UNIVERSIDADE ESTADUAL DE CAMPINAS
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MORFOLOGIA E EXPRESSÃO DOS FATORES DE REGULAÇÃO MIOGÊNICA (MRFS) E IGF-1 NO MÚSCULO ESQUELÉTICO DE RATOS SUBMETIDOS AO TREINAMENTO RESISTIDO

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profa. Dra. Maeli Dal Pai Silva

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Índice

1. LISTA DE SIGLAS E ABREVIATURAS

AKT: *Serine-Threonine Kinase* – quinase serina-treonina

AMT: microtrauma adaptativo

ANOVA: *Analysis Of Variance* – análise de variância

AST: área de secção transversal ou CSA: *cross-sectional area*

bHLH: *basic Helix-Loop-Helix*

C12: *group non-trained for 12 weeks* – grupo não treinado por 12 semanas

C8: *group non-trained for 8 weeks* – grupo não treinado por 8 semanas

cDNA: ácido desoxirribonucléico (DNA) complementar

Co: *control group* – grupo controle

CS: célula satélite

DNA: ácido desoxirribonucléico

DR: *deep-red portion* – porção profunda-vermelha

FGF: *Fibroblast Growth Factor* - fator de crescimento de fibroblasto

GH - *Growth Hormone* – hormônio do crescimento

H.E.: coloração de hematoxilina e eosina

HGF: *Hepatocyte Growth Factor* – fator de crescimento de hepatócito

HMM: *Heavy Meromyosin* – meromiosina pesada

HPRT: *Hypoxanthine phosphoribosyl-transferase 1*

IGF-1: *Insulin Growth Factor-1* - fator de crescimento semelhante à insulina-1

IL-6: *Interleukin-6* – interleucina 6

IL-15: *Interleukin-15* – interleucina 15

IRS: *Insulin Response Substrate* – substrato responsivo à insulina

LMM: *Light Meromyosin* – meromiosina leve

MAFbx: *atrogin-1/Muscle Atrophy F-box*

mATPase: *myofibrillar Adenosine TriPhosphatase* – miofibrilar adenosina trifosfatase

MHC: *Myosin Heavy Chain* - cadeia pesada da miosina

MLC: *Myosin Light Chain* - cadeia leve da miosina

MRF: *Myogenic Regulatory Factor* - fator regulador miogênico

mRNA: ácido ribonucléico mensageiro

mTOR: *mammalian Target Of Rapamycin*

MuRF1: *Muscle-specific RING Finger protein 1*

NF-κβ: *Nuclear Factor-κβ* - fator nuclear-κβ

OD: *Optical Density* - densidade óptica

OTS: *overtraining syndrome*

p70S6K: *p70S6 kinase*

PDU: *Proteasomal Dependent Ubiquitin* - sistema proteossomal dependente de ubiquitina

PI3K: *Phosphatidyl-Inositol-3 Kinase* – quinase fosfatidil-inositol-3

PST: pré-sobretreinamento

qPCR: *quantitative real-time PCR* – PCR quantitativo em tempo real

RNA: ácido ribonucléico

SD: *Standard Deviation* – desvio padrão

SDS-PAGE: *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis* – eletroforese em gel de poliacrilamida – dodecil sulfato de sódio

ST: sobretreinamento

SW: *superficial-white portion* – porção superficial-branca

T12: *group trained for 12 weeks* – grupo treinado por 12 semanas

T8: *group trained for 8 weeks* – grupo treinado por 8 semanas

TGF-β: *Transforming Growth Factor-beta* – fator de crescimento e transformação - β

TBP: *Tata Box Binding Protein*

TNF-α: *Tumor Necrosis Factor-α* - fator de necrose tumoral-α

Tr: *trained group* – grupo treinado

TR: treinamento resistido

2. RESUMO

O treinamento físico pode promover adaptações benéficas ao músculo esquelético. Entretanto, o treinamento de alta intensidade associado com um tempo insuficiente de recuperação, similar às condições de sobretreinamento, pode ocasionar efeitos prejudiciais. Os fatores reguladores miogênicos (MRFs) e o fator de crescimento IGF-1 são importantes reguladores da massa muscular no treinamento físico. Neste contexto, testamos a hipótese que o treinamento de alta intensidade com curto tempo de recuperação, poderia influenciar a morfologia, as isoformas da cadeia pesada de miosina (MHC), e a expressão dos MRFs MyoD e Miogenina e do IGF-1, no músculo esquelético de ratos. Ratos *Wistar* machos (200-250g) foram divididos em 4 grupos: treinado 8 semanas (T8), controle 8 semanas (C8), treinado 12 semanas (T12) e controle 12 semanas (C12). Os grupos T8 e T12 realizaram um programa de treinamento resistido de alta intensidade (5 dias/semana), envolvendo sessões de saltos em uma cuba contendo água. Ao término de cada período, os animais foram sacrificados e o músculo plantar retirado e submetido às análises morfológica e histoquímica, análises das MHCs e expressão gênica da MyoD, Miogenina e IGF-1. Do início ao final do experimento todos os grupos aumentaram o peso corporal, no entanto, o grupo T12 foi estatisticamente menor em relação ao C12. Com relação à área de secção transversal, observou-se uma redução das fibras IIC e IIAD no grupo T8 e IIA e IID no grupo T12 em relação aos seus controles. O grupo treinado por 12 semanas apresentou um aumento da frequência das fibras IIBD e redução nas frequências das fibras I, IIA e IID, em relação ao grupo controle; esses dados ainda foram corroborados pela redução das isoformas MHCI e MHCIIa e aumento da MHCIIb. A MHCIId não mostrou diferença significativa. A expressão gênica do grupo T12 apontou uma diminuição da MyoD e um aumento do IGF-1 comparado com o grupo C12; já, a expressão da Miogenina foi semelhante entre os grupos. Estes dados mostram que o modelo utilizado, semelhante às condições do sobretreinamento, promoveu a atrofia muscular e a transição das fibras musculares para uma atividade contrátil mais rápida. Estes fatos podem estar associados a uma menor atividade das células satélites. Em adição, o aumento da expressão do IGF-1, decorrente do treinamento, pode ter ocorrido na tentativa de prevenir o processo atrófico.

3. ABSTRACT

Physical training can promote beneficial changes in skeletal muscle. However, the high-intensity resistance training associated with insufficient recovery time may cause harmful effects. Myogenic regulatory factors (MRFs) and the growth factor IGF-1 are important mediators of muscle mass during physical training. In this context, we tested the hypothesis that high-intensity resistance training with short recovery time, similar to overtraining conditions, could influence the morphology, the myosin heavy chain (MHC) isoforms and the expression of MRFs MyoD and myogenin, and IGF-1 in skeletal muscle of rats. Male *Wistar* rats (200-250 g) were divided into 4 groups: trained 8 weeks (T8), control 8 weeks (C8), trained 12 weeks (T12) and control 12 weeks (C12). T8 and T12 groups were subjected to a high-intesnsity resistance training program (5 days/week), involving jumps sessions into water, carrying progressive overload equivalent to percentage of body weight. At the end of each period the animals were sacrificed and the plantaris muscles were removed and submited to morphological and histochemical analysis, myosin heavy chain (MHC) analysis and the gene expression of MyoD, Myogenin and IGF-1. From beginning to end of the experiment all groups increased body weight, however, in T12 body weight was lower compared to the C12. Regarding the cross-sectional area, there was a significant reduction of the IIC fibers and IIAD in T8 group and IIA and IID in T12 compared to their controls. The group trained by 12 weeks showed an increase in the IIBD, accompanied by a reduction in the I, IIA and IID muscle fibers frequency, compared to control group; these data have been corroborated by the reduction of MHCI and MHCIIa isoforms and increased of MHCIIb isoform. The MHCIId showed no significant differences. The gene expression of the T12 group showed a decrease in MyoD and an increase in IGF-1 compared with the C12 group; already, the expression of Myogenin was similar between groups. These data show that the model used, similar to the conditions of overtraining, promoted muscular atrophy and muscle fiber transition to a faster contractile activity. These facts may be associated with a lower activity of satellite cells. In addition, increased expression of IGF-1, due to training, may have occurred in an attempt to prevent the atrophic process.

4. INTRODUÇÃO

O músculo estriado esquelético exibe alta plasticidade, o que habilita este tecido a alterar suas características morfológicas, metabólicas e funcionais, em resposta a estímulos específicos (Pette e Staron 2000; Fluck e Hoppeler 2003). Neste contexto, as adaptações fenotípicas musculares podem ocorrer em várias situações, como no envelhecimento (McArdle et al. 2002), na imobilização (Dodd et al. 2009), na microgravidade (Schuenke et al. 2009) e no treinamento físico (Campos et al. 2002).

O treinamento resistido promove um aumento da força e da massa muscular, além de induzir a modulação dos tipos de fibras e das isoformas das cadeias pesadas da miosina (MHC) do tipo MHCIIb para MHCIIa (Campos et al. 2002). Por outro lado, o treinamento aeróbico leva a pequenas mudanças na massa muscular, entretanto, induz ao aumento do número de mitocôndrias, da atividade das enzimas do metabolismo oxidativo (Hawley 2002) e da captação de oxigênio em exercício submáximo (Trappe et al. 2006). Desta forma, para atender a demanda mecânica e metabólica do exercício/treinamento físico, ocorrem ajustes e/ou adaptações musculares que resultam em modificações na expressão gênica e tradução de proteínas músculo-específicas (Saltin e Gollnick 1983; Psilander et al. 2003). Assim, o controle da massa muscular é mediado pelas vias intracelulares que afetam a taxa de síntese e degradação de proteínas. Uma maior taxa de síntese do que degradação leva a uma maior massa muscular, enquanto o oposto conduz à diminuição da mesma (Baar et al. 2006).

4.1. Características gerais do músculo esquelético

O músculo estriado esquelético é um tecido versátil e heterogêneo, constituído por células multinucleadas especializadas, as fibras musculares, que apresentam núcleos localizados na região periférica da fibra, abaixo da membrana plasmática (Figura 1). A

disposição altamente organizada dos diferentes tipos de fibras nos músculos confere a este tecido uma ampla diversidade estrutural, metabólica e funcional (Schiaffino e Reggiani 1994; Pette e Staron 2000).

