME), *(iii)* T (trauma), *(iv)* $_{L}$-NAME (trauma + $_{L}$-NAME). Right gastrocnemius injury was induced by a single impact blunt trauma with a press developed by the Centro Industrial de Equipamentos de Ensino e Pesquisa Ltda (CIDEP/RS, Brazil) using the procedure of Lech et al.(16). Briefly, injury was produced by a metal mass (0.459 kg) falling through a metal guide from a height of 18 cm on the middle third of the gastrocnemius muscle belly. The impact kinetic energy delivered was 0.811 J (17).

Experimental procedures. During the procedure, rats were anesthetized with ketamine chlorhidrate (Ketalar, Parke Davis, 100 mg/kg) and xilazine 2% (Rompun, Bayer, 50 mg/kg) cocktail i.p. Sham trauma rats were also anesthetized to ensure standardization, but did not received muscle trauma. The animals of the ST and $_{L}$-NAME groups received a 100mg/kg dosage of $_{L}$-NAME, intraperitoneally, 2 hours after the trauma. Rats were killed 24 hours or 7 days later for molecular evaluation

and muscle histological analysis. The animals were anesthetized with ketamine chlorhidrate and xilazine 2% cocktail i.p. The gastrocnemius muscle was rapidly removed from both legs, snap frozen in liquid nitrogen, and stored at -80°C until analysis. The entire surgical procedure took less than 10 minutes. The gastrocnemius left paw was used as internal control. However, since the results were similar to the sham trauma controls, they will not be presented.

Histology. For histological examination a section of the muscle of all animals was trimmed and fixed by immersion in 10% buffered formalin for 24 hours. The blocks were dehydrated in a graded series of ethanol and embedded in paraffin wax. Serial 4-mm sections were stained with hematoxylin and eosin or sirius red. Five sections from each sample were analyzed by two independent pathologists who had no prior knowledge of the animal groups.

Morphometric image analysis. The collagen quantification was realized by morphometric image analysis through the specific software. Briefly, from each sample, 10 images were captured from randomly selected high-power fields (magnification, 100X) and saved in TIFF format for later analysis. The halogen lamp voltage was kept constant through voltage stabilization. Collagen staining was examined quantitatively. Morphometric measurements were performed using the Adobe Photoshop CS3 Extended 10.0 (Adobe Systems Inc, San Jose, CA) computer program on every muscle structure in each captured image. Every image exhibited on the monitor was adjusted to the same threshold level, and the area of structures

was measured in pixels using the “magic wand” tool. The total amount of pixels per image remained constant in all fields. The percentage of fibrosis in each image was then calculated using the ratio of the fibrosis area to the total amount of pixels per image (18).

Real-Time RT-PCR. Total RNA was obtained by Promega Kit (Promega, Madison, WI) and quantified by Nanodrop Technologies Spectrophotometer (ND–1000 UV/VIS). First-standard cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). The negative control (no transcriptase control) was performed in parallel. cDNA was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI 7000 (Applied Biosystems). Commercially available TaqMan-Gene Expression Assays (Applied Biosystems, Weiterstadt, Germany) for HGF (GenBank accession no D90102.1 and Rn00566673_m1), iNOS (GenBank accession no D12520.1 and Rn00561646_m1), MMP-2 (GenBank accession no U65656.1 and Rn01538170_m1), and the housekeeping gene hypoxanthine phosphoribosyl-transferase (HPRT - GenBank accession no M63983.1 and Rn01527840_m1) genes were used. Relative changes in gene expression levels were determined using the $^{2-\Delta\Delta CT}$ method as described previously (19). The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of HPRT detection, referred to as ΔCT.

