UNIVERSIDADE FEDERAL DOS VALES DO JEQUITINHONHA E MUCURI

Programa de Pós-Graduação em Reabilitação e Desempenho Funcional

Andrea Ferreira Lemes de Morais

MÚSCULO ESQUELÉTICO DO MODELO MDX SUBMETIDO A UM TREINO DE BAIXA INTENSIDADE REVELA ADAPTAÇÕES NA FIBROSE E FUNÇÃO MUSCULAR AO LONGO DO TEMPO

Diamantina

2019

Andrea Ferreira Lemes de Morais

MÚSCULO ESQUELÉTICO DO MODELO MDX SUBMETIDO A UM TREINO DE BAIXA INTENSIDADE REVELA ADAPTAÇÕES NA FIBROSE E FUNÇÃO MUSCULAR AO LONGO DO TEMPO

Dissertação apresentada ao programa de Pós-Graduação em Reabilitação e Desempenho Funcional da Universidade Federal dos Vales do Jequitinhonha e Mucuri, como requisito para obtenção do título de Mestre.

Orientadora: Prof.ª Drª. Thaís Peixoto Gaiad Machado.

Diamantina

2019

Elaborado com os dados fornecidos pelo(a) autor(a).

M827m Morais, Andrea Ferreira Lemes de.
Músculo esquelético do modelo mdx submetido a um treino de baixa intensidade revela adaptações na fibrose e função muscular ao longo do tempo / Andrea Ferreira Lemes de Morais, 2019.
59 p. : il.

Orientadora: Thaís Peixoto Gaiad Machado

Dissertação (Mestrado – Programa de Pós-Graduação em Reabilitação e Desempenho Funcional) - Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, 2019.

1. Distrofia muscular de Duchenne. 2. Modelo mdx. 3. Exercício de baixa intensidade. 4. Fibrose. I. Machado, Thaís Peixoto Gaiad. II. Título. III. Universidade Federal dos Vales do Jequitinhonha e Mucuri.

CDD 615.82

Ficha Catalográfica – Sistema de Bibliotecas/UFVJM
Bibliotecária: Jullyele Hubner Costa – CRB6/2972

ANDREA FERREIRA LEMES DE MORAIS

"Músculo esquelético do modelo *mdx* submetido a um treino de baixa intensidade revela adaptações na fibrose e função muscular ao longo do tempo".

Dissertação apresentada ao MESTRADO EM REABILITAÇÃO E DESEMPENHO FUNCIONAL, nivel de MESTRADO como parte dos requisitos para obtenção do título de MESTRA EM REABILITAÇÃO E DESEMPENHO FUNCIONAL

Orientador (a): Prof.ª Dr.ª Thais Peixoto Gaiad Machado

Data da aprovação : 20/09/2019

Prof.Dr.ª THAIS PEIXOTO GAIAD MACHADO - UFVJM

Prof.Dr.ª ANA PAULA SANTOS - UFVJM

Prof.Dr. ALEX SANDER DIAS MACHADO - UFVJM

Agradecimentos

Agradeço a Deus, ao meu guia espiritual pelos dias de luz nos momentos mais difíceis.

Agradeço a minha orientadora Prof.ª Dr.ª Thais Gaiad por compartilhar seu conhecimento, pela confiança, atenção e cuidado durante o desenvolvimento deste trabalho que me proporcionou crescer, não só profissionalmente.

Ao Prof. Dr. Alex Machado, agradeço pela disponibilidade, sua contribuição foi imprescindível para realização deste trabalho, sempre muito atencioso e positivo ao dividir suas experiências. Bem como à Prof.ª Dr.ª Ana Paula que, gentil e carinhosamente, fez parte da minha banca, muito contribuindo para abrilhantar mais este trabalho.

À minha família agradeço os votos de confiança e por entenderem que minha ausência se fazia porque estava plantando sonhos em outros lugares. Hoje colhemos a realização, obrigada! Amo vocês!

Agradeço ao Grupo *mdx*-colônia por me ajudar a entender aquele "quadro doido" dos cruzamentos, por estarem nos dias longos de coleta. Agradeço ao André, Jéssica e Marcílio pela certeza de sempre estarem lá.

Agradeço a Paula por estar comigo no início do experimento, pelo carinho e cuidado, pelo incentivo quando tudo parecia não dar certo, à Natália por todo auxílio no laboratório, sempre solícita. Agradeço a Pollyane pela ajuda de sempre e amizade.

1 MÚSCULO ESQUELÉTICO DO MODELO MDX SUBMETIDO A UM TREINO DE BAIXA INTENSIDADE REVELA ADAPTAÇÕES NA FIBROSE E FUNÇÃO MUSCULAR AO LONGO DO TEMPO

RESUMO

O objetivo do estudo foi investigar os efeitos de um protocolo de treinamento de baixa intensidade na fibrose muscular e na função do modelo *mdx*. Camundongos do modelo *mdx*, machos, com 8 semanas de idade (T0; n = 8) foram submetidos a um protocolo de oito semanas de treinamento em esteira horizontal (9m / min, 3x / semana, 30min / dia). Os camundongos foram alocados aleatoriamente no grupo Treinado (*mdx*-T, n = 8) e outros oito animais *mdx* foram sedentários (*mdx*-NT, n = 8). As medições funcionais in vivo de força e desempenho foram avaliadas ao longo do tempo do protocolo (T0, T4, T8). A análise do TGF-β1 e a histomorfometria da área das fibras de colágeno intramusculares utilizando vermelho picrossirius sob luz polarizada, foram realizadas no músculo tibial anterior (TA) e Soleus (SOL). Como resultado, houve diminuição de força após 4 semanas de treinamento (T4), que foi recuperada no T8 no *mdx*-T. A porcentagem de área de fibras de colágeno intramuscular diminuiu no músculo SOL de *mdx*-T em T4 quando comparado a T0 (p = 0,025) e TA de *mdx*-T teve uma área menor em T8 quando comparado a TA do grupo *mdx*-NT (p = 0,002). O TGF-β1 foi observado no sarcoplasma dos músculos TA e SOL do grupo *mdx*-NT, com alteração dependente da idade. O treinamento de baixa intensidade em esteira provocou adaptação da fibrose muscular distrófica e manteve a força de preensão do modelo *mdx*.

Palavras-chave: Distrofia muscular de Duchenne, modelo *mdx*, exercício de baixa intensidade, fibrose, TGF-β1.

1 *MDX* SKELETAL MUSCLE SUBJECT TO LOW INTENSITY TRAINING REVEALS FIBROSIS ADAPTATION AND MUSCULAR FUNCTION OVER TIME

ABSTRACT

The objective of this study was to investigate the effects of a low-intensity training protocol on muscle fibrosis and function of the *mdx* model. *Mdx* male animals with 8 weeks of age were (T0; n=8) underwent a protocol of eight weeks at a horizontal treadmill (9m/min, 3x/week, 30min/day). *Mdx* animals were randomly allocated at the Trained group (*mdx*-T, n=8) and other eight *mdx* animals were sedentary (*mdx*-NT, n=8). In vivo functional measurements of strength and performance were assessed over time of protocol (T0, T4, T8). Analysis of TGF-β1 and histomorphometry of the area of intramuscular collagen fibers using picrossirius red under polarized light were done on tibial anterior (TA) and Soleus (SOL). Strength decreased after 4 weeks of training (T4) and was regained at T8 at *mdx*-T. The percentage of intramuscular collagen fibers area decreased at SOL muscle of *mdx*-T at T4 when compared to T0 (p=0,025) and TA of *mdx*-T had a lesser area at T8 when compared to TA of *mdx*-NT group (p=0,002). TGF-β1 was observed at the sarcoplasm of TA and SOL muscles of *mdx*-NT group, with an age-dependent change. Treadmill low-intensity training provoked dystrophic skeletal muscle adaptation of fibrosis and maintained grip strength of the *mdx* model.

Key-words: Duchenne muscular dystrophy, *mdx* model, low-intensity training, treadmill, fibrosis, TGF-β1.

LISTA DE ILUSTRAÇÕES

ARTIGO CIENTÍFICO – CAPÍTULO 1

LISTA DE TABELAS

ARTIGO CIENTÍFICO – CAPÍTULO 1

SUMÁRIO

REFERENCIAL TEÓRICO

1 INTRODUÇÃO

As distrofias musculares são doenças hereditárias e os distúrbios musculares progressivos são caracterizados pela degeneração e necrose das fibras musculares (NAKAMURA; TAKEDA, 2011). São causadas por informações genéticas incorretas ou ausentes que impedem o corpo de produzir as proteínas necessárias para construir e manter os músculos saudáveis (GIANOLLA *et al*., 2013). O curso clínico varia de formas graves com início na primeira década e progressão rápida para formas mais suaves com início posterior e curso mais lento (HAUERSLEV *et al*., 2012).

1.1 Distrofia muscular de Duchenne

A Distrofia Muscular de Duchenne (DMD) é um transtorno progressivo que afeta os músculos estriados e cardíacos (PERTL *et al*, 2013). É caracterizada por progressão clínica rápida e severa, sendo a forma mais comum de doença muscular hereditária na infância (MAH *et al*., 2014), afetando meninos numa proporção de 1: 3.500 nascidos vivos (JARRAH *et. al*., 2014).

A maioria dos meninos com DMD apresenta, entre 3 e 5 anos de idade, atraso motor grosso, anormalidades da marcha, dificuldade para se levantar do chão e quedas frequentes (YIU; KORNBERG, 2015). A fraqueza muscular progressiva leva à perda da mobilidade independente no início da adolescência (FRAYSSE *et al*., 2017). A fraqueza muscular nos músculos respiratórios, bem como a cardiomiopatia, se desenvolvem no início da adolescência (HASEGAWA *et al*., 2017).

Intervenções respiratórias, cardíacas, ortopédicas, reabilitadoras e o uso de corticosteroides levaram a melhorias na função, qualidade de vida, saúde e longevidade. Crianças que hoje são diagnosticadas têm a possibilidade de uma expectativa de vida de quatro décadas (BUSHBY *et al*., 2010), tendendo ao óbito como resultado de insuficiência cardíaca e/ou respiratória (NAKAMURA; TAKEDA, 2011).

1.1.1 Fisiopatologia

A DMD é uma doença recessiva ligada ao cromossomo X causada por mutações do gene da distrofina (KHARRAZ *et al*., 2014), uma proteína de membrana que participa, juntamente com outras proteínas, do complexo distrofina-glicoproteínas (DGC), com função de proporcionar integridade e rigidez à fibra (TIMPANI; HAYES; RYBALK, 2015) (FIG 1). Segundo Thakur e colaboradores (2018), a falta de distrofina nos músculos dos pacientes com DMD e modelos murinos bem caracterizados de DMD tornam as fibras musculares frágeis e propensas a lesões, e resultam em perturbações significativas na homeostase do $Ca2^{+}$.

A elevação prolongada do $[Ca^{2+}]$ intracelular desencadeia a inflamação crônica, ciclos repetidos de degeneração com regeneração progressiva e ineficaz que levam à perda de fibras musculares e à infiltração de material fibrótico e outros materiais não contráteis (SERRANO; MUÑOZ-CÁNOVES, 2017). O enfraquecimento do sarcolema (NAKAMURA; TAKEDA, 2011) especialmente durante a atividade contráctil intensa (MANN *et al*., 2011), e os ciclos de degeneração e regeneração resultam na substituição de miofibras funcionais por conjuntivo fibroso e adiposo (BACH; MARTINEZ, 2011).