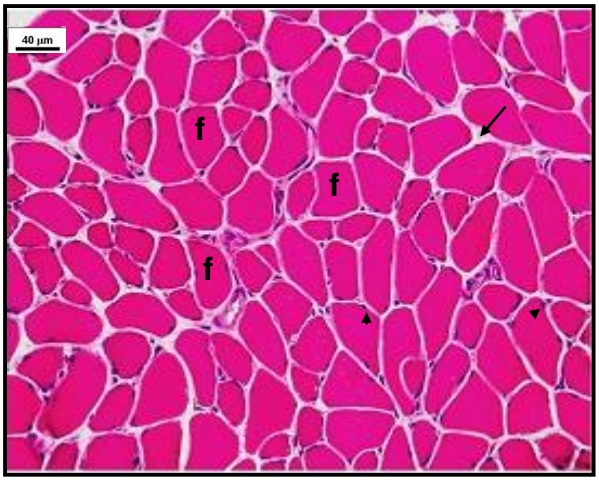

Figura 1. Corte transversal das fibras do músculo sóleo de ratos. Fibras musculares (f); Endomísio (⟶) e Mionúcleos (►). Coloração HE.

As fibras musculares são constituídas por estruturas repetidas, os sarcômeros, unidades contráteis da fibra muscular. Cada sarcômero é formado por várias proteínas, as proteínas contráteis: miosina (filamento grosso) e actina (filamento fino), além das proteínas estruturais, responsáveis pela organização e integridade funcional do sarcômero. O filamento fino é formado pela actina e duas proteínas reguladoras, a troponina e tropomiosina. O filamento grosso é formado pela polimerização de 200 a 300 moléculas de miosina da classe II (Huxley 1969).

A miosina é um hexâmero formado por seis polipeptídeos: duas cadeias pesadas da miosina (*Myosin Heavy Chain*, MHC), enroladas em α-hélice e quatro cadeias leves da miosina (*Myosin Light Chain*, MLC) (Lowey et al. 1969; Weeds e Lowey 1971; Elliot e Offer 1978; Scott et al. 2001). Cada cadeia pesada pode ser separada em duas porções: meromiosina

leve (*Light Meromyosin*, LMM), em forma de bastão, e meromiosina pesada (*Heavy Meromyosin*, HMM), conhecida como porção globosa (globular) da miosina, que contém um sítio de interação com a actina e uma região capaz de ligar-se à molécula de ATP e hidrolisá-la (atividade ATPásica) (Huxley 1969; Lowey et al. 1969). As HMM podem ser subdivididas nas porções S1 (parte globular com atividade ATPásica) e S2 (pequeno fragmento também em forma de bastão). As cadeias leves estão dispostas na proporção de duas cadeias (uma essencial e uma reguladora) para cada subfragmento S1 (Huxley 1969; Lowey et al. 1969; Dal Pai-Silva et al. 2005) (Figura 2). A contração muscular ocorre através da interação entre os filamentos finos (actina) e os filamentos grossos (porção globular da molécula de miosina) do sarcômero (Huxley 1969; Huxley 1971), após a hidrólise do ATP, pela miosina ATPase (mATPase) (Lowey et al. 1969).

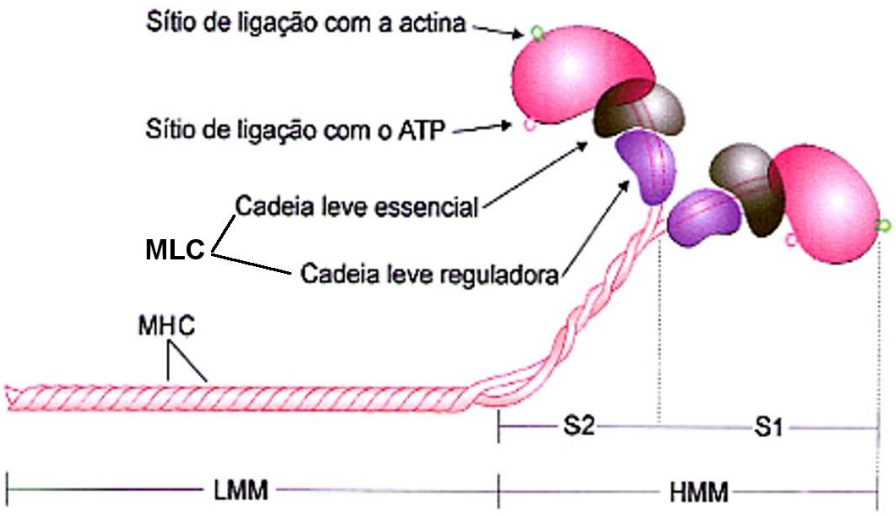

Figura 2. Esquema da molécula de miosina da classe II. Cada molécula de miosina é composta por duas cadeias pesadas da miosina (MHC) e quatro cadeias leves da miosina (MLC). As MHC podem ser clivadas e gerar as meromiosina leve (LMM) e meromiosina pesada (HMM). As HMM são compostas pela porção globosa S1 e pela porção α-hélice em forma de bastão, S2. As MLC estão dispostas na proporção de duas cadeias (uma essencial e uma reguladora) para cada subfragmento S1 (Dal Pai-Silva et al. 2005).

Segundo Talmadge e Roy (1993), a velocidade de contração de um músculo é diretamente proporcional a atividade da mATPase, da porção globular da miosina. Este evento foi demonstrado em análises de fibras isoladas, que revelaram uma alta correlação entre o tipo de fibra, baseado na atividade da mATPase, com a especificidade da cadeia pesada da miosina (MHC) (Barany 1967; Pette e Staron 2000). Pette e Staron (2001), utilizando-se da reação histoquímica para a ATPase miofibrilar, da imunohistoquímica utilizando anticorpos específicos para as isoformas de miosina (MHC) e da eletroforese em gel de poliacrilamida (SDS-PAGE), classificaram os tipos de fibras musculares em fibras de contração lenta – Tipo I (*Slow Fibers*), expressando MHCI e fibras de contração rápida – Tipo II (*Fast Fibers*), subdivididas em tipo IIA, expressando MHCIIa; tipo IID, expressando MHCIId e tipo IIB, expressando MHCIIb. As fibras do tipo IID (MHCIId) se equivalem as fibras IIX (MHCIIx), descritas em ratos (Schiaffino e Reggiani 1994). Os autores classificaram estas fibras como puras, uma vez que, expressavam uma única isoforma de miosina de cadeia pesada (Figura 3).

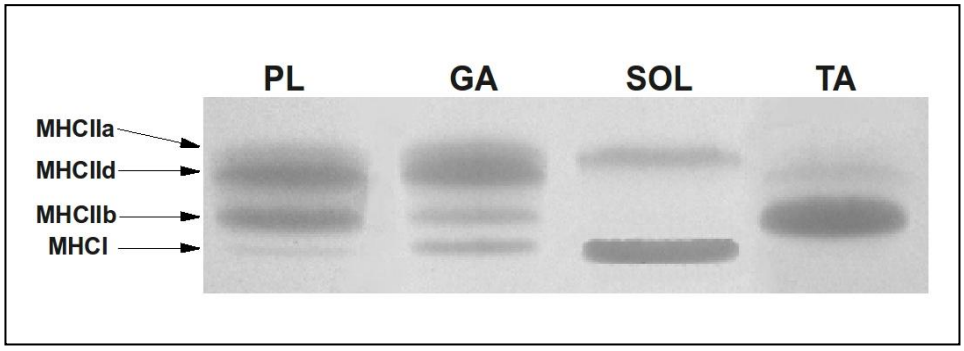

Figura 3. Separação das isoformas de miosina de cadeia pesada (MHC), segundo Pette e Staron (2001), em diferentes músculos de ratos através de SDS-PAGE. Expressão das isoformas de MHCIIa, MHCIId, MHCIIb e MHCI. *PL*, plantar; *GA*, gastrocnêmio; *SOL*, sóleo; *TA*, tibial anterior.

Além das fibras puras (Tipo I , Tipo IIA, Tipo IID e Tipo IIB), existem as fibras "híbridas", que expressam duas ou mais isoformas de miosina. Estas fibras foram classificadas

em Tipo IC (MHC I > MHC IIa), tipo IIC (MHC IIa > MHC I), tipo IIAD (MHC IIa > MHC IId), tipo IIDA (MHCIId > MHCIIa), tipo IIDB (MHC IId > MHC IIb) e tipo IIBD (MHC IIb > MHCIId) (Pette e Staron 2000; Pette e Staron 2001). A associação entre as fibras puras e híbridas constitui um contínuo de fibras lentas em direção as fibras rápidas: tipo I – tipo I/IIA – tipo IIA/I – tipo IIA – tipo IIA/D – tipo IID/A – tipo IID – tipo IID/B – tipo IIB/D – tipo IIB (Pette et al. 1999). Deste modo, assume-se uma organização seqüencial das fibras puras, as quais são intermediadas por fibras híbridas (I ↔ IC ↔ IIC ↔ IIA ↔ IIAD ↔ IID ↔ IIDB ↔ IIB) (Pette e Staron 2000) (Figura 4).