Western Blot Analysis. For Western blot analysis of satellite cell, muscle homogenates were prepared on cytosolic extracts based on Rizzi *et al*. (17) and

protein concentration was measured by the Bradford assay. Protein extracts (25-50μg) were separated by 10-12% sodium dodecyl sulphatepolyacrylamide gel electrophoresis and transferred electrically to polyvinyllidene difluoride membranes (Millipore, USA). The membranes were incubated overnight at 4ºC with monoclonal anti-satellite cell (SIGMA-ALDRICH, St. Louis, MO). Subsequent, the membranes were incubated with secondary HRP conjugated antibody (Dako, Glostrup, Denmark), and visualized using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden). Polyclonal anti-βactin antibody was used to confirm equal loading of protein (1:1.000, SIGMA-ALDRICH, St. Louis, MO). The density of the specific bands was quantified with an imaging densitometer (Scion Image, Maryland, MA, USA).

Immunohistochemistry. The muscle sections were washed with Tris-buffered saline (TBS), and treated with dilute normal serum for 15 min. The slides were drained and incubated with anti-iNOS (SIGMA-ALDRICH, St. Louis, MO – 1:100), anti-MMP-2 (SIGMA-ALDRICH, St. Louis, MO – 1:250), and anti-PCNA (Zymed, San Francisco, California, USA – 1:1000), for 1 h at room temperature. After been washed three times with TBS for 5 min, the slides were treated with biotin-conjugated secondary antibody for 45 min at room temperature. Subsequent to three further 5-min washes with TBS, the sections were incubated with alkaline phosphate-conjugated extravidin (SIGMA-ALDRICH, St. Louis, MO) at a dilution of 1:100 for 30 min. The sections were washed three times in TBS and then visualized using fast 5-bromo-4-chloro-3 indolyl phosphate/nitro blue tetrazolium substrate (SIGMA-ALDRICH, St. Louis, MO); known positive and negative tissue biopsies were used to control the observations.

Statistical Analysis. Results are expressed as mean values with 95% confidence intervals (95%IC) when symmetric variables were obtained and as median and percentiles (25% and 75%) when variables were asymmetric. The data were compared by analysis of variance (ANOVA); when the analysis indicated the presence of a significant difference, the means were compared with the Tukey test. Significance was accepted at $p<0.05$. Values were analyzed using the statistical package SPSS 13.0 (SPSS, Inc., Chicago, IL).

RESULTS:

Histological findings and morphometric image analysis

Histology showed a loss of normal architecture, with inflammatory reaction, angiogenesis, and vasodilatation 24 hours after trauma (figure 1A). A single administration of the nitric oxide synthase inhibitor $_{L}$-NAME (100mg/kg i.p. 2 hour after the trauma) markedly attenuated all the inflammatory histological abnormalities at 24 hours (figure 1B). On the 7^{th} day post-trauma there was distinct diffuse and poorly organized fibrosis and increased collagen concentration in the trauma group (figure 1C). The administration of $_{L}$-NAME was associated with more intense and focal formation of fibrotic tissue (figure 1D). The collagen is refractile under polarized light. Sirius red sections examined under polarized light demonstrated higher collagen concentration in $_{L}$-NAME group (figure 1F) when compared with the trauma group (figure E). The collagen quantification by morphometric image analysis

demonstrated increased collagen concentration in both trauma groups, as compared to the control group, however, it was significantly higher in L-NAME group (801.3±219 pixels) than in the trauma group (456.7±88 pixels) ($p<0.05$).

iNOS transcription and expression

Since L-NAME exposure appears to decrease the intensity of acute inflammation, we evaluated its impact on iNOS tissue transcription and expression. The control group showed no transcription or expression of iNOS, as expected. The trauma group demonstrated an increase in the iNOS transcription (table 1) and expression at 24 hours in the muscle fiber (figure 2A). On the order hand, the L-NAME group demonstrated a decrease of about 34% in the iNOS transcription (table 1) and expression at 24 hours (figure 2B). However, at 7 days both the trauma and L-NAME treated groups did not present any detectable iNOS transcription or expression (data no shown).