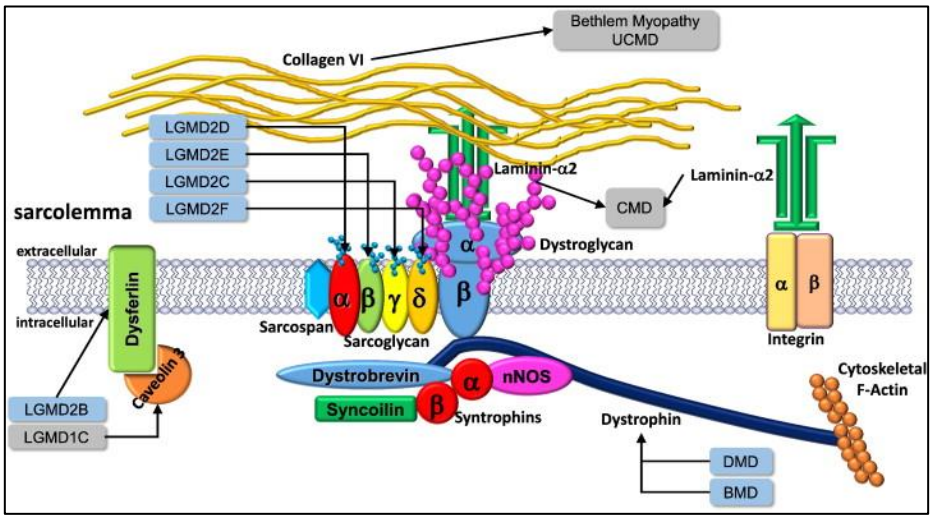

Fig. 1 – Expressão da distrofina celular. Fonte: NIGRO; PILUSO, 2014.

A manutenção do músculo esquelético é essencial para a preservação da saúde metabólica e da função contrátil e depende da remodelação contínua do tecido muscular iniciado tanto por atividades extrínsecas (por exemplo, atividade física ou suplementos alimentares) quanto por mecanismos intrínsecos (por exemplo, efeitos de moléculas endógenas liberadas de vários tecidos / células). Um aspecto essencial do remodelamento e reparo muscular é uma população funcional de células-tronco específicas do músculo quiescentes, comumente chamadas de células satélites (SCs) (FARUP *et al.*, 2014).

Após um processo de lesão o músculo esquelético tem a capacidade de se reparar (JUNQUEIRA; CARNEIRO, 2013). A regeneração muscular bem-sucedida começa com uma infiltração inflamatória inicial que resulta em degeneração muscular, fagocitose de miofibras lesadas e deposição de elementos extracelulares, maturação e remodelamento das miofibras e finalmente restauração da capacidade funcional do músculo reparado (FERRARI *et al*., 2005). As SCs associadas às miofibras são a principal fonte de células progenitoras, que proliferam e se diferenciam para reparar o tecido lesado (SEALE *et al*., 2000). No músculo ausente de distrofina a capacidade de se regenerar é subsequentemente perdida devido à exaustão destas células, que resulta na formação de cicatrizes permanentes (MANN *et al.,* 2011) o que também dificulta o movimento destas células durante o processo da regeneração (LUZ; MARQUES; SANTO NETO, 2002).

As células inflamatórias que se infiltram no local da necrose são uma fonte rica de fator de crescimento transformador beta (TGF-β). O TGF-β exerce então o seu efeito profibrótico nos fibroblastos, que aumentam a produção de proteínas da matriz extracelular (MEC). Uma quantidade excessiva de produção da MEC leva ao eventual aparecimento de fibrose (DESGUERRE *et al*., 2009).

O TGF-β é uma citocina multifuncional que atua em vários tipos celulares diferentes. Em particular, no músculo esquelético, inibe as respostas miogênicas, regula a remodelação da MEC e estimula a fibrose (KIM *et. al*., 2005; MASSAGUE, 2012) promovendo a expressão de genes profibróticos como o colágeno I (ISMAEEL *et al*, 2019) podendo modular a reparação muscular, não só por causar impacto nos fibroblastos responsáveis pela deposição excessiva da MEC, mas também por

influenciar as células estaminais e as células inflamatórias presentes e ativas na área lesionada (CISTERNAS *et al.*, 2014; DELANEY *et al.*, 2017).

Os níveis de expressão de TGF-β1 estão significativamente correlacionados com o grau de patologia e gravidade clínica da DMD (SONG *et al.*, 2017). Assim, a inibição do TGF-β1 modifica a proliferação de mioblastos, fibroblastos ou células inflamatórias encontradas no músculo lesionado. Também evita o acúmulo de componentes da MEC, restabelecendo o equilíbrio entre a deposição e a degradação de proteínas e proteoglicanos da MEC e permitindo a reparação eficiente do tecido (DELANEY *et al.*, 2017).

A manutenção da aquiescência das SCs células-tronco musculares residentes (MuSCs), é crucial uma vez que a ativação prematura das MuSCs pode levar à sua exaustão e, assim, prejudicar o potencial regenerativo (CHEUNG; RANDO, 2013), bem como a proteção contra danos oxidativos e estresses metabólicos e biomecânicos (THAKUR *et al*., 2018). Proteínas de choque térmico (HSPs) interagem com os principais reguladores de muitas vias de transdução de sinal que controlam a homeostase celular, proliferação, diferenciação e morte celular (MAYER; BUKAU, 2005).

1.1.2 Fibrose Muscular

Fibrose é definida como a deposição excessiva ou não regulamentada de componentes de MEC e é uma marca especial da DMD (KHARRAZ *et al*., 2014). A fibrose tem consequências negativas para o tratamento potencial da DMD, pois altera a função muscular devido à consequente perda da flexibilidade (característica do tecido muscular), dificulta a revascularização no tecido (NGUYEN *et al*., 2005; ALEXAKIS; PARTRIDGE; BOU-GHARIOS, 2007) além de reduzir a quantidade de músculo alvo disponível para terapia e reparo (KHARRAZ *et al*., 2014).

A principal fibra de reposição tecidual e que gera a fibrose muscular é o colágeno dos tipos I, III e IV. De acordo com Mackey, Donnelly e Roper (2005), os colágenos do tipo I e III coexistem no mesmo tecido ao mesmo tempo e suas proporções

relativas definem as propriedades funcionais deste tecido. Na predominância do colágeno do tipo I, o tecido apresenta uma alta resistência à tensão. Já tecidos com maiores proporções de colágeno do tipo III possuem grande flexibilidade. O colágeno tipo IV é o componente estrutural mais abundante da membrana basal, fornecendo suporte mecânico à mesma. Em certas regiões desempenha o papel de filtro, isto pode ser evidenciado nos rins (CALVI *et al*., 2012).

1.1.3 Tratamento para distrofia muscular de Duchenne

Pesquisas vêm sendo realizadas desde 1987 com diferentes estratégias terapêuticas, como as terapias celulares, gênicas e farmacológicas, entretanto a doença permanece sem cura até os dias atuais (TIMPANI; HAYES; RYBALK, 2015). Os tratamentos atuais para DMD são sintomáticos e objetivam melhorar a longevidade e a qualidade de vida, mas dificilmente a prevenir perda de função muscular (PERTL *et al*., 2013).

1.1.3.1 Exercício Terapêutico

Tem sido debatido por muitos anos se o exercício muscular é benéfico ou nocivo para pacientes com transtornos miopáticos (GIANOLA *et al*., 2013). O exercício tem sido proposto como tratamento para DMD, para manter a força muscular e evitar contraturas; no entanto, essa recomendação não foi aceita por unanimidade, pois o exercício pode danificar os músculos distróficos (HYZEWICZ; RUEGG; TAKEDA, 2015).

A atividade muscular adequada mantém um estado saudável e previne a inflamação crônica e doenças como obesidade e distúrbios cardiovasculares. O equilíbrio delicado entre os efeitos positivos e nocivos do exercício é muito menos claro progressivamente em miopatias degenerativas como a DMD (CAMERINO, 2014). Hoje

sabemos que o treinamento de força não é recomendado, sendo o exercício aeróbico submáximo (como a natação) mais apropriado (YIU *et al.* 2015).

A fisioterapia tem sido fortemente utilizada a fim de se retardar os danos causados pela DMD ao tecido muscular, porém não existe um consenso baseado em evidências sobre o melhor tipo ou intensidade do exercício terapêutico (MARKERT *et al*., 2011). De acordo com as considerações de cuidado para DMD em Birnkrant e colaboradores (2018), atividade ou exercício muscular excêntrico e exercícios de alta resistência ou treinamento de força devem ser evitados. Por outro lado, o exercício ou atividade aeróbica submáxima é recomendado, especialmente no início da doença - evitando excesso de esforço e excesso de trabalho e permitindo descanso adequado. A natação é altamente recomendada desde o estágio ambulatorial inicial e pode ser frequentemente continuada até a idade adulta. Já o Ciclismo tem sido recomendado como uma forma aeróbica de atividade submáxima, e o ciclismo assistido e o movimento assistido por robótica podem ser usados na idade adulta. A atividade física segura pode ser apoiada por equipamento adaptativo apropriado e tecnologia assistiva.

1.2. Modelos experimentais para estudos pré-clínicos

Modelos de animais são necessários para elucidação da patogênese e avaliação da eficácia e toxicidade durante o desenvolvimento de terapias. Na DMD, dentre os diversos modelos pré-clínicos, o camundongo *mdx* é o mais comum, ainda que seu fenótipo tenha progressão moderada, possui homologia genética e bioquímica com humanos (VAINZOF *et al*., 2008) e pode ser reproduzido de forma fácil e confiável (NAKAMURA; TAKEDA, 2011). No camundongo *mdx*, o início agudo da necrose da miofibra ocorre cerca de 21 dias de vida (PERTL *et al*., 2013), entre 3 a 6 semanas de idade sofre uma necrose surpreendente (McGREEVY *et al*., 2015); os músculos continuam passando por ciclos de necrose e regeneração ao longo da vida do camundongo e isso só diminui, sendo mais suave, depois de 12 semanas (NAKAMURA; TAKEDA, 2011).

Metodologias e equipamentos variados são utilizados por pesquisadores para determinar a eficácia de novas terapias avaliando o grau de gravidade e progressão da doença (LOPES, 2010). Certamente, o objetivo final de qualquer abordagem terapêutica potencial direcionada ao tratamento da DMD é a melhoria da função muscular. O benefício de potenciais terapias desenvolvidas em modelos animais deve necessariamente ser demonstrado por meio de testes funcionais (KLEIN *et al*.,2012). O teste de força de preensão do membro anterior avalia a força muscular única e, através do teste de corrida no rotarod, a força muscular, a coordenação, o equilíbrio podem ser determinados de maneira confiável e reproduzível, com base nos procedimentos operacionais padrão disponíveis da rede TREAT-NMD (AARTSMA-RUS; VAN PUTTEN, 2014).

Em todas as espécies o exercício tem um impacto diferente em diferentes músculos dos membros, dependendo de quais músculos são recrutados para os regimes de exercício específicos (GROUNDS *et al*., 2008). Os músculos de contração rápida são geralmente os mais suscetíveis à distrofia muscular (e também ao envelhecimento) (GROUNDS *et al*., 2014).

O equilíbrio entre os efeitos musculares positivos e prejudiciais do exercício para o músculo comprometido em miopatias progressivamente degenerativas, como a DMD, com importantes implicações para o estabelecimento de terapias físicas apropriadas, é muito delicado. Os parâmetros para avaliação destas modalidades de exercício foram descritas por Grounds *et al*. (2008) a fim de favorecer a padronização e troca de informações entre diferentes grupos de pesquisa.