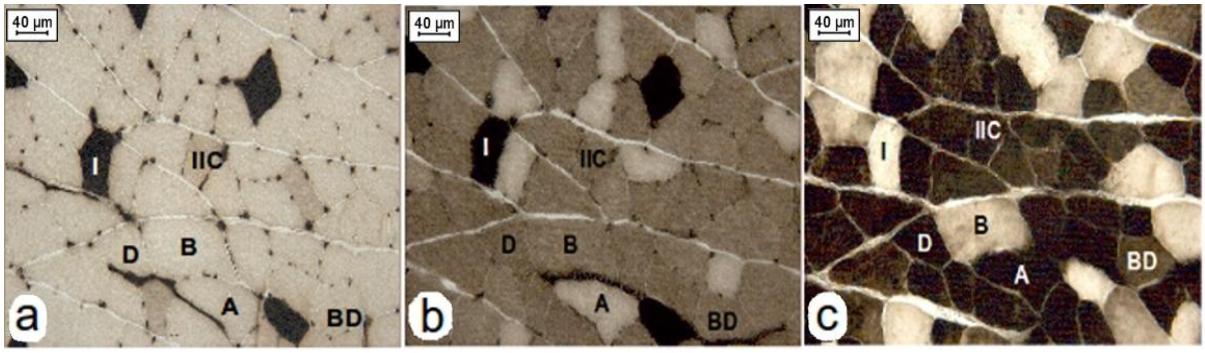

Figura 4. Corte transversal seriado do músculo plantar de rato. Análise histoquímica da mATPase em pHs 4.2 (a), 4.5 (b) e 10.6 (c). *I* - Fibra muscular tipo I; *IIC* - fibra muscular tipo IIC; *A* - fibra muscular tipo IIA, *D* - fibra muscular tipo IID; *BD* - fibra muscular tipo IIBD e *B* - fibra muscular tipo IIB.

A diversidade funcional e metabólica dos diferentes tipos de fibras confere ao músculo esquelético uma alta capacidade para realizar uma variedade de demandas funcionais (Campos et al. 2002). Neste contexto, a análise da plasticidade muscular, bem como as possíveis adaptações do músculo esquelético em resposta ao exercício físico têm sido amplamente investigadas (Williamson et al. 2000; Sharman et al. 2001; Parcell et al. 2003; Harber et al. 2004). Os estímulos sucessivos dos diferentes modelos de treinamento físico podem provocar ajustes específicos no fenótipo das fibras musculares, a fim de suprir as necessidades do organismo, e, tentar otimizar o desempenho físico (Siu et al. 2004).

4.2. Mecanismos envolvidos na hipertrofia do músculo esquelético no treinamento resistido

O treinamento resistido (TR) é considerado um potente estímulo, capaz de promover hipertrofia e induzir à modulação dos tipos de fibras e das isoformas de MHC, aumentando a força e a potência muscular (Campos et al. 2002; Kraemer et al. 2002; Bickel et al. 2005). A magnitude e especificidade das adaptações musculares aos diversos modelos e protocolos de TR (freqüência, intensidade, duração), são dependentes de alterações na expressão gênica (conteúdo de mRNA) e na tradução de proteínas músculo-específicas (Saltin e Gollnick 1983; Psilander et al. 2003), que promovem o aumento da síntese de proteínas miofibrilares.

O conteúdo de proteínas miofibrilares representa aproximadamente 85% do volume da fibra muscular (Hoppeler 1986). Assim, as alterações no balanço entre a síntese e degradação de proteínas miofibrilares podem contribuir para o aumento ou redução da massa muscular. Vários trabalhos apontam um aumento da síntese protéica, após uma única sessão de treinamento resistido, que pode permanecer elevada por até 24h (Rennie et al. 2004), 36h (McDougall et al. 1995) e 48h (Philips et al. 1997). Laurent et al. (1978) sugerem que os estágios iniciais de aumento da síntese protéica, são mediados por uma maior eficiência traducional. Posteriormente, o aumento de mRNA torna-se crítico para a continuidade deste processo (Adams 1998). Em humanos, Welle et al. (1999), demonstraram que o exercício pode estimular a síntese protéica sem alterar as concentrações de RNA total e mRNA. Os autores argumentam que se houver uma maior disponibilidade de ribossomos e de fatores traducionais (fatores de iniciação e elongação) para traduzir os mRNAs, o aumento da concentração de mRNA pode levar ao aumento da síntese protéica. Neste sentido, Psilander et al. (2003) sugerem que o aumento da síntese protéica seja resultado de uma melhora transcricional, de uma maior estabilidade das moléculas de mRNA, de uma maior taxa de tradução, ou a

combinação desses processos.

Consistente com o aumento da síntese protéica, Staron et al. (1991), mostraram um aumento de 10 a 30% na área de secção transversal (AST) das fibras musculares de indivíduos sedentários, após 12 semanas de treinamento resistido. Em adição, vários trabalhos relatam que o treinamento resistido crônico pode afetar positivamente a resposta aguda de síntese protéica muscular, após uma única sessão de exercício resistido (Phillips et al. 2002; Rasmussen e Phillips 2003), favorecendo uma maior resposta anabólica muscular.

Teoricamente, o número de mionúcleos é o fator determinante da taxa de síntese protéica, uma vez que fornecem a quantidade necessária de DNA para suportar o aumento da transcrição. A quantidade de citoplasma (área da fibra) controlada por um único mionúcleo caracteriza o conceito de domínio mionuclear (Cheek 1985). Em humanos, Petrella et al. (2006, 2008) sugerem que até um limite moderado de hipertrofia, que alcance um tamanho máximo de ~ 2.000-2.250 μm^2 na área do domínio mionuclear, o aumento na AST das fibras pode ocorrer, sem a necessidade de acrescentar novos mionúcleos. Tal fato ocorre devido à capacidade dos mionúcleos existentes na fibra de intensificar o processo de tradução e, assim, promover um aumento da síntese protéica. Porém, em modelos de treinamento resistido de longo prazo, que envolvem sessões repetidas de exercício, o aumento da AST das fibras pode exceder um volume citoplasmático suportável pelo mionúcleo. Neste caso, a adição de novos mionúcleos é necessária para suprir a hipertrofia das fibras musculares (Allen et al. 1995; Kadi e Thornell 2000).

Com base no conhecimento de que os mionúcleos das fibras musculares maduras são considerados pós-mitóticos (não apresentam capacidade de divisão), sugere-se que a adição de novos mionúcleos seja realizada somente pela atividade das células satélites (CS), que são precursores miogênicos com intensa atividade mitogênica (Figura 5). Essas células, quando

ativadas, proliferam e se diferenciam fornecendo núcleos para a regeneração da microlesão muscular (miotrauma adaptativo), contribuindo assim para a hipertrofia da miofibra (Bischoff 1994; Hawke e Garry 2001; Chargé e Rudnicki 2004).

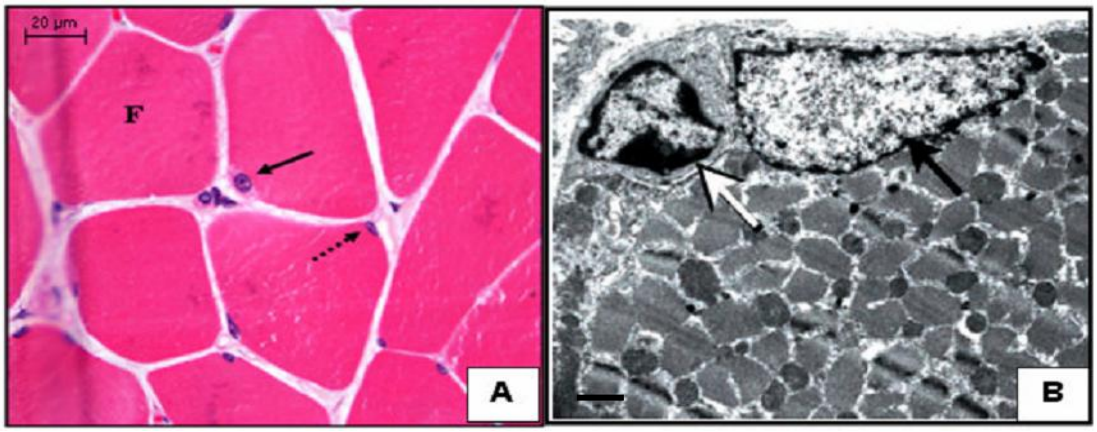

Figura 5. A: Corte transversal de músculo gastrocnêmio de rato. Localização periférica da célula satélite (seta contínua), Fibra muscular (F) e Mionúcleo (seta descontínua). Coloração H.E. B: Ultra-estrutura de uma fibra muscular. Célula satélite (seta branca) e Mionúcleo (seta preta). Bar = 2 µm.

O número de CS nos músculos é dependente da espécie, da idade e do tipo de fibra considerado, e sua freqüência varia ao longo da fibra muscular, sendo 20% maior na região da junção mioneural e próximo de capilares sanguíneos (Kraemer et al. 2002). Além disso, em ratos, os músculos de contração rápida apresentam menor porcentagem de CS, em relação aos músculos de contração lenta (Gibson e Schultz 1982).

As CS podem ser ativadas em resposta a miotraumas severos ou adaptativos, que requerem a regeneração muscular. Este mecanismo envolve a proliferação e/ou diferenciação das CS (Hawke e Garry 2001; Chargé e Rudnicki 2004), que participam da formação de novas fibras (apenas em situações de necrose seguida de regeneração), originam novas células satélites ou diferenciam-se em novos mionúcleos. Consistente com a participação das CS no processo de hipertrofia, Kadi et al. (2004) observaram, em humanos, um aumento do número

de CS, após 30 (19%) e 90 (31 %) dias de treinamento resistido, porém, o número de mionúcleos permaneceu inalterável por todo período de treinamento. Os autores argumentam que as mudanças moderadas no tamanho (~ 20%) da fibra muscular podem ser alcançadas, sem a adição de novos mionúcleos. Já, em músculo humano suportando acentuada hipertrofia (AST da fibra > 25%), a adição de novos mionúcleos, via recrutamento de células satélites, parece ser necessária para suportar o aumento da AST das fibras (Petrella et al. 2008). Segundo os autores, a presença basal de CS é o fator determinante, em resposta ao treinamento, para aumentar a quantidade de CS, incorporar novos núcleos e atingir maior hipertrofia muscular.