Growth factors transcription and expression

We also studied the influence of L-NAME exposure in MMP-2 transcription and expression. This protein is involved in the breakdown of extracellular matrix in normal physiological remodeling processes. There was no expression or transcription detected in the control (untraumatized) animals. The trauma group presented moderate expression (figure 3A) and an amplified transcription (table 1) at 24h. L-NAME treated group had similar expression levels as the trauma group, but with a

different expression pattern that was more restricted (figure 3B), with less positive cells as compared to untreated mice. After 7 days, the transcription decreased significantly in both groups (data no shown), however, the cellular expression was persistent in the trauma group and absent in the group $_{L}$-NAME-treated (figure 3C and 3D).

We analyzed the influence of $_{L}$-NAME exposure in HGF transcription. The control group showed no transcription of HGF, as expected. The trauma group presented an increase of the transcription at 24h. Nevertheless, the $_{L}$-NAME group showed decreased transcription at 24h (table 1). There was no difference between these groups after 7 days.

Total cell proliferation

We investigated the influence of $_{L}$-NAME exposure on the glocal cell proliferation of the injured muscle. The PCNA is a marker widely used for cell proliferation, including satellite cell proliferantion. In our study, the control group showed no expression of PCNA. The trauma group demonstrated an increase in proliferation activity at the injury site at 24 hours after trauma (figure 4A). The administration of $_{L}$-NAME decreased proliferative activity by about 34% at 24 hours (figure 4B). After 7 days, both groups demonstrated similar cellular proliferation at the injury site, which was still increased in relation to the control group (figure 4C and 4D).

Satellite cell expression

Finally, we studied the influence of L-NAME in satellite cell activation. The PAX-7 is a member of the paired box family of transcription factors, which is a marker of activated SC. In our study, the control group showed no SC activation. The trauma group demonstrated an increase in activation at 24 hours after trauma, while the administration of L-NAME significantly inhibited this increment in the activation. After 7 days, both groups demonstrated similar levels of activation (figure 5).

Discussion

The importance of satellite cell activity in skeletal muscle regeneration has been considered for some time. It is known that muscle repair is initiated by mechanical or chemical stimuli, which lead to the activation of quiescent satellite cells. On activation, they enter the cell cycle, divide, differentiate and fuse with muscle fibers to repair damaged regions of muscle fibers (4). In our study, total cell proliferation was analyzed by anti-PCNA immunohistochemistry, and SC proliferation by PAX-7 expression. The trauma group presented increased cell proliferation and SC expression at 24 hours post-trauma, but a single L-NAME administration, a NOS inhibitor, was able to decrease the activation of these cells.

These data, in conjunct with the findings of the histological study (sirius red), where the L-NAME-treated showed greater collagen concentration (figure 1), .

Allen *et al.* (9) first demonstrated that HGF could act as an activator of quiescent SC *in vitro.* In further experiments, Tatsumi *et al.* (20), Sheehan *et al.* (21) Tatsumi *et al.* (22) have demonstrated that HGF can be released from muscle matrix upon injury, and has the ability to activate early division of adult SC in culture and in

muscle tissue. These authors have also demonstrated that HGF mRNA is expressed in adult SC and can act in autocrine fashion. Regulation of HGF function in muscle can occur both in a transcriptional level and by its high-affinity binding to heparin sulfate proteoglycans of the tissue matrix. The mechanism of HGF release from the tissue matrix in response to injury may be an example of a common cellular response to injury or mechanical perturbation.

Our experiments demonstrated *in vivo* that the release of HGF was dependent on NO synthesis (table 1). Tatsumi *et al.* (23), based in an *in vitro* experiment, proposed that mechanical stretch stimuli triggers an intracellular cascade of events in the muscle fibers, which is pH-dependent and involves NOS activation, eventually leading to HGF release from the extracellular compartment and subsequent SC activation. Wozniak *et al.* (10) tested the hypothesis that NO and HGF are required to regulate SC activation on normal fiber, and that NOS-I (nNOS) down-regulation alters the response to stretch Suzuki *et al.* (24) investigated possible involvement of HGF from non-muscle organs during muscle regeneration. This study showed that in rat femoral muscle, HGF protein levels were elevated within 1 hour after muscle injury, with a simultaneous proteolytic activation of HGF protein. Semiquantitative RT-PCR analysis revealed an elevation of HGF mRNA expression after muscle injury in the liver and spleen, and also an increase of HGF protein levels in the spleen, suggesting the presence of endocrine HGF-inducing factor(s) during muscle regeneration.