O exercício contínuo forçado ou em esteira em declive pode ser usado para piorar o fenótipo *mdx* (TANIGUTI 2010; GRANGE *et al.,* 2015). Um protocolo de 30 minutos correndo em uma esteira horizontal a uma velocidade de 12 m / min, duas vezes por semana durante pelo menos 4 semanas, causa uma aumento da distrofia em adultos *mdx* e é amplamente utilizado na pesquisa pré-clínica (RADLEY-CRABB *et al.*, 2012; DE LUCCA *et al.*, 2014).

Exercícios voluntários como natação ou corrida em roda, parecem retardar a progressão da doença; entretanto, respostas específicas do músculo a determinados estímulos mecânicos podem ocorrer em relação à idade do camundongo e à duração do

período de treinamento (RADLEY-CRABB *et al.*, 2012 ; CAPOGROSSO *et al*., 2017). Em 2007, Kacsor e colaboradores estudaram os efeitos do treino de baixa intensidade, em esteira, no músculo esquelético do camundongo *mdx* e foi avaliado o comportamento de marcadores de stress oxidativo. Os autores sugerem que a baixa intensidade parece ser benéfica para o músculo distrófico, uma vez que favoreceu o movimento sem aumentar os marcadores de stress oxidativo. Hyzewich *et al*., em 2015, também atestaram efeitos benéficos do exercício analisando os parâmetros de stress oxidativo com o treinamento de baixa intensidade em piscina. Kogelman e colaboradores (2018) avaliaram o efeito da corrida voluntária durante 8 semanas, em animais idosos, sobre a função e patologia do músculo esquelético e coração e demonstraram que houve melhora do desempenho muscular ao mesmo tempo em que os níveis plasmáticos de creatinoquinase CK e a morfologia do quadríceps e do diafragma em camundongos *mdx* não foram afetados negativamente pelo exercício.

O exercício adequadamente prescrito é essencial para manter a saúde geral e tem múltiplos benefícios terapêuticos nos distúrbios metabólicos e cardiovasculares. O exercício também é o principal regulador da plasticidade do músculo esquelético. A sinalização mecanossensitiva é modulada pela intensidade, frequência e duração da atividade muscular, resultando em adaptações na função, morfologia e metabolismo em relação à demanda (CAPOGROSSO *et al.*, 2017). Assim, este estudo investigou os efeitos do treinamento de baixa intensidade em esteira, ao longo do tempo, sobre a função e reparação do musculo esquelético em modelo animal da DMD. Nossa hipótese é que esta intensidade de treinamento levaria a um equilíbrio entre movimentação ativa e lesão muscular, evitando o desuso sem aumentar a lesão do músculo com ausência da distrofina.

2 OBJETIVOS

2.1 Objetivo geral

Investigar se um treino de baixa intensidade em esteira exacerba a lesão do músculo esquelético distrófico do modelo *mdx* analisando o comportamento de parâmetros funcionais e morfológicos ao longo do período de intervenção.

2.2 Objetivos específicos

Para elucidar o comportamento dos parâmetros funcionais e morfológicos dos animais submetidos ao treino de baixa intensidade em esteira, este estudo pretende:

- Analisar a função muscular por meio da força de preensão dos animais e do desempenho no Rotarod;

- Analisar a morfologia da fibra muscular pelo estudo histopatológico;

- Determinar a razão entre a área de fibrose e a área muscular total por morfometria sob luz polarizada;

- Analisar a localização proteica do marcador de reparação- TGF-β1, pela análise imunohistoquímica.

3 REFERENCIAS

ALEXAKIS, C.; PARTRIDGE, T.; BOU-GHARIOS, G. Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen over production. **American Journal of Physiology- Cell physiology**, v. 293, p. 661-669, 2007.

BACH, J.; MARTINEZ, D. Duchenne muscular dystrophy: continuous noninvasive ventilatory support prolongs survival. **Respiratory Care**, v.56, p.744-750, 2011. https://doi.org/10.4187/respcare.00831

BIRNKRANT, D. et al. Diagnosis and management of Duchenne muscular dystrophy, part 2: **respiratory, cardiac, bone health, and orthopaedic management**. Lancet Neurol. vol. 17, n. 4, p. 347-361, 2018. doi:10.1016/S1474-4422(18)30025-5.

BUSHBY, K. et al. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. **Lancet Neurol**, v. 9, n.1, p. 77- 93, 2010.

CALVI, E. N. et al. **An experimental model for the study of collagen fibers in skeletal muscle**. Acta Cir Bras., v. 27, n. 10, 2012.

http://dx.doi.org/10.1590/S0102-86502012001000003

CAMERINO, G. M. et al. Gene expression in mdx mouse muscle in relation to age and exercise: **aberrant mechanical–metabolic coupling and implications for pre-clinical studies in Duchenne muscular dystrophy.** Human Molecular Genetics, v. 22, p. 1-13, 2014.

CAPOGROSSO, R. F. et at. Contractile efficiency of dystrophic mdx mouse muscle: in vivo and ex vivo assessment of adaptation to exercise of functional end points. **Journal Appl Physiol**., v. 122, n. 5, p.828- 843, 2017.

CHEUNG, T. H.; RANDO, T. A. Molecular regulation of stem cell quiescence. **Nature Reviews Molecular Cell Biology,** v. 14, p. 329- 340, 2013.

CISTERNAS, P. et al. Role of wnt signaling in tissue fibrosis, lessons from skeletal muscle and kidney**. Curr Mol Med.,** v. 14, n. 4, p. 510-522, 2014.

DELANEY, K. et al. The role of TGF-b1 during skeletal muscle regeneration. **Cell Biology International**, v. 41, p. 706-715, 2017.

DE LUCA, A. et al. **Use of treadmill and wheel exercise for impact on mdx mice phenotype**. Treat-NMD neuromuscular network, p. 1-9, 2014.

DE LUCA, A. et al. **Use of grip strength meter to assess the limb strength of mdx mice**. Treat-NMD neuromuscular network, p. 1-9, 2014.

DESGUERRE, I. et al. Endomysial fibrosis in Duchenne muscular dystrophy: a marker of poor outcome associated with macrophage alternative activation. **J Neuropathol Exp Neurol.**, v. 68, n.7, p. 762-773, 2009.

FARUP, J. et al. Influence of exercise contraction mode and protein supplementation on human skeletal muscle satellite cell content and muscle fiber growth. **Journal of Applied Physiology** (Bethesda, MD: 1985), v. 117, n. 8, p. 898-909, 2014.

Doi: 10.1152 / japplphysiol.00261.2014

FERRARI, et al. Regeneration process in the muscle injury: **a review**. Fisioterapia em movimento, Curitiba, v.18, n.2, p. 63-71, 2005.

FRAYSSE, et al. Gait characterization in golden retriever muscular dystrophy dogs using linear discriminant analysis. **BMC Musculoskeletal Disorders**, v. 18, n. 153, p. 1-9, 2017.

GIANOLA, S. et al. Efficacy of muscle exercise in patients with muscular dystrophy: A systematic review showing a missed opportunity to improve. **Plos One**, v. 8, p. 1-9, 2013.

GRANGE, R. W. et al. **Use of treadmill and wheel exercise to assess dystrophic state**. Treat-NMD Neuromuscular Network, p. 1-12, 2015.

GROUNDS, M. D. et al. Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. **Neurobiol Disease**, v. 31, p. 1- 19, 2008.

GROUNDS, M. D. et al. **Quantification of histopathology in haemotoxylin and eosin stained muscle sections**. Treat-NMD neuromuscular network, p. 1-13, 2014. http://www.treatnmd.eu/research/preclinical/dmd-sops/

HASEGAWA, S. Molecular hydrogen alleviates motor deficits and muscle degeneration in *mdx* mice. **Redox report**, v. 22, n. 1, 2017.

HAUERSLEV, S. et al. Calpain 3 is important for muscle regeneration: evidence from patients with limb girdle muscular dystrophies. **BMC Musculoskeletal disorders**, v. 13, n. 43, p. 1-11, 2012. http://www.biomedcentral.com/1471-2474/13/43

HYZEWICZ, J. et al. **Low intensity training of mdx mice reduces carbonylation and increases expression levels of proteins involved in energy metabolism and muscle contraction**. Free Radic Biol Med., v. 82, p. 122- 136, 2015.

ISMAEEL, A. et al. Role of transforming growth factor-β in skeletal muscle fibrosis: a review. **Int. Journal Mol. Sci**., n. 20, p. 2446- 2019. http:/doi:10.3390/ijms20102446

JARRAH, A. S. et al. **A mathematical model of skeletal muscle disease and immune response in the mdx mouse**. BioMed Research International, p.1-11, 2014. http://dx.doi.org/10.1155/2014/871810

JUNQUEIRA, L. C.; CARNEIRO, J. Tecido Muscular. In: **Histologia Básica: Texto e Atlas**. 12 ed. Rio de Janeiro: Guanabara Koogan, p.177-198, 2013.

KACSOR, J. J. et al. **Low intensity training decreases markers of oxidative stress in skeletal muscle of mdx mouse**. Free Radic Biol Med, v. 43, n. 1, p. 145-154, 2007.

KHARRAZ, Y. et al. **Understanding the process of fibrosis in Duchenne muscular dystrophy**. BioMed Research International, p. 1-12, 2014. http://dx.doi.org/10.1155/2014/965631

KLEIN, S. M. et al. Noninvasive in vivo assessment of muscle impairment in the *mdx* mouse model – a comparison of two common wire hanging methods with two different results**. Journal of Neuroscience Methods**, v. 203, p. 292-297, 2012.

KIM, H. S. et al. **Doxycycline inhibits TGF-beta1-induced MMP-9 via Smad and MAPK pathways in human corneal epithelial cells.** Invest Ophthalmol Vis Sci, v. 46, n. 3, p. 840-848, 2005.

KOGELMAN, B. et al. Voluntary exercise improves muscle function and does not exacerbate muscle and heart pathology in aged Duchenne muscular dystrophy mice. **Journal of Mol and Cell Cardiology**, v. 125, p 29- 38, 2018.

LUZ, M. A. M.; MARQUES, M. J.; SANTO NETO, H. Impaired regeneration of dystrophindeficient muscle fibers is caused by exhaustion of myogenic cells. **Brazilian Journal of Medical and Biol Research**, v.35, n.6, p. 69- 695, 2002.

MACKEY, A. L.; DONNELLY, A. E.; ROPER, H. P. Muscle connective tissue contente of endurance-trained and inactive individuals. **Scandinavian Journal of Med & Sci in Sports**, v. 15, n. 6, p. 402- 408, 2005.

MASSAGUE, J. **TGFβ signalling in context**. Nature Reviews Molecular Cell Biology, v. 13, p. 616- 630, 2012.

MAYER, M. P.; BUKAU, B. Hsp70 chaperones: **cellular functions and molecular mechanism**. CMLS, Cell. Mol. Life Sci., v. 62, p. 670- 684, 2005.

McGREEVY, J. W. et al. Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. **Journal List. Dis Model. Mech**., v. 8, p. 195- 213, 2015.

MAH, J. K. et al. A systematic review and meta-analysis on the epidemiology of Duchenne and Becker muscular dystrophy. **Neuromuscular Disorders**, p. 1-10, 2014. http://dx.doi.org/10.1016/j.nmd.2014.03.008

MANN, C. J. et al. **Aberrant repair and fibrosis development in skeletal muscle**. Skeletal Muscle, v. 1, n. 1, p. 21, 2011.

MARKERT, C. D. et al. **Exercise and Duchenne muscular dystrophy: toward evidenced based exercise prescription**. Muscle &Nerve, v. 43, p. 464- 478, 2011.