O controle e ativação das CS podem ser influenciados por vários fatores de crescimento, que atuam como reguladores positivos, como os hormônios (GH - *growth hormone*, insulina, e testosterona), os fatores de crescimento (IGF-1, *insulin-like growth factor-1*; HGF, *hepatocyte growth factor*; FGF, *fibroblast growth factor*) e as citocinas (IL-6, *interleukin-6*; IL-15, *Interleukin-15*); e/ou reguladores negativos como o TGF-β, *transforming growth factor-beta* (Chargé e Rudnick 2004). Além disso, as CS tanto no estado quiescente como no estado ativado, expressam marcadores miogênicos (Hawke e Garry 2001), que controlam as fases de ativação, proliferação e diferenciação durante o processo de crescimento ou reparo muscular (Seale e Rudnick 2000). Vários marcadores para CS foram identificados, entre eles o *Pax7*, o *Sox8*, o *c-Met*, a proteína *CD34* (Cornelison et al. 2000; Michal et al. 2002; Schmidt et al. 2003; Chargé e Rudnick 2004) e os fatores de regulação miogênica (*MRFs*, *myogenic regulatory factors*) (Seale e Rudnick 2000).

Os MRFs são fatores transcricionais expressos no músculo esquelético durante a miogênese e crescimento muscular, nos processos de reparação, sendo importantes também para a manutenção do fenótipo do músculo. Fazem parte dos MRFs a MyoD, Miogenina, Myf5 e MRF4. Essas proteínas nucleares contêm um domínio altamente conservado, conhecido como "basic helix-loop-helix" (bHLH). Os MRFs reconhecem, através de seu domínio básico ("basic"), uma seqüência consenso no DNA conhecida como E-box (5´-CANNTG-3´), presente na região promotora da maioria dos genes músculo-específicos. A região "helix-loop-helix" do MRF constitui o domínio de ligação dessa molécula com proteínas E, como E12 e E47. A ligação do heterodímero MRF-proteína E à seqüência E-box ativa a transcrição dos genes músculo-específicos, levando à sua expressão (Rudnick e Jaenisch 1995; Molkentin e Olson 1996; Sabourin e Rudnick 2000). Além disso, essa interação pode iniciar a transcrição dos genes dos próprios MRFs durante o crescimento muscular (Murre et al. 1989; Sasson 1993) (Figura 6).

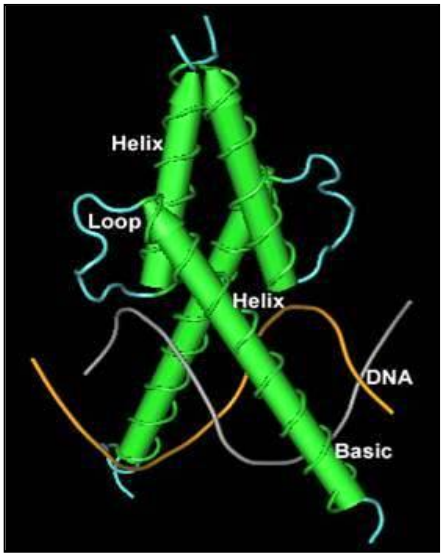

Figura 6. Estrutura cristalográfica do complexo formado pelo dímero do fator transcricional da família "basic Helix-Loop-Helix" (bHLH) MyoD e o DNA (adaptado de Ma et al. 1994).

A MyoD e o Myf5 são conhecidos como fatores primários, sendo expressos em mioblastos na fase de proliferação, que antecede a de diferenciação, enquanto que a miogenina e o MRF4 são expressos em células na fase de fusão e diferenciação em fibras musculares imaturas (Megeney e Rudnick 1995) (Figura 7). A miogenina e a MyoD também podem estar envolvidas na manutenção do fenótipo da fibra muscular adulta, como rápido ou lento. A miogenina é expressa em níveis superiores aos da MyoD em músculos lentos, enquanto que o oposto é verdadeiro para músculos rápidos (Hughes et al. 1993; Voytik et al. 1993). Os MRFs também estão envolvidos com as adaptações musculares em resposta ao treinamento físico, Bickel et al. (2005) e Psilander et al. (2003) mostraram um aumento na expressão de mRNA para MyoD, miogenina e MRF4, no músculo vasto lateral, após uma única sessão de treinamento resistido.

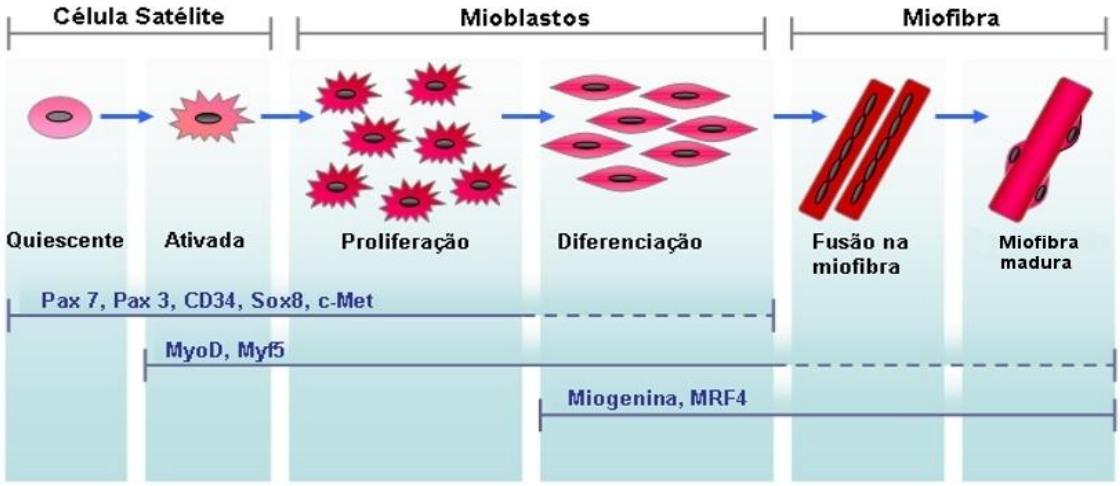

Figura 7. Representação esquemática das células satélites no crescimento muscular. Células satélites quiescentes expressam os marcadores Pax 7, Pax 3, CD 34, Sox 8 e c-Met. Após sua ativação, proliferam e passam a expressar os MRFs MyoD e Myf-5, tornando-se mioblastos. A expressão de Miogenina e MRF4 controla a diferenciação dos mioblastos em miotubos, que posteriormente se diferenciam para formar as miofibras maduras (adaptado de Zammit et al. 2006).

Em resposta a estímulos adaptativos (miotrauma do treinamento resistido) as CS são ativadas, proliferam-se e se diferenciam em mioblastos (Hawke e Garry 2001). Uma pequena proporção de células satélites que proliferaram retornam ao estado quiescente e restabelecem a população de células satélites, pelo processo de auto-renovação (Deasy et al. 2001) (Figura 7). Já, os mioblastos comprometidos com a regeneração migram para a região danificada e fundem-se à fibra muscular pré-existente para reparar o local da microlesão e/ou adicionar núcleos para ampliar a capacidade de síntese protéica e, assim, promover a hipertrofia. Entretanto, em casos de lesões mais severas onde ocorre a necrose da fibra (ação de toxinas e distrofia muscular), os mioblastos poderão alinhar-se e fundir-se entre si, para formar uma nova miofibra (Hawke e Garry 2001; Chargé e Rudnick 2004) (Figura 8).

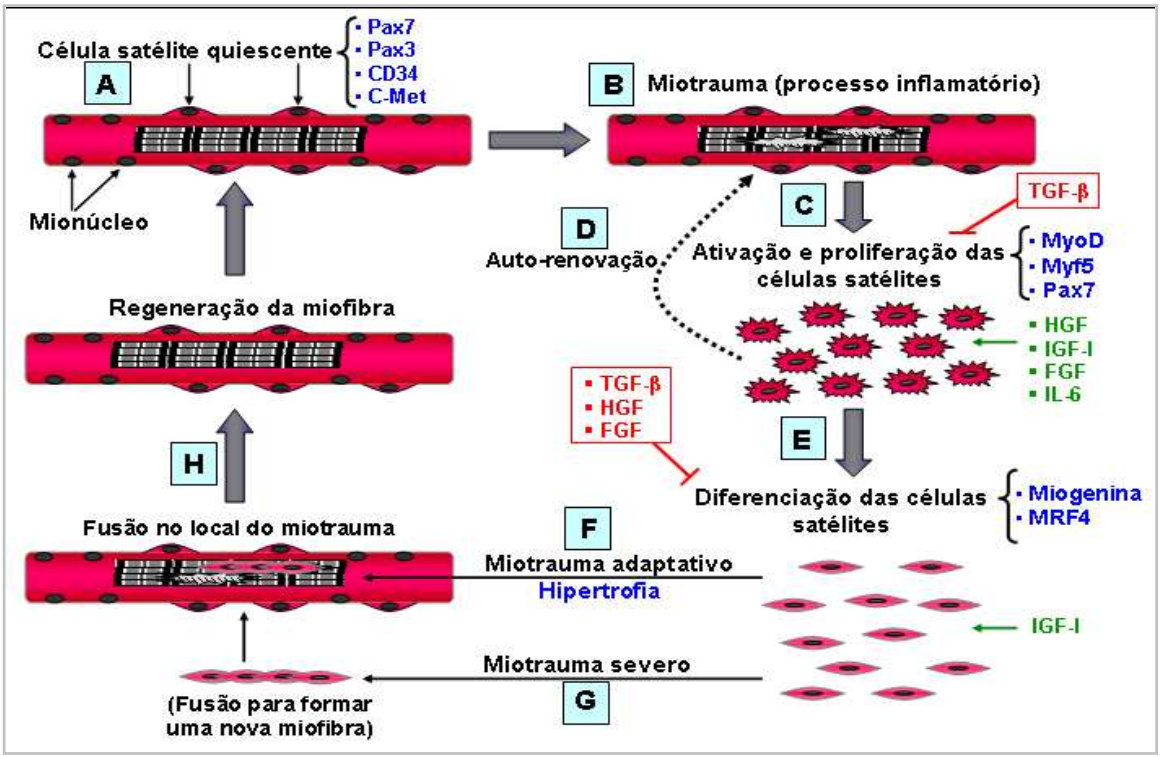

Figura 8. Esquema demonstrando o processo de regeneração muscular. Fibra muscular normal com célula satélite quiescente e mionúcleo (A). Após um miotrauma (B), as células satélites quiescentes são ativadas, proliferam-se (C) e se diferenciam em mioblastos (E). No miotrauma adaptativo do exercício físico, os mioblastos migram para a região danificada e fundem-se à fibra muscular pré-existente para reparar o local da microlesão e/ou adicionar núcleos para ampliar a taxa síntese protéica (hipertrofia) (F). Porém, em situações de miotraumas severos que ocorra necrose das fibras (ação de toxinas e distrofia), os mioblastos poderão se alinhar e fundir-se entre si, para formar uma nova miofibra (G), e reparar o dano da fibra muscular (H). Durante o processo de regeneração, alguns mioblastos retornam ao estado quiescente e restabelecem a população de células satélites (D). A fase de ativação das células satélites é caracterizada pela alta expressão de MyoD e Myf5, e na diferenciação ocorre um aumento na expressão de miogenina e MRF4 (Azul). Cada estágio do processo de regeneração é mediado por fatores de crescimento, que atuam como reguladores positivos (verde) ou reguladores negativos (vermelho). Adaptado de Chargé e Rudnick (2004).