The possible candidate as the NO-dependent factor for HGF-matrix release is the matrix metalloproteinases (MMP). Yamada *et al.* (15) recently provided evidence that MMPs mediate HGF release from extracellular matrix of SC and that this step in

the pathway is downstream from NO synthesis. In the present study, we demonstrated that MMP-2 is present in skeletal muscle injury 24 hours after trauma by mRNA RT-PCR and immunohistochemistry (table 1 and figure 2). Wang *et al.* (25) demonstrated, using *in vitro* and *in vivo* experiments, that myoblast transplantation was greatly improved after MMP-1 treatment within the dystrophic skeletal muscles of MDX mice. Thus, MMP may be able to improve muscle function recovery after injury or disease by increasing both the number of myofibers that are generated by activated myoblasts and the size of myoblast coverage area by promoting migration. However, it has not been determined how NO stimulates the enzymatic activity of MMP2 after muscle injury. Yamada *et al.* (4) in experiments with stretch-stimulated SC cultures, demonstrated that exposure to a NO donor induced the conversion of the inactive form of MMP (proMMP2) to the active MMP2. Therefore, NO is a key signal responsible for SC activation and growth factors production such as MMP and HGF after skeletal muscle injury. In our experiments it was demonstrated that there is overexpression of iNOS, both by RT-PCR and immunohistochemistry, at 24 hours after trauma. At the cellular level, shear stress generated by damaged fibers within the basal lamina is thought to stimulate synthesis of NO by neuronal and endothelial NOS (26). Numerous studies have demonstrated increase iNOS in different inflammatory processes (17, 27). There are some evidences that calcium-calmodulin is involved in the activation pathway for NO synthesis. Tatsumi et al. (28) studied cultures that were treated with a calcium ionophore for 2h activated cultured SC and contained active HGF, similar to the effect of mechanical stretch or NO donor treatments. The response was abolished by addition of calmodulin inhibitors or $_{L}$-NAME. Therefore, results from these experiments provide an additional insight that

calcium-calmodulin mediates HGF release from the matrix via increased NO synthesis.

In summary, we presented *in vivo* data that support the notion that the muscle regeneration process depends on initial NO production, with subsequent matrix metalloproteinase activation, release of hepatocyte growth factor from the extracellular matrix, increased total cell proliferation and increased of SC expression. Blocking early NO production, besides decreasing early cell proliferation and SC activation, induced increased collagen deposition, indicating that this molecule has an important role in the regeneration/fibrosis balance of the muscle repair process. Since regeneration is important for full functional recovery after muscle injury, better comprehension of the diverse factors regulating this balance may lead to innovative therapeutic approaches to muscle trauma.