NAKAMURA, A.; TAKEDA, S. Mammalian models of Duchenne muscular dystrophy: pathological characteristics and therapeutic applications. **Journal of Biomedicine and Biotechnology**, v.143, p.1040- 1042, 2011.

NGUYEN, F. et al. **Microvessel density in muscles of dogs with golden retriever muscular dystrophy**. **Neuromuscular Disorders**, v. 15, p. 154- 163, 2005.

NIGRO, V.; PILUSO, G. Spectrum of muscular dystrophies associated with sarcolemmal-protein genetic defects. **Biochimica et Biophysica Acta**, v. 1852, p. 585-593, 2015.

PERTL, C. et al. A new web-based method for automated analysis of muscle histology. **BMC Musculoskelet Disorders**, v. 16, p. 14- 26, 2013.

RADLEY-CRABB, H. et al. A single 30 min treadmill exercise session is suitable for ‘proofof concept studies’ in adult mdx mice: a comparison of the early consequences of two different treadmill protocols. **Neuromuscular Disorders**, v. 22, p. 170-182, 2012.

SEALE, P. et al. **Pax7 is required for the specification of myogenic satellite cells**. Cell, v. 102, p. 777- 786, 2000

SERRANO A. L.; MUÑOZ-CÁNOVES P. Fibrosis development in early-onset musculardystrophies: mechanisms and translational implication. **Seminars in Cell Developmental Biology**, v.64, p. 181-190, 2017

SONG, Y. et al. **Expression levels of tgf-β1 and ctgf are associated with the severity of Duchenne muscular dystrophy**. Experimental and Therapeutic Medicine n. 13, p. 1209-1214, 2017.

TANIGUTI, A. P. T. Fibrose muscular em camundongos *mdx*: **efeitos do exercício físico e agente anti-fibrótico**. São Paulo, Campinas [s.n.], 2011.

THAKUR, S. S. et al. **Therapeutic potential of heat shock protein induction for muscular dystrophy and other muscle wasting conditions**. Philos Trans R Soc Bio Sci ,v. 373,n. 1738,p.1- 11, 2018.

TIMPANI, C. A.; HAYES, A.; RYBALK, E. Revisiting the dystrophin-ATP connection: How half a century of research still implicates mitochondrial dysfunction in Duchenne muscular dystrophy aetiology. **Medical Hypotheses**, v. 85, n. 6, p. 1021-1033, 2015.

VAINZOF, M. et al. Animal models for genetic neuromuscular diseases. **Journal of Mol. Neuroscience**, v. 34, n. 3, p. 241-248, 2008.

YIU, M. E.; KORNBERG, A. J. Duchenne muscular dystrophy. **Journal of Paediatrics and Child Health**, p. 1- 6, 2015.

ARTIGO

Treadmill low-intensity training mediates fibrosis and maintains the function of dystrophic muscle

Andrea Ferreira Lemes de Morais[1], Alex Sander Dias Machado[1], André Nobre Evangelista[1], Priscilla Avelino Ferreira Pinto[1], Thais Peixoto Gaiad Machado[1]

[1]Post-graduate Programm of Physical therapy and functional performance - PPGReab. Department of Physiotherapy, Universidade Federal dos Vales do Jequitinhonha e Mucuri, UFVJM – Campus JK - Diamantina, MG, Brazil.

* Corresponding author.

Thais Peixoto Gaiad Machado

Address: Federal University of Jequitinhonha and Mucuri Valleys, UFVJM, Campus JK - Diamantina/MG Rodovia MGT 367 - Km 583, nº 5000 - Alto da Jacuba. Zip Code: 39100-000.

Tel: +55 (38) 3532-1239.

thaispgm@gmail.com

Conflict of interest: No potential conflict of interest relevant to this article was reported.

Blind Title Page

Treadmill low-intensity training mediates fibrosis and maintains the function of dystrophic muscle

Abstract

Objective: Investigate the effects of a low-intensity training protocol on muscle fibrosis and function of the *mdx* model.

Design: *Mdx* male animals with 8 weeks of age were (T0; n=8) underwent a protocol of eight weeks at a horizontal treadmill (9m/min, 3x/week, 30min/day). *Mdx* animals were randomly allocated at the Trained group (*mdx*-T, n=8) and other eight *mdx* animals were sedentary (*mdx*-NT, n=8). In vivo functional measurements of strength and performance were assessed over time of protocol (T0, T4, T8). Analysis of TGF-β1 and histomorphometry of the area of intramuscular collagen fibers using picrossirius red under polarized light were done on tibial anterior (TA) and Soleus (SOL).

Results: Strength decreased after 4 weeks of training (T4) and was regained at T8 at *mdx*-T. The percentage of intramuscular collagen fibers area decreased at SOL muscle of *mdx*-T at T4 when compared to T0 (p=0,025) and TA of *mdx*-T had a lesser area at T8 when compared to TA of *mdx*-NT group (p=0,002). TGF-β1 was observed at the sarcoplasm of TA and SOL muscles of *mdx*-NT group, with an age-dependent change.

Conclusion: Treadmill low-intensity training provoked dystrophic skeletal muscle adaptation of fibrosis and maintained grip strength of the *mdx* model.

Key-words: Duchenne muscular dystrophy, *mdx* model, low-intensity training, treadmill, fibrosis, TGF-β1.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common muscular dystrophy that affects 1:3500 boys due to an x linked disorder with genetic inheritance. Mutations in the dystrophin gene lead to protein expression deficiency resulting in muscular degeneration, necrosis and atrophy (Song et al., 2017). Dystrophin is typically expressed on skeletal, smooth and cardiac muscles and on the brain but its absence on DMD is particularly observed on skeletal and cardiac muscles (Grange, Gainer, Marschner, Talmadge e Stu, 2002).

Dystrophic skeletal muscle undergoes a fast degeneration and this chronic cycle progressively leads to exhaustion of satellite cells pools. As regeneration decline and cannot follow the fast muscle degeneration, myofibres are changed to adipous and fibrotic tissue (Rodrigues, Echigoya, Fukada e Yokoda, 2016). Inflammatory cells that infiltrate at necrosis place are a source of transforming growth factor beta (TGFβ).

TGF-β is a multifunctional cytokine that acts in different cellular types. Its role on the regulation of skeletal muscle inflammatory response, inhibition of regeneration, regulation of the extracellular matrix remodeling and fibrosis promotion is well established on literature (Kim & Lee, 2017), and also is positively regulated on mdx muscle (Ismael, Kim, Sirk, Smith, Bohannon & Koutakis 2019).

Skeletal muscle fibrosis increases the area of extracellular matrix (ECM) on muscle sections and, as a result of the high deposition of collagen fibers, increases of muscle stiffness and contractibility; it limits muscle function and mobility. Muscle fibrosis also limits the amount of available muscle tissue to therapy and repair (Ismael, Kim, Kirk, Smith, Bohannon e Koutakis, 2019).

Constant cycles of muscle fibers degeneration/regeneration and incomplete regeneration leads to a severe and progressive muscle loss, weakness and premature death (Bushby, Finkel, Birnkrant, Case, Clemens e Cripe et al., 2010).

Several important events of DMD natural history were clarified by preclinical studies using the mdx model. They include the sarcolemma mechanical fragility (Nakamura & Takeda, 2014), aberrant calcium homeostasis (Serrano & Muñoz-

Cánoves, 2017), mitochondrial distress, imbalanced oxidative stress (Thakur, Widerski, Ryall e Lynch, 2018) and chronic inflammation (Hyzewicz, Ruegg e Takeda, 2015a).

The aerobic training was proposed to enhance muscle strength and avoid contractures on boys with DMD, but the impact and beneficial mechanisms due to the aerobic exercise regimen need more research (Hyzewicz et al., 2015a; Zelikovich, Quattrocelli, Salamone, Kuntz e McNally, 2019) once the studies with mdx model produced inconsistent results.

High intensity training has been suggested to exacerbate muscular dystrophy (Camerino et al, 2014; De Luca et al., 2003; Hyzewicz et al., 2015a; Zelikovich, Quattrocelli, Salamone, Kuntz e McNally, 2019). In the other hand, voluntary exercise as swimming and voluntary wheel running seems to delay the disease progression (Yiu & Kornberg, 2015; Zelikovich, Quattrocelli, Salamone, Kuntz e McNally, 2019).

This study investigated the effects of chronic low-intensity training on function and regeneration of skeletal muscle of the mdx model over time.

METODOLOGY

Forty male dystrophic *mdx* mice (C57BL/10ScSn- Dmdmdx/J) acquired from UniABC, São Paulo, Brazil (CQB-172/02) were studied. Animals were maintained in cages on a 12h day / 12h dark inverted cycle with ambient temperature controlled at 22 ºC and supplied with food and water ad libitum. Thirthy two *mdx* animals were randomly assigned into two groups: Trained (*mdx*-T) or No-Trained (*mdx*-NT) group. The other eight animals were control of the inicial time T0. This research was approved by the Ethics Committee on Animal Use of the University Federal dos Vales do Jequitinhonha e Mucuri (CEUA/UFVJM), protocol nº 025/15.

Design

Training protocol began after the first assessment (T0). One group had trained for 4 weeks (n=16: *mdx*-T=8 e *mdx*-NT=8) and another one had trained for 8 weeks (n=16; *mdx*-T=8 e *mdx*-NT=8) (figure 1). T0 was determined at the time dystrophic animals had 8 weeks of age. The age of 8 to 12 weeks is considered a morphological stable phase of the disease to *mdx* model once animals have already suffered an important cycle of degeneration/regeneration (Grounds et al., 2008).

Insert Figure 1 here.

Training protocol

After an adaptation protocol (table 1), *mdx*-T group were stimulated to run at a motorized treadmill (EP 131; Insight®, Brazil) at 9m/min once this speed is already this speed is already standardized for low-intensity training to *mdx* model (De Luca et al., 2014). The protocol consisted of 30min/day, 3x/week during 4 weeks (Short protocol - T4) or 8 weeks (Long protocol – T8). Animals of *mdx*-NT group were placed at the horizontal treadmill with speed at 0m/min aiming to be exposed to the same environmental conditions.

Insert Table 1 here.

Functional tests

Grip Strength and Rotarod were used as functional measurements. They were assessed each 2 weeks of protocol over time (T0, T2, T4, T6 and T8) (see Figure 1).

Every moment of functional measurements animals were weighted at a semi-analytic balance (UX-420H 0,001g precision) to follow animals well-being and also to further use to normalization the values of grip strength avoiding the influence of weight on this variable as described by van Putten et al. (2010).

Rotarod

Performance at Rotarod was used to assess coordination, balance, muscle strength and condition over time of protocol (Aartsma-Rus & van Putten, 2014). An automated device was used (MP13977, Insight®, Brazil). After adaptation at the device (table 2), the mice were placed in the rotarod tube as it rotated at a slow and steady speed of 5 rpm. The running started as soon as all the mice were in position. The tube speed accelerated from 5 to 37 rpm and maintained that speed. The running time was stopped automatically when mice dropped from the tube, as this activated the time bar positioned below the tube, but the animals were repositioned immediately. The test session was terminated for mice capable of running for 500 seconds. The mice were given a maximum of two more attempts, which allowed them to improve their execution time when they fell earlier. The maximum execution time (ie the longest of the tests) was used for further analysis (Aartsma-Rus & van Putten, 2014).

Insert Table 2 here.