As adaptações fenotípicas musculares observadas em diferentes modelos de treinamento físico são dependentes da força, velocidade e duração do processo de contração

muscular (impulso nervoso), cuja magnitude está associada aos estímulos extrínsecos (carga ou estresse mecânico) e intrínsecos (níveis de cálcio intracelular, hipóxia e o estado redox) (Baar e Esser 1999). Os estímulos específicos (perturbações mecânicas, estiramento, microlesão/injúria e estresse celular), originados de diferentes tipos e protocolos de exercício físico são transduzidos por receptores de superfície celular (moléculas transmembranas), ativando vias intracelulares que integram esta informação (Wackerhage e Woods 2002), e assim, controlam as mudanças quantitativas e qualitativas no músculo, por meio da ativação ou repressão de genes músculo específicos (Bassel-Duby e Olson 2006). Dentre as vias intracelulares, destaca-se a via do crescimento semelhante à insulina-1 (IGF-1, *insulin-like growth factor-1*) (Tidball 2005).

O IGF-1, um polipeptídeo altamente conservado com 70 aminoácidos, similar na sua sequência estrutural com o IGF-2 e a insulina, desempenha uma função central na regulação do desenvolvimento e crescimento muscular pelas suas potentes ações mitogênicas e metabólicas (Clemmons 2009); estimula a proliferação e diferenciação de células precursoras musculares (mioblastos e células miossatélites) bem como a hipertrofia dos miócitos durante a regeneração muscular (Florini et al. 1996; Musaro et al. 1999). Sua origem predominante é o fígado, o qual fornece aproximadamente 75% de todo o IGF-1 circulante do corpo (Schwander et al. 1983). No entanto, a expressão de IGF-1 não é restrita ao fígado, podendo ser encontrada na maioria dos tecidos do corpo, inclusive o muscular (Roith 2003).

Vários estudos fornecem evidências da atuação do IGF-1 como um potente sinal anabólico no tecido muscular (Hobler et al. 1998; Barton-Davis et al. 1999; Glass 2003; Goldspink 2005). Os sinais mecânicos que atingem as células musculares, como por exemplo, a perturbação mecânica nas fibras musculares, ocasionada pelo processo de contração durante

o exercício físico (treinamento resistido), induz à liberação do IGF-1, que se liga ao receptor na superfície celular, e, assim, ativa uma "cascata" de eventos intracelulares, através da via IRS → PI3K → AKT → mTOR→p70S6K. O IGF-1 ligado ao receptor irá recrutar o IRS (substrato responsivo à insulina, *insulin response substrate*), causando a ativação da PI3K (quinase fosfatidil-inositol-3, *phosphatidyl-inositol-3*), que adiciona fosfato na AKT (*serine-threonine kinase*), tornando-a ativa para fosforilar o mTOR (mammalian target of rapamycin), e subsequentemente a p70S6K (*p70S6 Kinase*), que atua como um potente estimulador da síntese protéica (Glass 2005; Tidball 2005) (Figura 9).

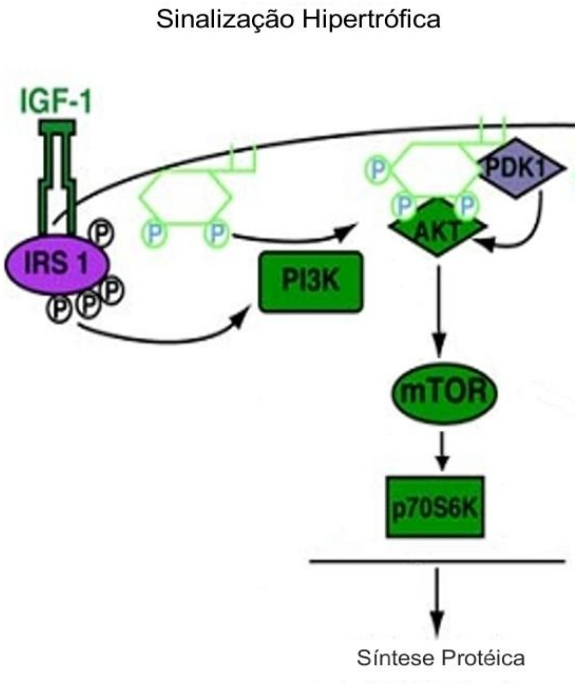

Figura 9. Via de sinalização intracelular envolvida na hipertrofia. Os sinais mecânicos que atingem a fibra muscular, como por exemplo, a perturbação mecânica ocasionada pelo processo de contração durante o exercício físico (treinamento resistido), induz à liberação do IGF-1, que se liga ao receptor na superfície celular, e, assim, ativa uma "cascata" de eventos intracelulares, através da via IRS→PI3K→AKT→mTOR→p70S6K, aumentando a síntese protéica (Adaptado de Glass 2005).

4.3. Sobretreinamento

Estudos mostram que o treinamento resistido (TR) pode provocar ajustes específicos no fenótipo das fibras musculares, alterando as propriedades morfofuncionais do músculo (Green et al. 1999; Sharman et al. 2001). Neste contexto, os efeitos benéficos do TR, incluem o aumento na área de secção transversal das fibras (hipertrofia), na força, na potência (Timson 1990) e na capacidade oxidativa do músculo esquelético (Holloszy & Coyle, 1984; Booth e Thomason 1991). No entanto, a adaptação do músculo esquelético ao treinamento geralmente envolve a aplicação da sobrecarga progressiva, além de um nível confortável para o praticante, de modo a otimizar o seu desempenho (Fry et al. 1991; Stone et al. 1991; Fry e Kraemer 1997; Smith e Miles 1999). Todavia, existe uma linha tênue entre a melhora e a piora do desempenho atlético. Assim, quando o treinamento proporciona um trauma muscular repetitivo devido à alta intensidade/volume, associada ao tempo insuficiente de repouso/recuperação, pode ocorrer uma estagnação ou mesmo a diminuição do desempenho (Small 2002), levando a um estado chamado de sobretreinamento (Snyder et al. 1995; Lehmann et al. 1999). A recuperação do sobretreinamento (ST) pode demorar semanas ou meses de repouso absoluto ou através do treinamento físico bastante reduzido. O termo pré-sobretreinamento (PST) ou sobretreinamento de "curto prazo" descreve a forma menos severa desta condição, em que a recuperação geralmente ocorre dentro de dias a semanas (Lehmann et al. 1993). O limite entre ST, o PST e o treinamento normal são de difícil determinação porque podem apresentar um ou mais dos seguintes sintomas: estado catabólico acentuado; alterações fisiológicas, imunológicas e bioquímicas; aumento da incidência de lesões e alterações de humor (Allen et al. 1995; Booth et al. 1998; Deasy et al. 2001; Hohl et al. 2009).

A avaliação da diminuição do desempenho específico ainda representa o melhor

padrão no diagnóstico do ST e necessita de testes específicos do esporte. Nesse sentido, testes máximos até a exaustão podem identificar uma diminuição no desempenho específico do esporte, pois atletas em ST normalmente apresentam uma diminuição no desempenho anaeróbico, uma redução no tempo de exaustão em testes de alta intensidade e uma pequena diminuição na freqüência cardíaca máxima. O lactato também apresenta uma diminuição durante o exercício submáximo e isto resulta num leve aumento do limiar anaeróbio (Baar et al. 1999). Nesse contexto, Fry et al. (1994) demonstraram que indivíduos submetidos a um programa de sobretreinamento anaeróbico (treinamento resistido de alta intensidade) de curto prazo, apresentaram diminuição do desempenho atlético. Em outro estudo, Halson et al. (2002) verificaram, em oito ciclistas, os efeitos cumulativos do estresse do exercício e sua subseqüente recuperação sobre o desempenho físico e os indicadores de fadiga. Foi observada uma diminuição significativa na potência, um aumento de 29% na alteração do humor, diminuição do desempenho físico, associada a uma redução de 9,3% da freqüência cardíaca máxima e 5% de redução no consumo de oxigênio.

Alguns fatores importantes complicam um diagnóstico imediato do ST: (1º) a variação intra-individual; (2º) os sintomas diferentes para diminuições agudas (PST) e crônicas (ST) do desempenho físico; (3º) o volume excessivo de treinamento afetando o organismo de maneira diferente da intensidade excessiva de treinamento; (4º) duas formas de ST, simpática e parassimpática, sendo que cada uma apresenta sintomas diferenciados; (5º) os sintomas do ST em atletas de resistência também são diferentes dos atletas de força (Fry e Kraemer 1997); (6º) são necessárias repetidas amostras de sangue antes e depois do exercício; (7º) o exercício causa alterações no volume plasmático e isto deve ser corrigido; (8º) os marcadores fisiológicos e bioquímicos apresentam características diferentes de acordo com o esporte e são influenciados por aspectos psicológicos, sociais ou culturais (Petibois et al. 2002).