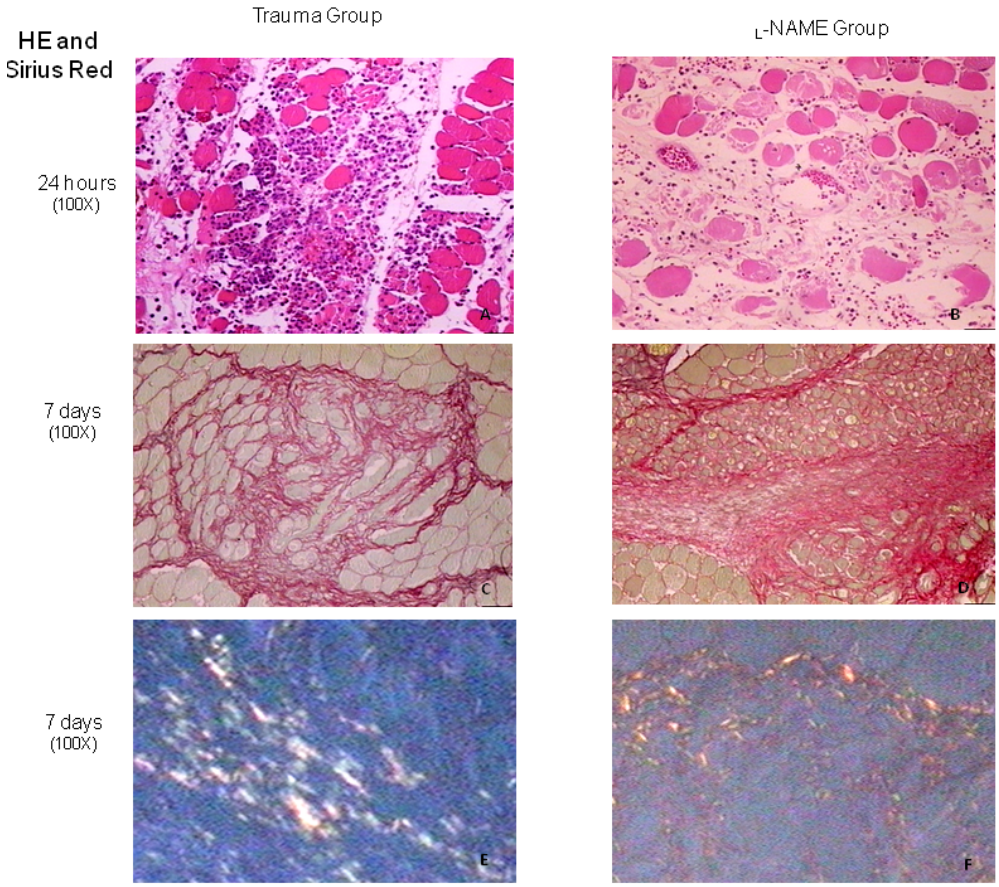

Figure 1 – Histological analysis in muscle section in coloration of the hematoxilin-eosin and picrossirius.

In A: the trauma group (HE – 100X) showed modifications of normal architecture, with angiogenesis, vasodilatation, edema and inflammatory reaction with important infiltrate of neutrophils. B :(HE – 100X) the treatment with $_{L}$-NAME decreased the inflammatory infiltrate. C: trauma group (HE – 100X) in 7 days after muscle injury with fewer fibrosis confirmed in E in picrossiruis stainning under polarized light. (100X). D: the $_{L}$-NAME group (HE- 100X) was associated with more intense and focal formation of fibrotic tissue. F: important increase of the deposition of collagen (picrossirius – 100X) under polarized light.

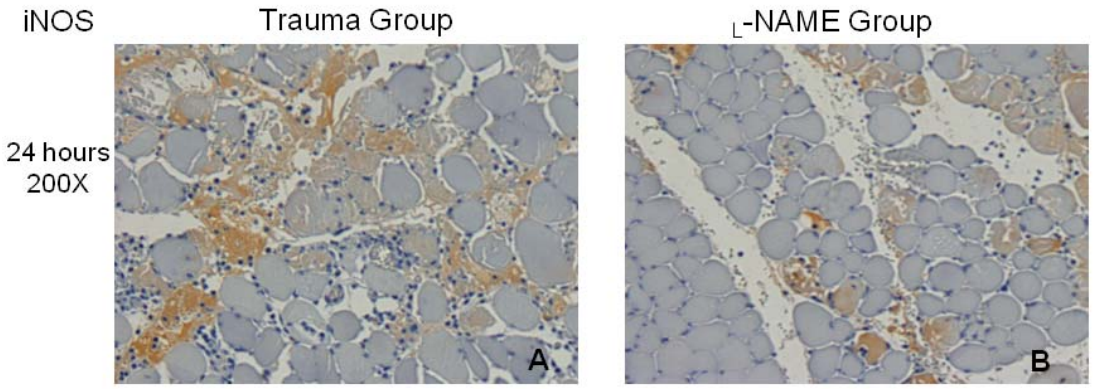

Figure 2 – Immunohistochemistry of iNOS at 24 hours after trauma.