Grip Strength test

The forelimb grip strength was performed with the Grip Strength Meter (PanLAb®, Brazil). Animals were suspended by the tail above the grid and after they grasped, they were pulled backwards. The value of maximal force was registered by the Grip Strength Meter. Each animal were tested five times with one minute interval between trials. The three highest values were averaged to calculate the absolute strength, which was divided by the corporal mass in grams (van Putten et al., 2010; De Luca et al., 2014). All measurements were made by the same blinded investigator to avoid bias.

Muscle tissue analysis

Animals were euthanaziated at each time of protocol: n=8 at T0, n=16 at T4 and n= 16 at T8 according to CEUA protocol nº025/15 seventy two hours after the final

exercise session. All animals were subjected to an overdose of ketamine hydrochloride (200 mg/kg) and xylazine hydrochloride (20 mg/kg), via intraperitoneal injection. Tibial anterior (TA) and Soleus (SOL) muscle were collected and fixed in paraformaldehyde solution at 4 % during 24h and further tranfered to phosphate buffer. They were treated with increasing ethanol concentrations (70 to 100 %) to dehydrate and with xylene to clear. The samples were then embedded in paraffin (Ervplast®) and sections of 5mm in thickness were obtained. The cross-sections were oven-dried (60 °C) at a horizontal position for better adhesion of the cuts. After deparaffinization protocol, the sections were stained using Hematoxylin-eosin (HE) according to conventional histological procedures to identify histopathological features. Histochemical reaction using Picrossirius red, a combination of Sirius red F3BA (Sigma-Aldrich, Color Index 35780) dissolved in a saturated picric acid solution, was used in order to distinguish collagen from the skeletal muscle fibers.

Intramuscular collagen fiber quantification

Slides reacted with picrossirius red were analyzed under polarized light in 400x. Photomicrographs of ~20 sequential images of each animal studied were performed to carry out the analysis of the whole section transverse muscle (Luz et al., 2002; Smith & Barton, 2014) totalizing an analysis of an area of 2000 to 3000 fibers/animal. The amount of deposition of the collagen fibers was calculated by the percentage of the area of collagen fibers in relation to the total area (57248.52 µm2) of each image, through binary analysis (black/white) and expressed in micrometers using ImageJ® software: “Process” > “Binary” > “Make Binary” > “Analyze” > “Set Measurements” > selected “Area’ + Area fraction” > “Analyze” > “Measure”.

Immunohistochemical analysis (IHC)

Primary polyclonal antibodies against TGFβ-1 (anti-human) (StressMarq Biosciences®), 1:750 were applied on TA and SOL muscle sections. Sections were immersed in citric acid solution at 0.01M, pH 6.0 and submitted to 95°C for 30 min to antigenic recovery. Next, the blockade of endogenous peroxidase with hydrogen peroxide at 3% for 40 min was performed. Primary antibodies were applied and incubated for 20 h in a damp chamber at 4°C. After three more rinses in buffered saline solution (PBS), secondary antibody (N-Histofine®) was applied and incubated for 30

min at room temperature (24°C). IHC reaction was revealed with DAB (Chromogen/Substrate Bulk Pack, ScyTek Laboratories) for 2 min. In the negative control, the primary antibody was omitted and all slides were counterstained with hematoxylin. Photo

All tissue photomicrographs for histological, histomorphometrical and IHC analyses were made under an optical microscope (LABOMED® LxPol) equipped with an Axio CAM HRc camera and Software Capture Pro 2.9.0.1.

Data analysis

Qualitative assessments of histological, histochemical and IHC of muscle samples were analyzed by observing three sections from each one of the animals (n = 8)/per group=5: T0, T4 *mdx*-T and *mdx*-NT, T8 *mdx*-T an *mdx*-NT. The descriptive statistical analysis was performed via mean and standard deviation calculations (grip strength, rotarod and intramuscular collagen fiber quantification). Analysis of the normality of the data was performed by the Shapiro-Wilk test and considering normal distribution the p value>0.05. To detect difference between groups the Student t test analysis was conducted and to detect difference intra groups an analysis of variance (ANOVA) was performed and the Tukey test was used as post hoc. The IBM SPSS Statistics ver. 22.0 (IBM Co., Armonk, NY, USA) was used with the level of significance set at $p<0.05$.

RESULTS

The *mdx*-T and *mdx*-NT groups presented an age dependent increased in corporal mass, without difference between groups. The mean values of the length of stay at Rotarod also have not shown difference between or intra groups (figure 2A).

The behavior of the normalized grip strength are shown at Figure 2B, C and D. No difference was observed between groups at any assessed times (2B). Both groups presented a decrease of the grip strength values at T4 not related to the protocol training. The group that underwent the protocol training (*mdx*-T) showed difference between T0 to T4 (p=0.036), T4 to T8 (p=0.005) and T6 to T8 (p=0.050) (Figure 2D). There was an increase of grip strength of the trained animals after the 4th week of training. The *mdx*-NT group showed difference between the values of T0 and T4 (p=0.001), T0 and T6 (p=0.000) and T0 and T8 p= 0.050) (figure 2C), but not between T4 to T8.

Insert Figure 2 here.

The area of collagen fibers of SOL muscle at T4 (0.31±0.04) was lower than T0 to trained animals (0.48 ±0.05) (p=0.04) (figure 3A). The TA muscle showed different percentage of area at T8 between groups with *mdx*-T (0.35±0.02) presenting lower values when compared to *mdx*-NT group (0.59±0.06) (p=0.021) (figure 3B).

Insert Figure 3 here.

Typical histopathological features of dystrophic skeletal muscle were observed at SOL and TA muscle of both groups. Collagen fibers were distributed between muscular fibers with thicker tracts at perimysium than at endomysium. The *mdx*-T group presented innumerous thinner tracts at endomysium, mainly at T4 with thicker tracts observed at T0 than at T8, time were the collagen fibers had covered a higher area. Tracts of *mdx*-NT group were thicker at perimysium at T0 when compared to T4 (figure 4).

Insert Figure 4 here.

TA muscle showed thinner collagen fibers at perimysium of *mdx*-T group with the thicker ones at the perimysium of *mdx*-NT group on all assessed moments, especially at T8 (figure 5).

Insert Figure 5 here.

Immunolocalization of TGF-β1 has shown the presence of this profibrotic marker in the endomysium and perimysium of the TA and SOL muscles at T0, T4 and T8. It was also observed in sarcoplasm at T0 and *mdx*-NT at T4 and T8 (figure 6).

Insert Figure 6 here.

DISCUSSION

The low-intensity protocol training had maintained grip strength, rotarod performance and favored a decline on the intramuscular collagen fibers area of tibial anterior muscle over time.

TGF-β1 stained at endomysium and perimysium tracts of muscle fibers, as expected to dystrophic muscle. And, sarcoplasm of sedentary animals was also stained to TGF-β1 at T8. According to Ismael et al. (2019) the expression of TGF-β1 increase progressively with the progression of the disease and is positively correlated to the collagen density.

The effect of the intensity and of the time of treadmill training protocol on forelimb strength in vivo and on corporal mass of mdx mice has been already studied (Camerino et al., 2014). Our results attest that a training of low-intensity did not worsened muscle function of mdx mice. It is interesting to note that at the moment were animals were 12 weeks old (at T4), the length of stay at Rotarod was lower than at the beginning or end of the 8 weeks of protocol. The same was observed to grip strength which suffered a decline at T4.

Some studies suggest that the strength of mdx mice decrease after 3 months of age (Grounds et al., 2008). It is related to degeneration/regeneration cycles that are present between 4 and 15 weeks of age of these animals (Van Putten et al., 2010). This result is in accordance with the forelimb strength decrease that was found in mdx mice of 5 to 12 weeks age that have not made other functional tests besides grip strength or treadmill running (De Luca et al., 2008). Capogrosso et al. (2017) submitted animals of 4 and 5 weeks age to a protocol of 12 weeks of treadmill training, at 12m/min and also found a decline on grip strength after 4 weeks of protocol.

Even that trained and not trained mice have not shown difference between their grip strength (inter group), there was a considered increase of grip strength of trained mice between T6 to T8 that attest an adaptation to trained that could not be observed on sedentary ones.

Proper muscle activity maintains a healthy status, prevent chronic inflammation (Ellingsgaard, Pernille e Bente, 2019) and cardiovascular disturbances (Pedersen &

Saltin, 2015). Exercise regulates function, morphology and metabolism of skeletal muscle through modulation of different signaling pathways (Camerino et al., 2014). Also, exercise decrease dysfunctional adipose tissue and improve oxygenation (Nieman & Wentz, 2019).

Physical exercise has been proposed as an adjuvant therapy to humans DMD aiming to maintain muscle strength and prevent contractures as long as possible. However, exercise practice remains controversial once dystrophic muscle can be damage due to excessive use (Hyzewicz et al., 2015a). According to recent systematic reviews, besides some studies have demonstrate the benefice of exercise in slow up motor function damage in young people with DMD, more research must be conducted to understand cardiac and respiratory function to exercise, the effect of long-term training protocol on dystrophic muscle and the effects of running and swimming on DMD (Hyzewicz et al., 2015a; Kostek & Bradley, 2018).

The intensity of training and age of dystrophic animals influence the effects of exercise on dystrophic muscle. High intensity training using forced treadmill or downhill running causes loss of muscle strength, myofibres necrosis (Terrill, Radley-Crabb, Grounds e Arthur, 2012) and fibrosis (Nakamura, Yoshida, Takeda, Dohi e Ikeda, 2002).

Moreover, damage-related genes as the transforming growth factor- β1 (TGF-β1), tumor necrosis factor (TNF-α) and tyrosine kinase c-src are severely increased, which reinforce lesion signs and muscle dysfunction (Camerino et al., 2014).

In the other hand, low-intensity training (LIT), as swimming or voluntary wheel running, reduce oxidative stress markers (Kaczor, Hall, Payne e Tarnopolsky, 2007), improves strength, muscle resistance (Baltgavis, Call, Cochrane, Lker, Yan e Lowe, 2012) and reduce inflammatory status (Hyzewicz et al., 2017).

Muscles are susceptible different to damage induced by exercise. Hindlimb muscles are directly involved in running exercises (Capogrosso et al., 2017). Soleus and tibial anterior muscle are hindlimb muscles and are constituted by different fibers types which explain their different answer to training. In general, tibial anterior muscle is more affected to treadmill training because it has predominantly type II fibers (Staron et al., 1998). The fibrosis of tibial anterior muscle was influenced by low-intensity training

protocol demonstrating reduce values at the end of the protocol in TA muscles of the animals that were submitted to exercise. We also observed a reduction of collagen fibers on soleus muscle after 4 weeks of protocol. Even in the dystrophic genotype, a muscle of slow type fibers is more resistant to mechanical challenge (Camerino et al., 2014).

When fibrosis occurs, collagen not only increases its amount but also suffers post transduction dysfunctions that alters its organization and contribute to tissue stiffness (Smith & Barton, 2014). Picrossirius red staining is one of the most known histochemical techniques able to selectively detect tracts of collagen and it becomes more specific when associated to the detection under polarized light (Rittié, 2017).

On *mdx* model the infiltration of immune cells reaches a peak between 4 and 8 weeks of age (Hyzewicz, Tanihata, Kuraoka, Ito, Miyagoe-Suzuki e Takeda, 2015b) and fibrosis can be seen after 10 weeks age (Hyzewicz et al., 2015a), which is in accordance to our finding on the muscle of mdx mice at T0 (8 weeks of age). In this way, we suggest that training had positively mediated collagen deposition in both studied muscle at T4 and had also kept smaller areas of fibrosis on trained mice in relation to sedentary mdx mice.