Nesta linha de investigação, a alta incidência do ST em atletas (McKenzie 1999; Kentta et al. 2001) tem atraído interesse dos pesquisadores nos últimos anos para identificar as possíveis causas e efeitos deste fenômeno. Várias hipóteses têm sido propostas, sendo que algumas permanecem viáveis, enquanto, outras não têm o mínimo suporte. Muitas evidências sugerem que o dano muscular é freqüentemente o iniciador do ST. Esta hipótese é fundamentada pelo rigoroso grau de lesão tecidual, devido ao treinamento intenso dos atletas. Naturalmente, o treinamento e a competição estão associados a um trauma tecidual leve seguido de sua recuperação (Smith 2000). Quando é permitido a adequada recuperação, ocorre um processo adaptativo, muitas vezes chamado de "microtrauma adaptativo" (Smith 2000). No entanto, quando a intensidade/volume de treinamento são aumentados abruptamente e o tempo de descanço/recuperação são insuficientes, um trauma leve pode evoluir para uma um trauma crônico do tecido. Assim, esta lesão subclínica pode levar ao catabolismo muscular, resultado do balaço protéico negativo (Philips et al. 1997). Numerosos estudos envolvendo os efeitos do sobretreinamento sobre o balanço protéico no músculo esquelético mostraram um predomínio do estado catabólico (Petibois et al. 2000; Wolfe 2006). Petibois et al. (2003) observaram em indivíduos sobretreinados um maior acúmulo de aminoácidos e menor conteúdo de proteínas no sangue, comparado a indivíduos bem treinados, sugerindo a ocorrência de um aumento na proteólise muscular, com concomitante atrofia muscular (Smith e Miles 1999).

Atualmente, tem sido sugerido que o ST pode induzir uma inflamação local aguda, evoluindo para uma inflamação crônica e produzindo uma inflamação sistêmica. Parte dessa inflamação sistêmica envolve a ativação dos monócitos circulantes, que podem sintetizar grandes quantidades de citocinas inflamatórias, entre elas o fator de necrose tumoral-α (TNF-α). Tem sido demonstrado que a atrofia da musculatura esquelética pode ser induzida pelo fator de necrose tumoral (TNF-α) (Beutler et al. 1985; Tisdale 1997; Argiles e Lopez-Soriano

1999). Além disso, há uma importante associação entre o IGF-1, que se destaca por ser um importante regulador da massa muscular e o mediador inflamatório TNF-α. Fan et al. (1995) demonstraram que o TNF-α diminui o IGF-1 no fígado e no músculo, enquanto que o pré-tratamento com anti-TNF-α previne completamente o decréscimo do IGF-1 no músculo. Além disso, o TNF-α pode ativar o fator nuclear Kappa β (NF-κβ), uma família de fatores transcricionais envolvidos em vários processos celulares como apoptose, imunidade/inflamação e desenvolvimento/diferenciação, sendo todos expressos no músculo esquelético (Hunter et al. 2002). Os genes alvos do NF-κβ, responsáveis pela atrofia da musculatura esquelética induzida pelo TNF-α, ainda são pouco conhecidos, porém esse processo parece resultar principalmente da proteólise específica de cadeia pesada de miosinas (MHC), mediada pela ativação do sistema proteossomal dependente de ubiquitina (PDU) pelo NF-κβ (Acharyya et al. 2004).

Embora a hipertrofia muscular observada após treinamento resistido esteja associada à modulação dos tipos de fibras e das isoformas de miosina (MHC), aumento a força e da potência muscular (Campos et al. 2002; Bickel et al. 2005) e uma maior expressão dos MRFs e do IGF-I (Tamaki et al. 2000; Psilander et al. 2003; Bickel et al. 2005), novos trabalhos são necessários para elucidar os efeitos desse treinamento, a longo prazo e associado a um tempo inadequado de repouso. Neste contexto, testamos a hipótese que o treinamento de alta intensidade com curto tempo de recuperação, poderia influenciar a morfologia, as expressões das isoformas da cadeia pesada de miosina (MHC), dos MRFs MyoD e Miogenina e do IGF-1, no músculo esquelético de ratos.

5. OBJETIVOS

Avaliar a influência do treinamento de alta intensidade com um tempo insufuciente de recuperação, sobre a morfologia, as expressões das isoformas da cadeia pesada de miosina (MHC), dos fatores reguladores miogênicos (MyoD e Miogenina) e do fator de crescimento IGF-1, no músculo esquelético de ratos.

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7. ARTIGO 1: Submetido para publicação na Revista European Journal of Applied Physiology

High-intensity resistance training with insufficient recovery time between bouts induce atrophy and alterations in myosin heavy chain content in rat skeletal muscle

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High-intensity resistance training with insufficient recovery time between bouts induces atrophy and alterations in rat skeletal muscle myosin heavy chain content

Abstract

The aim of this study was to test whether high-intensity resistance training with insufficient recovery time between bouts, could result in a decrease of muscle fiber cross-sectional area (CSA) and alter fiber type frequencies and myosin heavy chain (MHC) isoform content in rat skeletal muscle. *Wistar* rats were divided into 2 groups: trained (Tr) and control (Co). Tr group were subjected to a high-intensity resistance training program (5 days/week) for 12 weeks, involving jump bouts into water, carrying progressive overloads based on percentage body weight. At the end of experiment animals were sacrificed, superficial white (SW) and deep red (DR) portions of the plantaris muscle were removed and submitted to mATPase histochemical reaction and SDS-PAGE analysis. Throughout the experiment both groups increased body weight, but Tr was lower than Co. There was a significant reduction in IIA and IID muscle fiber CSA in the DR portion of Tr compared to Co. Muscle fiber-type frequencies showed a reduction in type I and IIA in the DR portion and IID in the SW portion of Tr compared to Co; there was an increase in types IIBD frequency in the DR portion. Change in muscle fiber type frequency was supported by a significant decrease in MHCI and MHCIIa isoforms accompanied by a significant increase in MHCIIb isoform content. MHCIId showed no significant differences between groups. These data show that high-intensity resistance training with insufficient recovery time between bouts promoted muscle atrophy and a transition from slow-to-fast in contractile activity in rat plantaris muscle.

Key words: Skeletal muscle · Resistance training · Myosin Heavy Chain · Atrophy · Rat

Introduction

Skeletal muscle plays an important role in determining success in competitive sports. This tissue show substantial heterogeneity as a result of distinct fiber types and myosin heavy chain (MHC) isoforms (Schiaffino and Reggiani 1996). Mammalian single muscle fiber analysis revealed the presence of pure (expressing a single MHC isoform) and hybrid fibers (expressing two or more MHC isoforms) in different muscles, which give this tissue a wide functional and metabolic diversity (Pette and Staron 2000). The following pure fiber types are based on human MHC isoform expression patterns: one slow - Type I expressing MHCI, and two fast - Type IIA with MHCIIa, and Type IIX/IID with MHCIIx/IId (Schiaffino and Reggiani 1994). However, adult rat limb muscles express four different MHC isoforms: Types I, IIa, IIx/IId, and IIb in fiber types I, IIA, IIX/IID, and IIB, respectively (Bar and Pette 1988). The differential expression of MHC isoforms in different muscles reflects their functional responses, such as contractile and metabolic behavior. These responses can also be affected by a variety of stimuli, including chronic stimulation, removal of a synergist muscle, endurance exercise, and heavy resistance training (Oakley and Gollnick 1985; Staron et al. 1990; Green et al. 1999; Sharman et al. 2001). It is generally accepted that skeletal muscle can adapt to progressive physical training via both a quantitative mechanism, based on changes in muscle mass and fiber size, and a qualitative mechanism, based on changes in fiber type and MHC content (Schiaffino and Reggiani 1996). In this context, many quantitative histochemical and biochemical studies have reported that resistance training promotes significant alteration in MHC content, enhancing physical performance (Sharman et al. 2001; Campos et al. 2002; Harber et al. 2004).

Skeletal muscle adaptation to athletic training generally involves applying a progressive overload, which implies workload beyond a comfortable level in order to optimize

performance (Fry et al. 1991; Stone et al. 1991). Unfortunately, there is a fine line between improved and reduced performance. When the exercise training leads to a repetitive muscle trauma, due to high intensity/volume training, associated with insufficient rest/recovery time between bouts, harmful effects can occur, in a state called overtraining (Kuipers and Keizer 1988). Recovery from overtraining syndrome (OTS) may require weeks to months of absolute rest or greatly reduced exercise training. The term overreaching or "short-time" overtraining describes the less severe form of overtraining, in which recovery generally occurs within days to weeks (Lehmann et al. 1993). The line between overreaching and OTS is difficult to determine because both can show one or more of the following symptoms: accentuated catabolic state; physiological, immunological, and biochemical alterations; and increased incidence of injury and mood alterations (Armstrong and VanHeest 2002; Meeusen et al. 2006).

In recent years, the high incidence of OTS in athletes has attracted interest from researchers to identify the possible causes and effects of this phenomenon (McKenzie 1999; Kentta et al. 2001). A variety of hypotheses have been proposed to account for OTS and considerable evidence suggests that muscle injury is frequently the initiator of OTS. This hypothesis reasons that a rigorous degree of tissue damage is due to hard training and competition for athletes. Naturally, training and competition are associated with a mild tissue trauma followed by recovery (Smith 1999). When adequate recovery is allowed, it results in an "overshoot" phenomenon, an adaptive process often called "adaptive microtrauma" (Smith 1999). However, when intensity/volume training is abruptly increased and rest/recovery time between bouts are insufficient, mild trauma could develop into a more chronic, severe form of tissue trauma. Thus, progression from the benign adaptive microtrauma stage may exacerbate the initial injury (Stone et al. 1991) resulting in a subclinical injury in the overtrained athlete.

Muscle injury induces a reduction in strength (Fatouros et al. 2006) due to extensive muscle damage (Seene et al. 1999), swelling in the injured area, soreness, edema (Jamurtas et al. 2000; Fatouros et al. 2006), and local inflammatory response (Smith 2004). Also, Petibois et al. (2003) demonstrated that overtrained individuals have higher amino acid and lower protein blood accumulation in response to exercise than well-trained individuals. This result strongly suggests a protein catabolism for amino acid supply during exercise in overtrained individuals. Similarly, Smith (1999) reported a transition from the carbohydrate and lipid metabolism towards the protein metabolism during prolonged overtraining conditioning. The author further reported that during overtraining, glutamine and other amino acids are released from skeletal muscle for uptake by tissues involved in immune response and tissue repair, resulting in negative protein balance. Moreover, a decrease in the testosterone/cortisol ratio has also been suggested as a marker of 'anabolic-catabolic balance' and as a tool in OTS diagnosis (Budgett 1998). Collectively, the results of these studies indicate that OTS can directly induce muscle mass loss.