In photomicrography A the trauma group (immunohistochemistry – 200X) showed increase of iNOS expression in the cytoplasm of the muscle fiber. In photomicrography B (immunohistochemistry – 200X) the treatment with $_{L}$-NAME decrease iNOS expression. At 7 days both trauma and $_{L}$-NAME treated group did not present any iNOS expression.

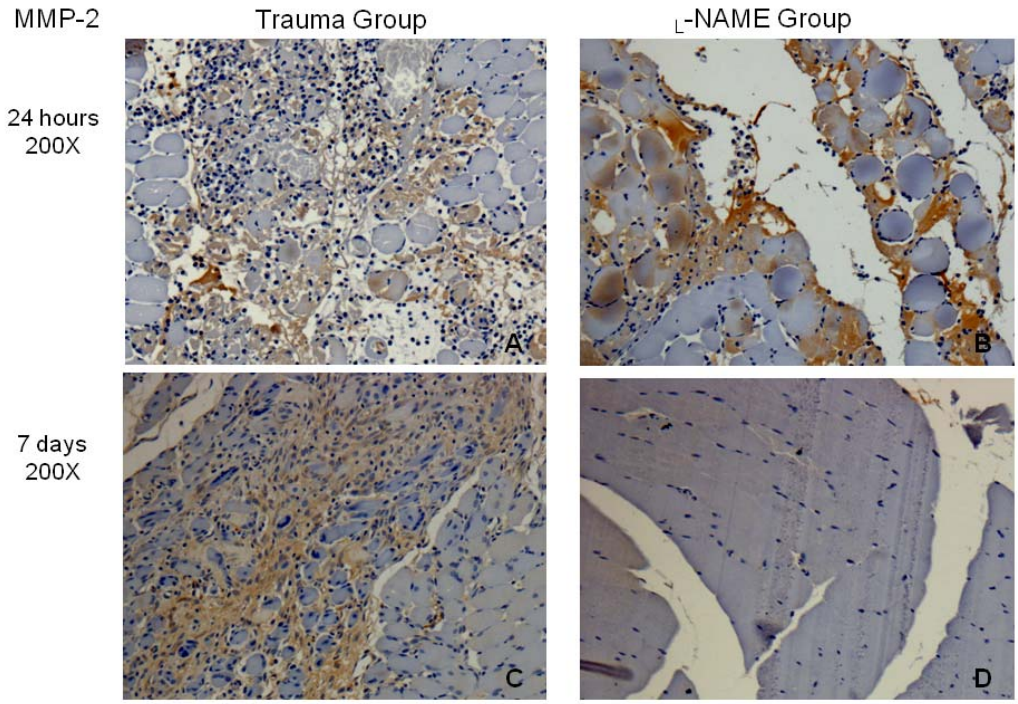

Figure 3 – Immunohistochemistry of MMP-2 at 24 hours and 7 days after trauma.

In photomicrography A the trauma group (immunohistochemistry – 200X) showed moderate expression. In photomicrography B (immunohistochemistry – 200X) the treatment with $_{L}$-NAME had similar expression levels as the trauma group, but with a different expression pattern that was more restricted. After 7 days the cellular expression was persistent in the trauma group (photomicrography C) and absent in the group $_{L}$-NAME-treated (photomicrography D).