Fibrosis was here studied by intramuscular collagen fibers morphometry using picrossirius red under polarized light and the immunolocalization of TGF-β1. It is possible to observe that TGF-β1 on mdx mice skeletal muscle is age dependent and is located at epimysium and perimysium. Also at sarcoplasm and around blood vessels of sedentary animals, features very close to intramuscular collagen fibers.

Sedentary mdx mice showed TGF-β1 localized at sarcoplasm of muscle fibers, mainly at T8, time where they were 16 weeks old. TGF-β1 is generally present at epimysium and perimysium of dystrophic muscle while on healthy muscle no stain can be seen to this pro fibrotic marker (Bernasconi et al., 1995).

Song et al. (2017) attested that the expression of TGF-β1 is correlated to the degree of pathology and clinic progression of DMD. Its expression is regulated specifically at sarcoplasm of muscle cells and at myenteric plexus of human with DMD.

Diverse animals' studies observed answers of TGF-β to muscle damage by eccentric muscle contraction, a modality of contraction proven harmful to the dystrophic

muscle (Grounds et al., 2008). Studies show that levels of TGF-β are increased after an eccentric muscle contraction (Kim & Lee, 2017).

CONCLUSION

As fibrosis reflects the final phase of a chronic inflammatory process typical of the dystrophic muscle, we suggest that low-intensity training can mediate the regeneration of muscle fibers of the hindlimb maintaining muscle strength during a chronic protocol of exercise. The limitation of this study is that inflammatory cells were not investigated, cardiac and diaphragm muscle were not analyzed and pro fibrotic marker was not quantified. The clinical contribution of these results is that DMD boys in the ambulatory phase of the disease can benefit of training considered of low-intensity. Further clinical studies are necessary to attest modalities of low-intensity training in humans at this phase and its real effects not only on clinical parameters but also on cardiac and respiratory muscle and limb muscles involved on functional activities.

REFERENCES

Aartsma-Rus A, van Putten M. 2014. Assessing functional performance in the *mdx* mouse model. *Journal Vis. Exp* (85): 1-11; doi:10.3791/51303

Baltgalvis KA, Call JA, Cochrane GD, Laker RC, Yan Z, Lowe DA. 2012. Exercise training improves plantarflexor muscle function in *mdx* mice. *Med Sci Sports Exerc* 44(9): 1671-1679; doi:10.1249/MSS.0b013e31825703f0

Bernasconi P et al. 1995. Expression of transforming growth factor-β1 in dystrophic patient muscles correlates with fibrosis. *Journal of Clinical Investigation* 96 (2): 1137-44.

Bushby K, Finkel R, Birnkrant DJ et al. 2010. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurol* 9 (1): 77-93.

Camerino GM, Cannone M, Giustino A et al. 2014. Gene expression in mdx mouse muscle in relation to age and exercise: aberrant mechanical–metabolic coupling and implications for pre-clinical studies in Duchenne muscular dystrophy. *Human Molecular Genetics* 22: 1-13.

Capogrosso RF, Mantuano P, Cozzoli A et al. 2017.Contractile efficiency of dystrophic mdx mouse muscle: in vivo and ex vivo assessment of adaptation to exercise of functional end points. *Journal Appl Physiol* 122 (5): 828-843.

Deacon RMJ. 2013. Measuring motor coordination in mice. *J. Vis. Exp*. (75), e2609, doi:10.3791/2609

Delp MD, Duan C. 1996. Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *Journal Appl Physiol* 80 (1): 261-70.

De Luca A, Nico B, Rolland JF et al. 2008. Gentamicin treatment in exercised mdx mice: Identification of dystrophin-sensitive pathways and evaluation of efficacy in work-loaded dystrophic muscle. *Neurobiol Disease* 32: 243-253.

Ellingsgaard H, Pernille H, Bente KP. 2019. Exercise and health- emerging roles of IL6. *Current Opinion in Physiology* 10: 49-54.

Grange RW, Gainer TG, Marschner KM, Talmadge RJ, Stu JT. 2002. Fast-twitch skeletal muscles of dystrophic mouse pups are resistant to injury from acute mechanical stress. *Am J Physiol Cell Physiol* 283: C1090–C1101; doi: 10.1152/ajpcell.00450.2001

Grounds MD, Radley HG, Lynch GS et al. 2008. Towards developing standard operating procedures for pre-clinical testing in the *mdx* mouse model of Duchenne muscular dystrophy. *Neurobiol Disease* 31(1): 1-19.

Hzewicz J, Ruegg UT, Takeda S. 2015. Comparison of experimental protocols of physical exercise for *mdx* Mice and Duchenne muscular dystrophy patients. *Journal of Neuromuscular Diseases* 2: 325-342; doi 10.3233/JND-150106

Hyzewicz, J, Tanihata J, Kuraoka M et al. 2015. Low-intensity training of mdx mice reduces carbonylation and increases expression levels of proteins involved in energy metabolism and muscle contraction. *Free Radic Biol Med* 82: 122-136.

Hyzewicz, J, Tanihata J, Kuraoka M et al. 2017. Low-intensity trining and the C5a complement antagonista NOX-D21 rescue the mdx phenotype through modulation of inflammation. *The American Journal of Pathology* 187(5): 1147-1161

Ismael A, Kim JS, Kirk JS, Smith RS, Bohannon W, Koutakis. 2019. Role of transforming growth factor-β in skeletal muscle fibrosis: a review. Int. *J. Mol. Sci.* 20: 2446; doi: 10.3390/ijms20102446

KACSOR JJ. et al. 2007. Low-intensity training decreases markers of oxidative stress in skeletal muscle of mdx mouse. Free Radic Biol Med 43(1): 145-154.

Kim HS, Luo L, Pflugfelder SC, Li DQ. 2005. Doxycycline inhibits TGF-beta1-induced MMP-9 via Smad and MAPK pathways in human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 4(3): 840-848.

Kim J, Lee J. 2017. Role of transforming growth factor-β in muscle damage and regeneration: focused on eccentric muscle contraction. *Journal of Exercise Rehabilitation* 13(6): 621-626

Kostek MC, Gordon B. 2018. Exercise Is an Adjuvant to Contemporary Dystrophy Treatments. *Exercise and Sport Sciences Reviews* 46(1):34-41; doi: 10.1249/JES.0000000000000131

Luz MAM, Marques MJ, Santo Neto H. 2002. Impaired regeneration of dystrophindeficient muscle fibers is caused by exhaustion of myogenic cells. *Brazilian Journal of Medical and Biol Research* 35 (6): 69- 695.

Massgue J. 2012. TGFβ signalling in context. *Nature Reviews Molecular Cell Biology* 13: 616- 630.

Pedersen BK, Saltin B. 2015. Exercise as medicine – evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sports* 3 Suppl. 25: 1-72. https://doi.org/10.1111/SMS.12581

Rradley-Crabb H, Terril J, Shavlakadze, Tonkin J, Arthur P, Gronds M. 2012. A single 30 min treadmill exercise session is suitable for ‘proofof concept studies’ in adult mdx mice: a comparison of the early consequences of two different treadmill protocols. *Neuromuscular Disorders* 22: 170-182.

Rittié L. 2017. Method for picrossiriu red-polarization detection of collagen fibers in tissue sections. In: Rittié L.(eds). *Methods in Molecular Biology* 1627. Humana Press, New York, NY.

Rodrigues M, Echigoya Y, Fukada S, Yokota T. 2016. Current Translational Research and Murine Models For Duchenne Muscular Dystrophy. *Journal of Neuromuscular Diseases* 3: 29-48; doi: 10.3233/JND-150113

Serrano AL, Muñoz-Cánoves P. 2017. Fibrosis development in early-onset muscular dystrophies: mechanisms and translational implications. *Semin. Cell Dev. Biol*. 64: 181-190.

Song Y et al. 2017. Expression levels of tgf-β1 and ctgf are associated with the severity of Duchenne muscular dystrophy. *Experimental and Therapeutic Medicine* 13: 1209-1214.

Staron RS, Kraemer WJ, Hikida RS et al.1999. Fiber type composition of four hindlimb muscles of adult Fisher 344 rats. *Histochem Cell Biol* 111 (2): 117-23.

Taniguti APT. 2011. Fibrose muscular em camundongos *mdx*: efeitos do exercício físico e agente anti-fibrótico. São Paulo, Campinas [s.n.].

Terril JR, Radley-Crabb HG, Grounds M, Arthur PG. 2012. *N*-Acetylcysteine treatment of dystrophic *mdx* mice results in protein thiol modifications and inhibition of exercise induced myofibre necrosis. *Neuromuscula Disorders* 22(5): 427-434.

Van Puten M, Winter C, Roon-Mom WV et al. 2010. A 3 months mild functional test regime does not affect disease parameters in young mdx mice. *Neuromuscular Disorders* 20: 273-280.

Zelikovich, Quattrocelli, Salamone, Kuntz, McNally. 2019. Moderate exercise improves function and increases adiponectin in the mouse model of muscular dystrophy. *Sci Rep* 9: 5770.

Figure, Tables and legends

Color figures are intended for color reproduction on the Web and in black-and-white in print.

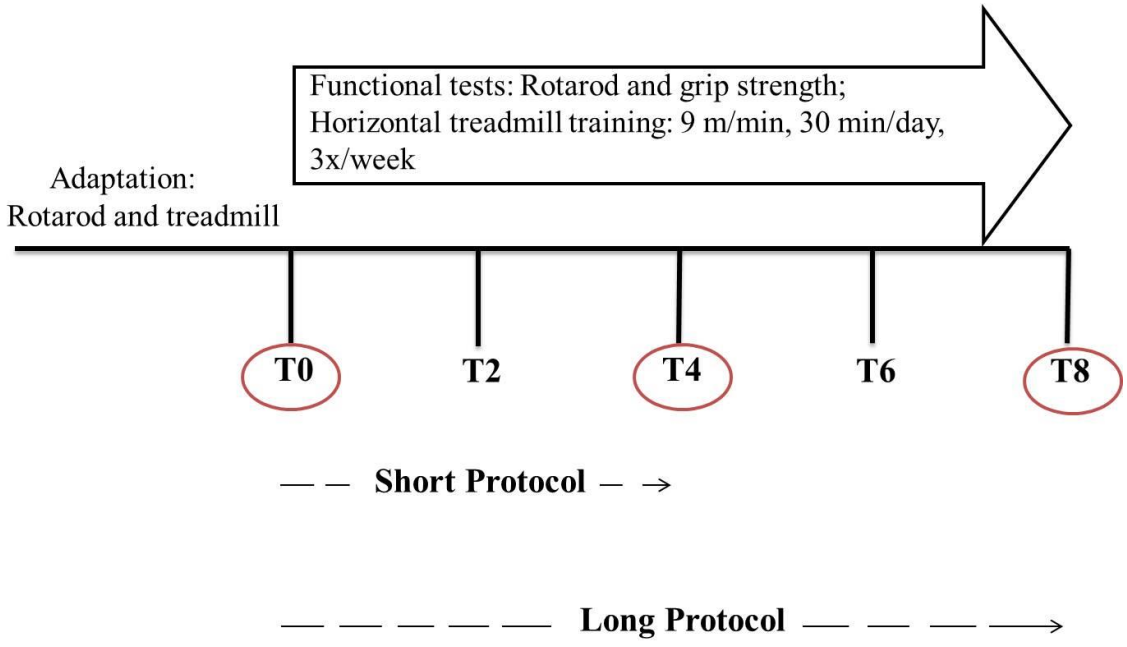

Fig 1 – Experimental design: Times of assessment and collection of biological collection. Functional measurements of mdx-T and mdx-NT: Rotarod and Grip Strength at T0, T2, T4, T6 e T8; Biological collection at T0, T4 e T8.