Although evidence suggests that OTS have a profound impact on skeletal muscle phenotype due to a change in the anabolism/catabolism balance, it remains poorly understood whether OTS induces morphological and contractile properties alterations of skeletal muscle. The purpose of this study was to test the hypothesis that high-intensity resistance training with insufficient recovery time between bouts, which could potentially lead to overtraining, may result in a decrease of fiber types cross-sectional area (CSA) and alter the muscle contractile activity observed through changes in fiber type frequencies and myosin heavy chain (MHC) isoform content in rat skeletal muscle. We analyzed the plantaris muscle because it is highly recruited in our model of high intensity resistance training and because it possesses a mixture of slow and fast twitch MHC-based phenotype. Previous studies involving humans and

animals strongly linked MHC expression and CSA with muscle contractile function and a variety of physiological stimuli, such as resistance training (Pette and Staron, 2000; Seene et al., 2003). To our knowledge, few studies have investigated contractile changes, specifically MHC expression and skeletal muscle CSA during overtraining.

Methods

Research Design

An animal model was used to test the hypothesis that high-intensity resistance training associated with insufficient recovery time between bouts could result in harmful effects on skeletal muscle. Training protocol intensity and volume throughout the experiment period were progressive and provided an effective manner of investigating skeletal muscle results. We used independent variable high-intensity resistance training to examine the effects on muscle parameters (dependent variables). Two days after the end of training, morphometric and biochemical analyses were performed to assess muscle fiber type and MHC isoform content in a single isolated muscle. The animal model provided an accurate method for isolating single muscles and performing analysis on whole muscle preparations to reflect total muscle response.

Animals

All experimental procedures were approved by the Biosciences Institute Ethics Committee, UNESP, Botucatu, SP, Brazil (Protocol No. 018/08-CEEA) and were conducted according to the policy statement of the U.S. Department of Health, Education, and Welfare and the American Physiological Society on research with experimental animals. Male *Wistar* rats (200-250g) were obtained from the Multidisciplinary Center for Biological Investigation

(CEMIB, UNICAMP, Campinas, São Paulo, Brazil). They were housed in collective polypropylene cages (three animals per cage) covered with metal grids, in a temperature-controlled room (22-24ºC) under a 12:12 hour light-dark photoperiod and provided with unlimited access to standard rat chow and water. Rats were randomly divided into two groups: non-trained (Co, n=9) and trained (Tr, n=9).

Training protocol

Trained group was submitted to a 12-week high-intensity resistance training program, similar to that described by Cunha et al. (2005) (Table 1). Before the initial training program, animals underwent a one-week pre-training (once a day) to familiarize them with the water and exercise. In this phase, rats individually performed jumping bouts into a 38cm deep vat of water at 28-32°C (Fig. 1). Animals jumped into the water and surfaced to breathe without needing any direct stimulus to complete the jumping bouts. The depth was appropriate to allow each animal to breathe on the surface of the water during successive jumps. A jump was counted when the animal reached the surface and returned to the bottom of the vat, a repeatedly performed movement. The adaptation protocol consisted of increasing the number of sets (two to four) and repetitions (five to ten), carrying an overload of 50% body weight strapped to a vest on the animal's chest (Fig. 1). After the adaptation period, the Tr group began the progressive high-intensity resistance training program for five consecutive days per week with 40 second rests between each set. The volume (sets x jumps) and intensity (overload) of the training protocol are presented in Table 1. This training emphasizes high workload with insufficient recovery time between bouts, and was designed to induce the muscle to support an overload beyond recommended to promote beneficial effects on muscle tissue. Bouts were performed between 2 and 3pm.

Table 1 Program of high-intensity resistance training

Week	Sets x jumps	Overload (% Body weight)
1	4 x 5	60
2	4 x 7	60
3	4 x 12	60
4	5 x 6	65
5	5 x 8	65
6	5 x 10	65
7	4 x 10	70
8	4 x 10	70
9	4 x 8	80
10	4 x 8	80
11	4 x 6	85
12	4 x 6	85

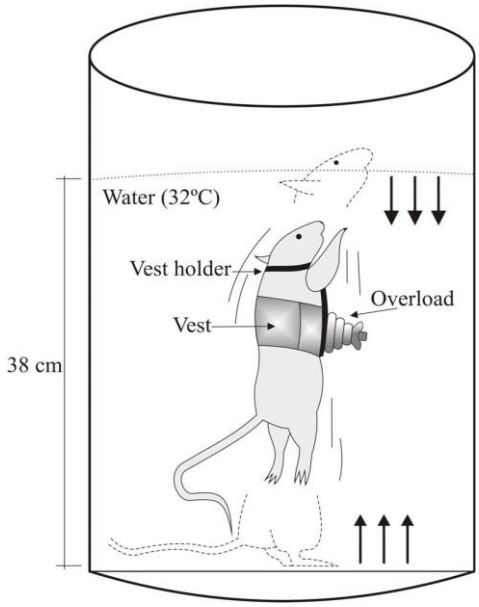

Fig 1. Sketch of the high intensity resistance training apparatus.

Histochemical and morphometric procedures

At the end of the experiment animals were anesthetized with pentobarbital sodium (40mg/kg IP) and sacrificed by decapitation. Measurements throughout the experiment included weekly animal body weight and food intake. Plantaris muscle was harvested and the middle portion

frozen in liquid nitrogen at -156°C. Samples were kept at -80°C until use. Histological sections (12µm thick) were obtained in a cryostat (JUNG CM1800, Leica Germany) at –24°C to determine muscle fiber-type frequency and cross sectional area (CSA) using myofibrillar adenosine triphosphatase (mATPase) histochemistry after preincubation at pH4.2, 4.5 and 10.6 (Guth and Samaha 1969; Brooke and Kaiser 1970). Pure (Type I, IIA, IID, and IIB) and hybrid muscle fibers (Type IIC, IIAD, and IIBD) were identified based on their staining intensities (Staron et al. 1999) (Fig. 2). No attempt was made to delineate subtypes IIDA and IIDB (Staron and Pette 1993). Muscle fiber type frequency and CSA of approximately 200 fibers from each animal were determined using an Image Analysis System Software (Leica QWin Plus, Germany). Two regions of the plantaris muscle were analyzed. A superficial-white (SW) portion with a higher percentage of slow fibers (types I and IIA) and the deep-red (DR) portion containing a higher percentage of fast fibers (type IIB) (Staron et al. 1999).

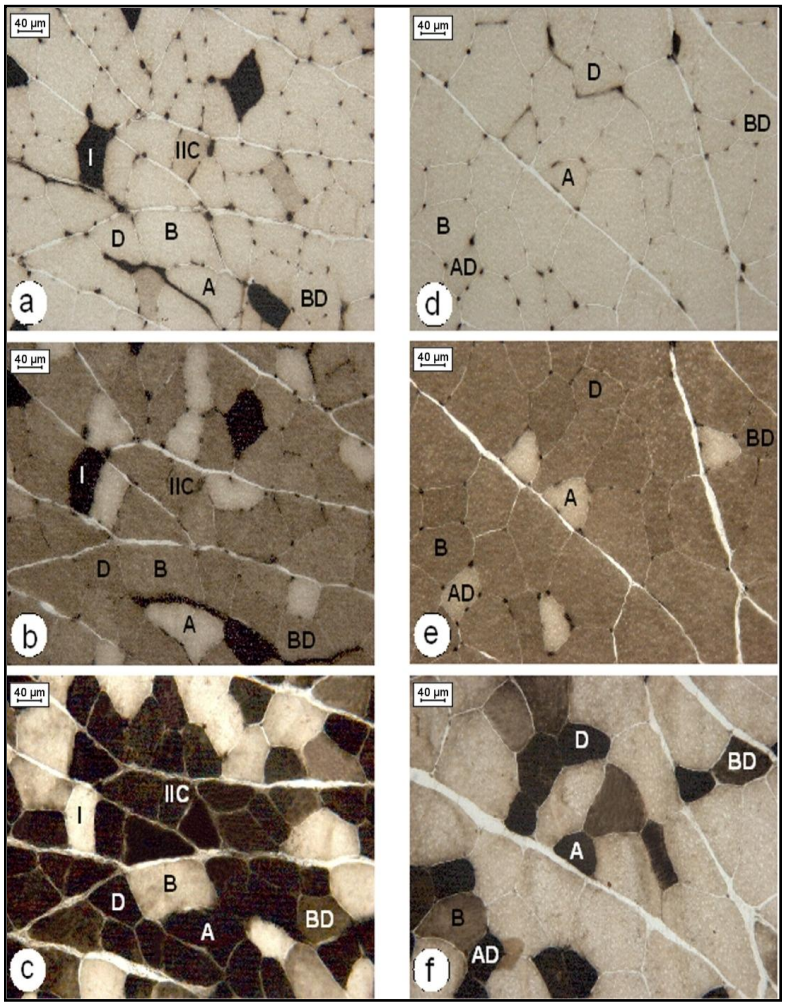

Fig 2. Serial cross sections of deep-red (a, b, c) and superficial-white (d, e, f) portions of plantaris muscle taken from a control animal demonstrating fiber-type distribution using myofibrillar adenosine triphosphatase (mATPase) reaction after preincubation at pH4.2 (a,d), 4.5 (b,e), and 10.6 (c,f). Pure (I, type I; A, type IIA; D, type IID; and B, type IIB) and hybrid (IIC, type IIC; AD, type IIAD; and BD, type IIBD) muscle fibers.