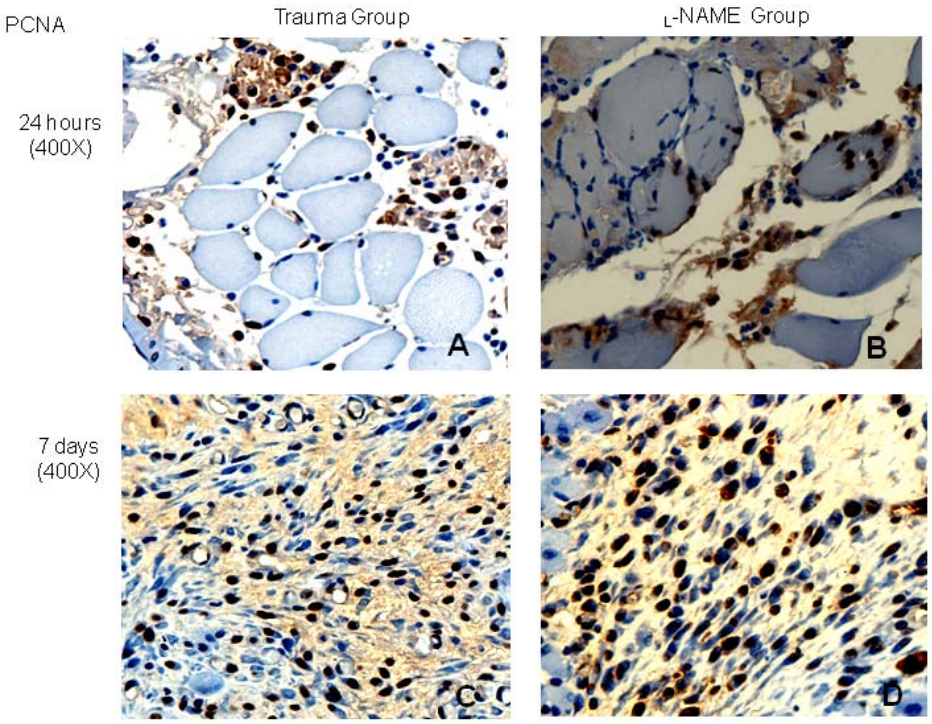

Figure 4 – Immunohistochemistry of PCNA at 24 hours and 7 days after trauma.

In photomicrography A the trauma group (immunohistochemistry – 200X) showed moderate total cell proliferation at 24 hours after trauma. In photomicrography B (immunohistochemistry – 200X) the treatment with $_{L}$-NAME decreased the expression levels of total cell proliferation. After 7 days the cell proliferation was persistent in both groups (photomicrography C and D).

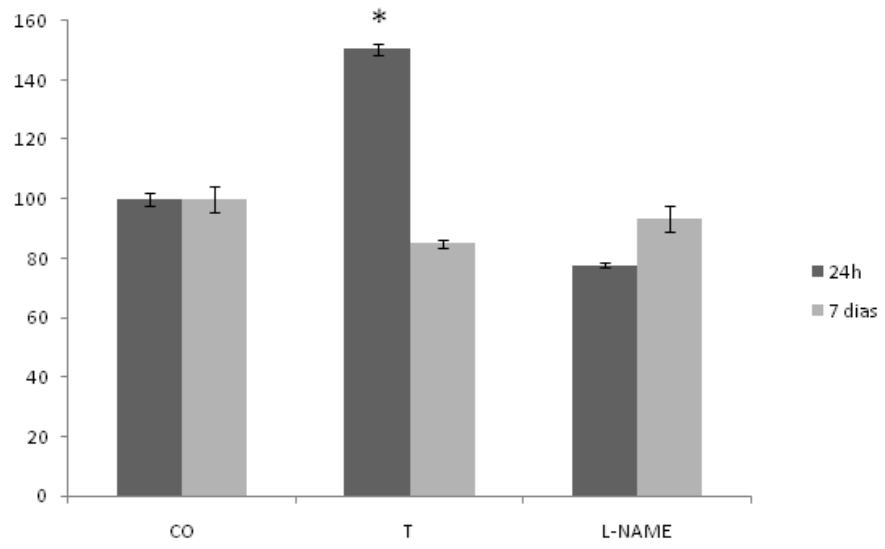

Figure 5 – Activation of satellite cell (see text).