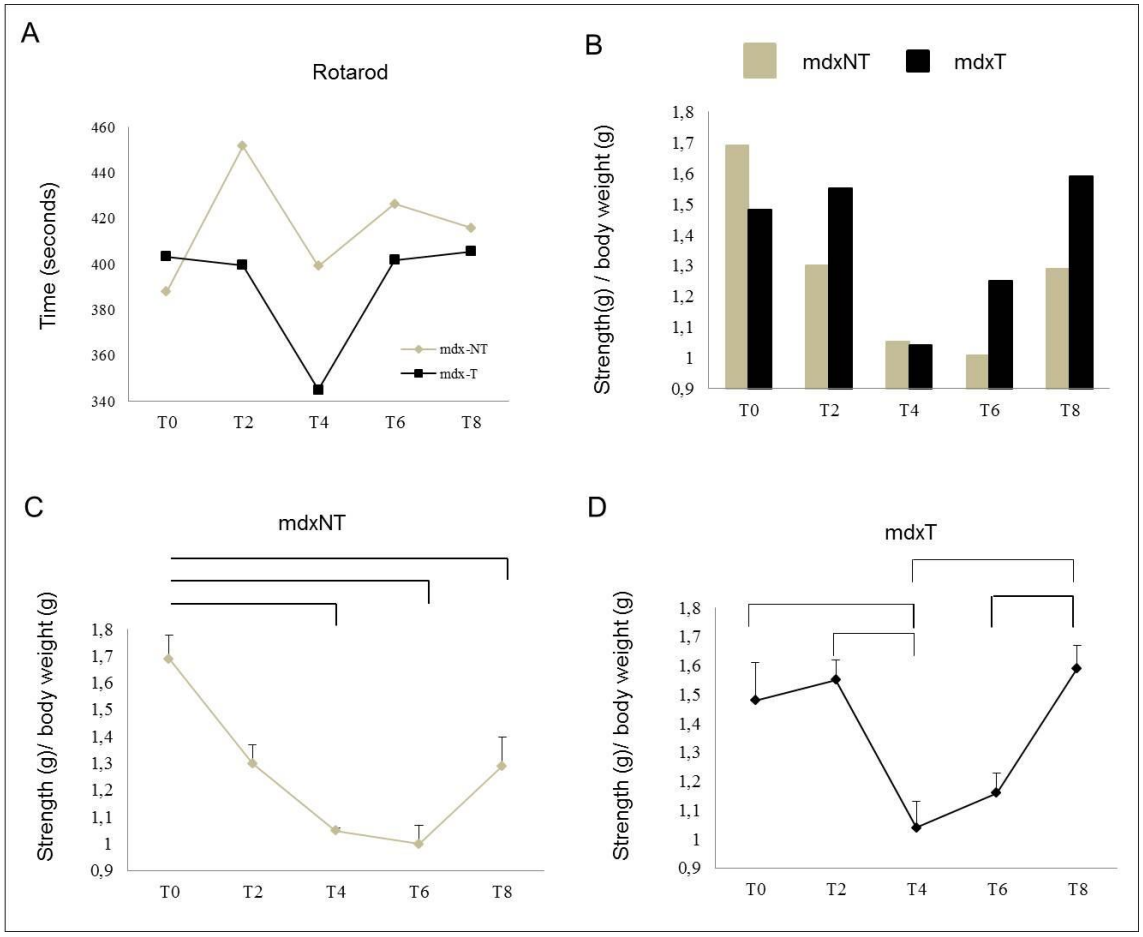

Fig 2 – A: Lenght of stay (seconds) at Rotarod over time of protocol training. B: Normalized Grip Strength over time comparing mdxT and mdxNT groups. C and D: Normalized Grip Strength of mdx-NT and mdx-T. *difference intra-groups: mdx-NT T0xT4 (p=0,001), T0xT6 (p=0,000) e T0xT8 (p= 0,050); mdx-T T0xT4 (p=0,036), T2xT4 (p=0,011), T4xT8 (p=0,005), T6xT8 (p=0,050).

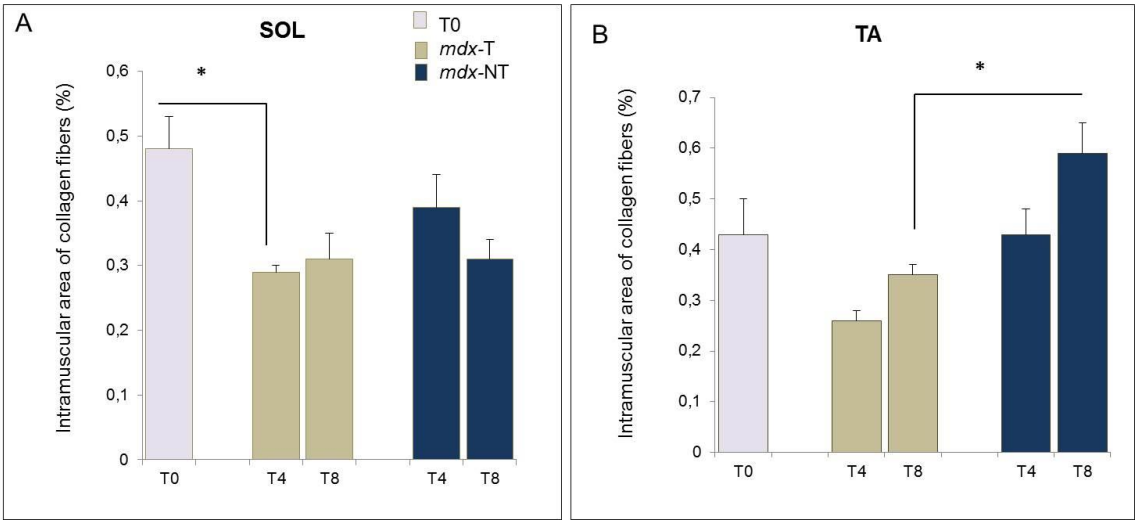

Fig 3 - Effect of low-intensity training on the percentage of intramuscular collagen fibers deposition. Morphometric analysis of the percentage of intramuscular collagen fiber deposition in soleus (A) and tibial anterior (B). * Significant difference between groups (ANOVA with post-hoc Tukey).

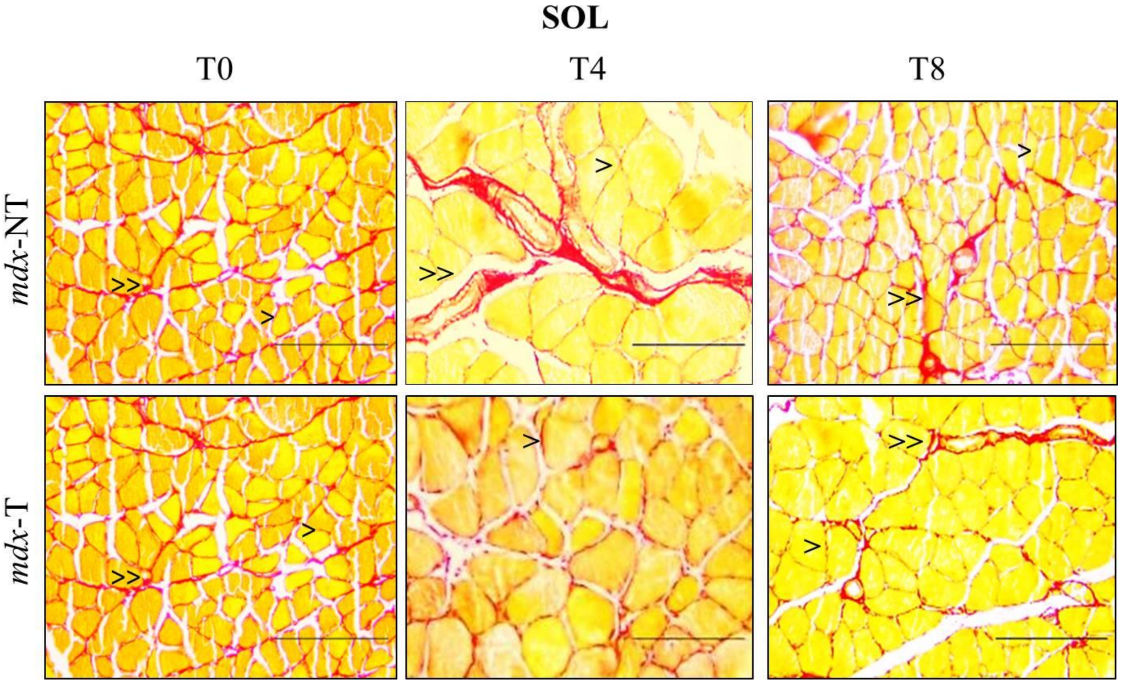

Fig 4 - Morphological analysis of the deposition of collagen fibers on soleus muscle of mdx-NT and mdx-T. Picrossirius red reaction. >>Deposition of collagen fibers in the perimysium. >Deposition of collagen fibers in endomysium. Scale bar=100 µm.

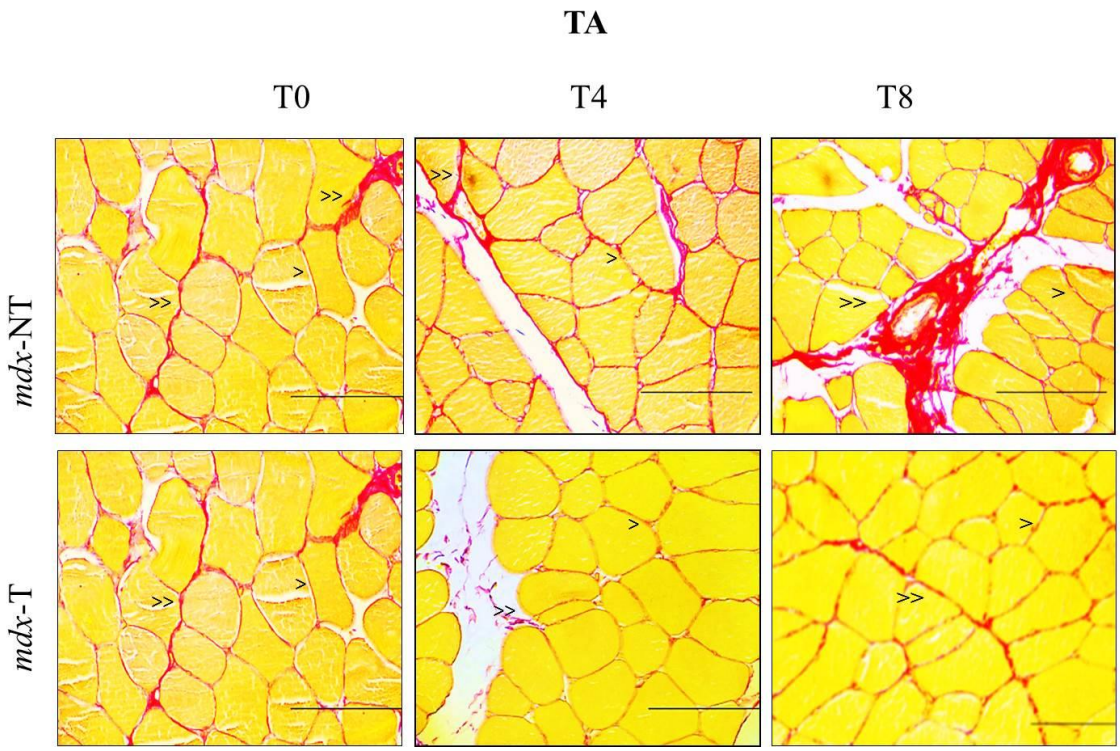

Fig 5 - Morphological analysis of the deposition of collagen fibers on tibial anterior muscle of mdx-NT and mdx-T. Picrossirius red reaction. >>Deposition of collagen fibers in the perimysium. >Deposition of collagen fibers in endomysium. Scale bar=100 µm.