Table 1 – Transcription mRNA of iNOS, MMP-2 and HGF markers in muscle injury rats and statistical significance in 24 hours after lesion

	CO (n=10)	T (n=10)	$_L$-NAME (n=10)	Significant
iNOS	100 (99.5 – 100.5)	1365.3 (1245.3 – 1485.3)*	366.5 (347.1 – 385.7)	*p<0,05
MMP-2	100 (99.4 – 100.6)	202.8 (181.8 – 223.8)*	167.4 (166.3 – 168.6)	*p<0,05
HGF	100 (98.6 – 101.4)	142.1 (124.3 – 159.8)*	69.04 (58.9 – 79.2)	*p<0,05

Data were shown by mean ± 95%IC values

*p<0.05 higher versus control and $_L$-NAME groups

**p<0.05 higher versus control group

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8 CONSIDERAÇÕES FINAIS

Considerando-se que a regeneração muscular inadequada acarreta incapacidades funcionais, laborais e principalmente predisposição a novas lesões, é essencial que todas as medidas no sentido de diminuir suas sequelas sejam tomadas. Neste estudo experimental demonstramos, pela primeira vez, *in vivo,* que o óxido nítrico parece ser indispensável para regeneração muscular.

- Foi demonstrado no presente estudo que trauma direto sobre o músculo gastrocnêmio alterou a arquitetura muscular, com presença de células inflamatórias, edema e degeneração de células musculares

- A presença de células inflamatórias foi comprovada pela expressão da mieloperoxidase por imunoistoquímica que demonstrou significativo infiltrado de macrófagos local;

- A presença da lesão muscular gerou uma produção maciça de radicais livres de oxigênio e nitrogênio no local da lesão;

- O processo inflamatório muscular estava relacionado à ativação do fator de transcrição NF-κB;

- Esse processo inflamatório local foi acompanhado por aumento na expressão do mRNA no tecido muscular de citocinas pró-inflamatórias (IL-1β, IL-6), bem como iNOS;

- A expressão da iNOS foi confirmada por imunoistoquímica;

- O tratamento com $_{L}$-NAME foi capaz de amenizar as alterações morfológicas e moleculares inflamatórias causadas pelo trauma;

- A proliferação celular foi avaliada pela expressão da proteína PCNA que demonstrou estar aumentada no grupo trauma em 24 horas após a lesão quando comparada ao grupo tratado com $_{L}$-NAME. No 7° dia após a lesão ambos os grupos demonstraram intensa proliferação celular;

- A ativação das células satélites foi avaliada pela expressão do PAX-7, marcador específico de células satélites ativadas, que demonstrou estar aumentada no grupo trauma em 24 horas após a lesão quando comparada ao grupo tratado com $_{L}$-NAME. No 7° dia após a lesão ambos os grupos não demonstram ativação destas células;

- No 7° dia após a lesão foi observada grande produção de colágeno em ambos os grupos lesionados, sendo significativamente maior no grupo tratado com $_{L}$-NAME;

- Portanto, o bloqueio de NO em fases iniciais da lesão muscular parece alterar o equilíbrio regeneração/fibrose em favor da fibrose.

9 PERSPECTIVAS FUTURAS

Atualmente essa linha de pesquisa vem sendo desenvolvida e estruturada no Laboratório de Biologia Molecular em Doenças Autoimunes e Infecciosas do HCPA. Estudar a resposta das células satélites em modelo de inflamação crônica como a artrite experimental, parece ser um imenso desafio uma vez que as células satélites participam da regeneração muscular. Pacientes portadores de doenças inflamatórias crônicas, como artrite reumatóide, apresentam inúmeras complicações sendo algumas delas extra-articulares como a sarcopenia reumatóide, ainda pobremente estudada.

Estudar a influência de doadores de óxido nítrico sobre a regeneração do músculo esquelético também parece ser uma estratégia interessante, especialmente sobre a inflamação crônica.

Se confirmados esses dados em novos experimentos *in vivo* e em modelos experimentais de inflamação crônica podemos vislumbrar novas estratégias terapêuticas para pacientes com lesões musculares agudas e crônicas com processos de regeneração e respostas funcionais adequadas.

Com o objetivo de prosseguir com a realização de diversos estudos sobre o papel do óxido nítrico no reparo muscular, novo experimento deverá ser iniciado com utilização de doador e inibidor da produção de óxido nítrico. A partir disso, deseja-se desenvolver novos projetos de pesquisa, com a participação de alunos de graduação, mestrado e doutorado.