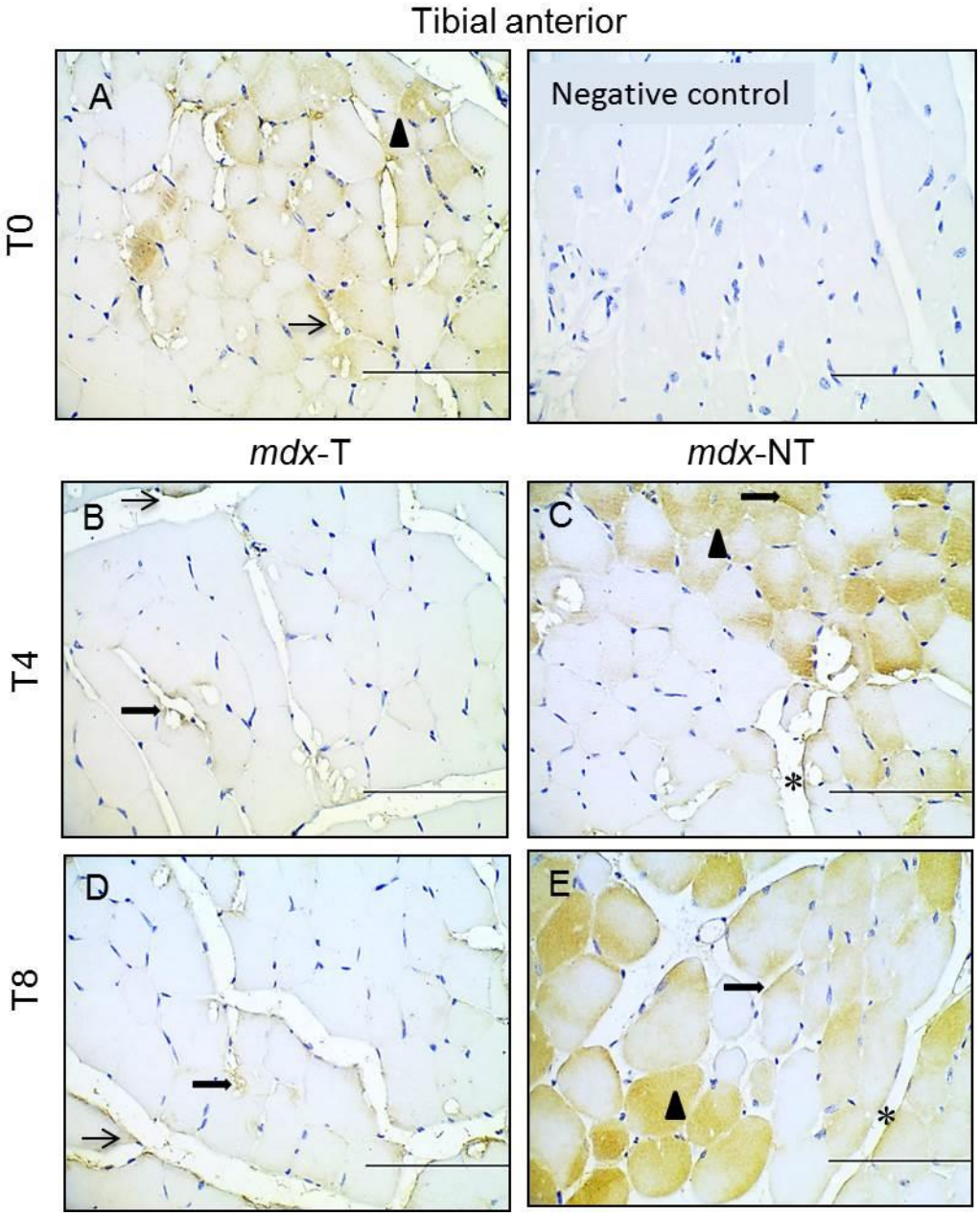

Fig 6 – Morphological analysis of tibial anterior muscle of mdxT and mdxNT groups at T0, T4 and T8, 400x, IHC. Immunolocalization of TGF-β1 and negative control of immunohistochemical reaction. On A: Tibial anterior muscle at T0; B (mdxT) and C (mdxNT) at T4; D (mdxT) and E (mdxNT) at T8. ∗ perimysium, → endomysium, ▲ sarcoplasm. Bar: 100µm.

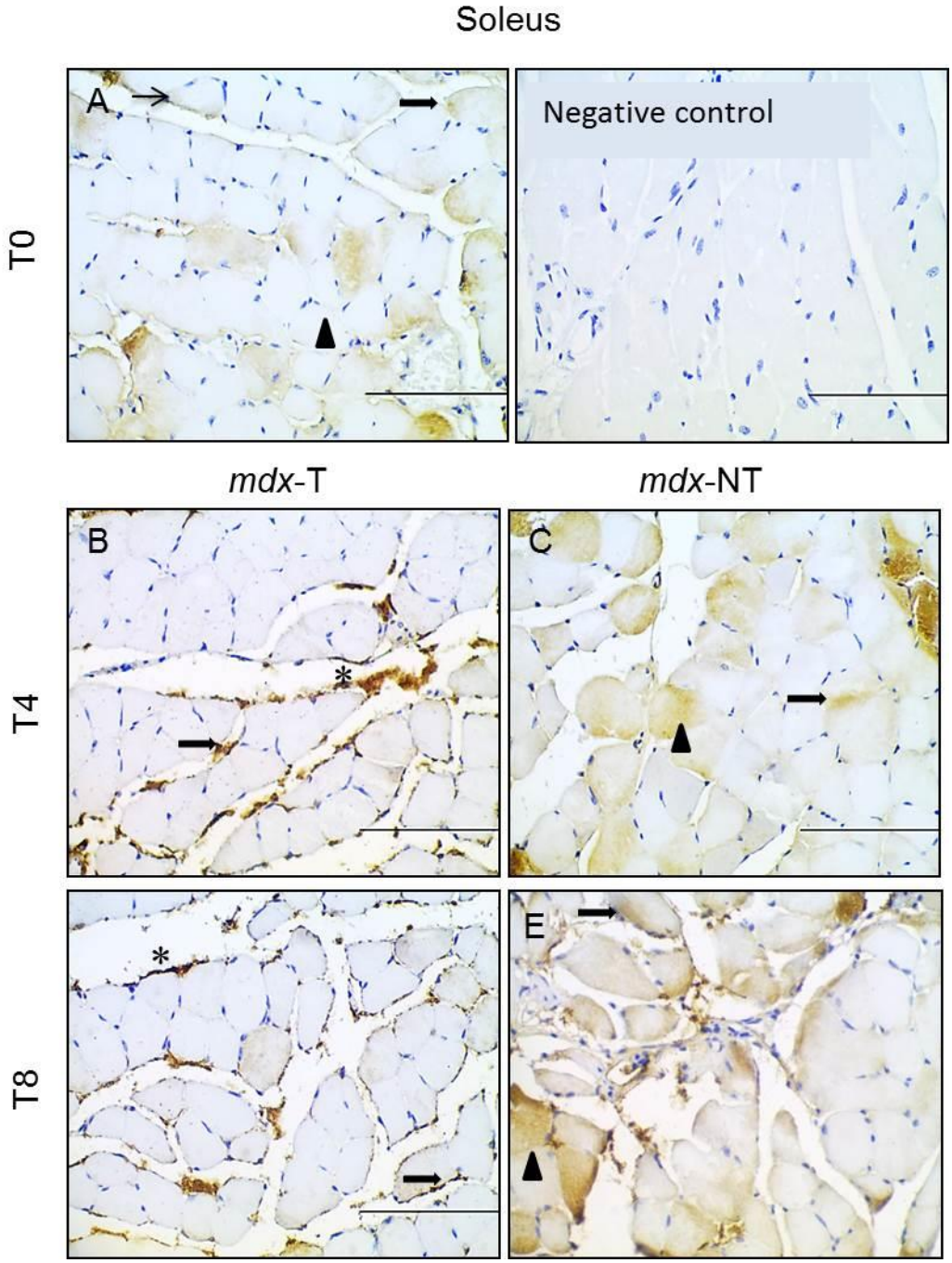

Fig 7 - Morphological analysis of Soleus muscle of mdxT and mdxNT groups at T0, T4 and T8, 400x, IHC. Immunolocalization of TGF-β1 and negative control of immunohistochemical reaction. On A: Soleus muscle at T0; B (mdxT) and C (mdxNT) at T4; D (mdxT) and E (mdxNT) at T8. ∗ perimysium, → endomysium, ▲ sarcoplasm. Bar: 100µm.

Table 1

Table 1. Adaptation protocol of *mdx*-T group at the treadmill

Time	Speed
4 min	2 m/min
15 min	4 m/min
30 min	6 m/min
15 min	9 m/min
30 min	9 m/min

* Adaptation protocol started at the week before the training protocol where mdx animals were 7 weeks of age and lasted 5 days long.

Table 2. Adaptation protocol to Rotarod

Time	Rotarod speed
250 sec	16 rpm
250 sec	25 rpm
500 sec	37 rpm

***** Rotarod adaptation started at the week before the training protocol where mdx animals were 7 weeks of age and lasted 3 days long.

ANEXO A – COMISSÃO DE ÉTICA E PESQUISA NO USO DE ANIMAIS

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DOS VALES DO JEQUITINHONHA E MUCURI
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UFVJM

CERTIFICADO

Diamantina, 18 de novembro de 2015

Certificamos que o projeto intitulado " Efeitos do exercício de baixa intensidade em esteira na reparação tecidual do modelo mdx: análise da localização e expressão do mRNA dos colágenos tipo I e III no músculo esquelético distrófico ", protocolo nº 025/2015, sob a responsabilidade de Thaís Peixoto Gaiad Machado - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADO** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA/UFVJM) DA UNIVERSIDADE FEDERAL DOS VALES DO JEQUITINHONHA E MUCURI, em reunião de 10/06/2015.

Vigência do Projeto	01/02/2016 a 01/02/2018
Espécie/linhagem	*Mus musculus / mdx*
Nº de animais	40
Peso/Idade	30 gramas/8 semanas
Sexo	Macho
Origem	FioCruz - RJ

Com o recebimento deste parecer, o responsável compromete-se a entregar o relatório final da proposta até 60 dias após o término. Em caso de planos de aula, a cada seis meses estes deverão ser revalidados.

Ressaltamos que, conforme a Resolução Normativa I, de 9 de Julho de 2010, qualquer alteração no protocolo previamente aprovado, na equipe técnica, bem como acidentes envolvendo os animais, competem ao responsável a comunicação a CEUA/UFVJM.

Cleube Andrade Boari
Coordenador(a) da Comissão de Ética no Uso de Animais / UFVJM

Campus JK
Comissão de Ética no Uso de Animais/ UFVJM
Pró-Reitoria de Pesquisa e Pós-Graduação - Rodovia MGT 367 - Km 583, nº 5000, Alto da Jacuba
Diamantina/MG - CEP 39100-000 Telefone: +55 (38) 3532-1200

CEUA Comissão de Ética no Uso de Animais

ANEXO B – **Normas da Revista Physical Therapy in Sports**.

https://www.elsevier.com/wps/find/journaldescription.cws_home/623067?generatepdf=true