SDS-PAGE

Myosin heavy chain (MHC) isoform analysis was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in triplicate (maximum 5% variation). The protocol for analyzing the specimens was based on Talmadge and Roy (1993) and Mizunoya et al. (2008) with modifications for single-fiber analyses (Staron 1991). Eight histological sections (12 µm thick) were collected from each whole muscle sample and placed in a solution

(0.5mL) containing glycerol 10% (w/vol), 2-mercaptoethanol 5% (vol/vol), and sodium dodecylsulfate (SDS) 2.3% (w/vol) in a Tris/HCl buffer 0.9% (pH 6.8) (w/vol). The final solution was shaken for 1 minute and heated for 10 minutes at 60°C (Campos et al. 2002). Portions (20µL) of the extracts were submitted to electrophoresis reaction (SDS-PAGE 8%) using a 4% stacking gel, for 26 hours at 180V, where the maximum current was limited to 13mA. The gels were stained with Coomassie Blue (Bar and Pette 1988) and used to identify the MHC isoforms according to their molecular weight showing bands at the MHCI, MHCIIa, MHCIId and MHCIIb levels (Fig. 3). The gels were photographed and images captured by VDS Software (Pharmacia Biotech). Finally, densitometry was performed using Image Master VDS Software (version 3.0), which determined relative MHC isoform content.

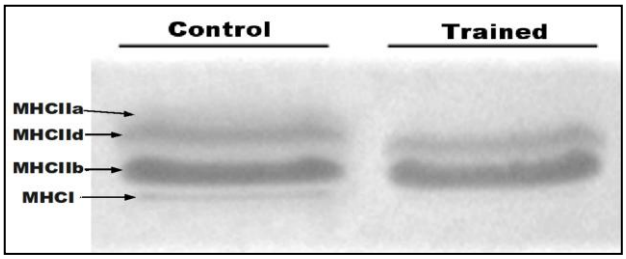

Fig 3. Electrophoretic separation of myosin heavy chain (MHC) isoforms from plantaris muscle from one representative animal in each group: non-trained (Co, n=9) and trained (Tr, n=9). MHCIIa, myosin heavy chain IIa; MHCIId, myosin heavy chain IId; MHCIIb, myosin heavy chain IIb; and MHCI, myosin heavy chain I.

Statistical Analysis

Fiber type frequency data were analyzed using the Goodman Test for contrasts between and within multinomial populations (Goodman 1964; Goodman 1965) to assess differences between the groups. Statistical comparisons between the groups were made using Analysis of

Variance for the two-factor model (Zar 1999) for each of body weight, food intake, and MHC isoform content values. When significant main effects were revealed, specific differences were assessed using Tukey's post hoc comparisons. Data are expressed as Mean ± Standard Deviation (SD). Differences were considered significant when $p<0.05$.

Results

Body weight

Final average body weights are shown in Table 2. Confirming that animals began the experiment with similar health status and physical activity level, no statistical difference was observed in initial body weight between groups (Table 2). After 12 weeks of experiment a significant decrease ($p<0.05$) in body weight was observed in Tr group against the Co group (Table 2). No statistical differences ($p>0.05$) in food intake were observed between the groups (data not shown).

Table 2 Initial and final body weight in experimental groups. Values are means ± SD. Non-trained (Co, n=9) and trained (Tr, n=9) groups.

Group	Initial body weight (g)	Final body weight (g)
Co	286.7 ± 18.9	498.6 ± 30.5 *
Tr	276.4 ± 15.5	440.9 ± 30.3 *#

* $p < 0.05$ compared to initial body weight;

# $p < 0.05$ compared to Co group (ANOVA, Tukey-test).

Muscle fiber type frequency and MHC content

The representative SDS-PAGE gel used to quantify MHC isoforms is shown in Fig. 3 and data from the two groups are summarized in Table 3. MHCI and MHCIIa percentages decreased and MHCIIb percentage increased ($p<0.05$). These data were supported by alterations in fiber type frequency. A representative mATPase histochemistry reaction used to measure fiber-type frequency is shown in Fig. 2 and the corresponding data are presented in Fig. 4. MHC isoform modulation: a significant decrease in MHCI and MHCIIa and increase in MHCIIb content in Tr were reflected by a significant ($p<0.05$) reduction in Type I and IIA muscle fiber frequency and an increase in type IIBD fibers; these consisted of more MHCIIb than MHCIId content (Pette and Staron 2000; Pette and Staron 2001) in the DR portion. On the other hand, the reduction in IID fiber type frequency in the Tr group SW portion was not associated with a significant ($p>0.05$) change in MHCIId content.

Table 3 Relative myosin heavy chain isoform percentages from homogenate muscle samples determined using sodium dodecylsulfate- polyacrylamide gel electrophoresis.

Group	MHCI	MHCIIa	MHCIId	MHCIIb
Co	4.6 ± 2.8	7.9 ± 3.5	33.4 ± 6.9	54.1 ± 9.5
Tr	0.9 ± 0.7 *	3.0 ± 2.1 *	29.6 ± 9.4	66.5 ± 10.3 *

MHCI, Myosin heavy chain I; MHCIIa, myosin heavy chain IIa; MHCIId, myosin heavy chain IId and MHCIIb, myosin heavy chain IIb. Values are means ± SD. Non-trained (Co, n=9) and trained (Tr, n=9) groups.* Significantly different from control group; $p < 0.05$ (ANOVA, Tukey-test).

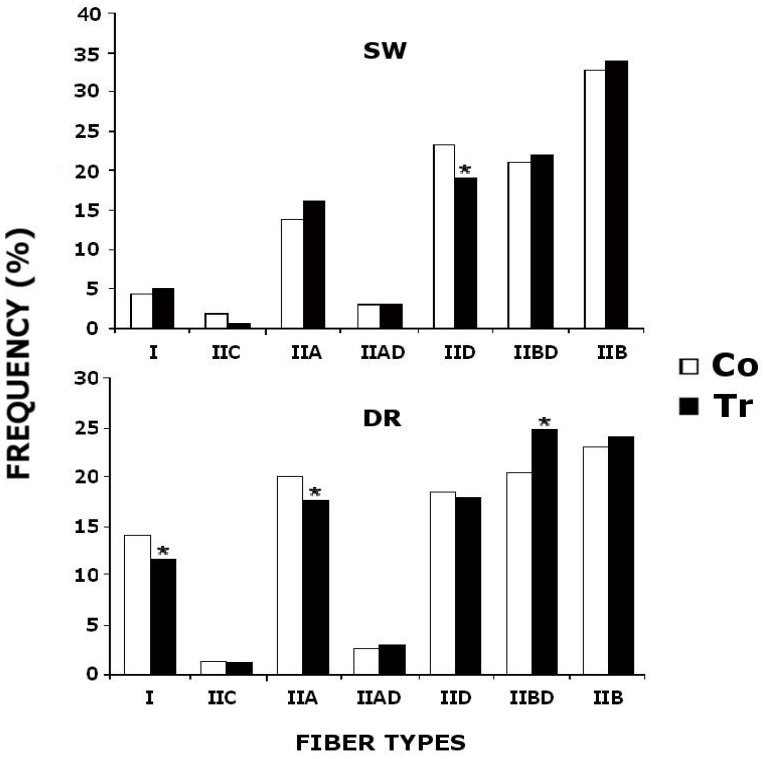

Fig 4. Plantaris muscle fiber type frequency in non-trained (Co, n=9) and trained (Tr, n=9) groups in superficial white (SW) and deep-red regions (DR). (I, Type I; IIC, type IIC; IIA, type IIA; IIAD, type IIAD; IID, type IID; IIBD, type IIBD; and IIB, type IIB). *Significantly different from control group; $p<0.05$ (Goodman Test).

Muscle fiber cross-sectional area

An atrophic effect was observed after high-intensity resistance training (Table 4). Tr group plantaris muscle DR portion muscle fiber types IIA and IID CSA's were significantly lower ($p<0.05$) than the Co group. Twelve weeks of high-intensity resistance training with insufficient recovery time promoted a decrease in CSA of approximately 15.6% for type IIA and 19.2% for type IID in Tr DR portion (Table 4). No significant changes in CSA were found in fiber types I and IIB in DR portion. However, there was a tendency for type I and IIB CSA

to decrease after the training protocol in the Tr group DR portion (p=0.08 and p=0.09, respectively).

Table 4 Cross-sectional area (μm^2) of muscle fiber types in different portions (SW, superficial white; DR deep red) of the plantaris muscle

Group	Mucle fiber type						
	I	IIC	IIA	IIAD	IID	IIBD	IIB
Co							
SW	1364.4 ± 206.1	1332.7 ± 183.8	1616.3 ± 152.8	1629.7 ± 213.9	1980.8 ± 251.7	2775.7 ± 548.3	5561.3 ± 1143.1
DR	2088.1 ± 428.4	1946.5 ± 385.2	2303.2 ± 390.9	2498.5 ± 490.9	2611.4 ± 587.0	3269.7 ± 451.1	4723.5 ± 883.1
Tr							
SW	1213.3 ± 191.5	1302.4 ± 295.8	1565.4 ± 198.0	1541.8 ± 324.9	1887.8 ± 291.0	2340.4 ± 221.0	4720.0 ± 692.6
DR	1780.8 ± 265.2	1649.5 ± 284.7	1944.8 ± 202.3 *	2095.2 ± 322.1	2109.0 ± 456.0 *	2867.8 ± 395.9	4099.2 ± 547.9

Values are means ± SD. Non-trained (Co, n=9) and trained (Tr, n=9) groups. * Significantly different from control group; $p<0.05$ (ANOVA + Tukey-test).

Discussion

Although it is easy to study resistance training in humans, it is difficult to determine the phenotypic muscle responses to this training. This is primarily due to the invasive nature of muscle biopsies and to the risks inherent in using human subjects. Considering the heterogeneity of muscle fibers in different muscle regions, a small muscle sample cannot accurately reflect total muscle response. To circumvent these problems, Tamaki et al. (1992) suggested a weight-lifting protocol designed to induce hypertrophy in rat limb muscles. Here, we used a model of resistance training in a liquid medium, suggested as a variation of the model proposed by Tamaki et al. (1992), and a standardized training protocol, which included an imbalance period between exercise bouts and rest. The advantage of our animal model is

that it provides the ability to perform analysis on whole muscle preparations, providing a more extensive examination of muscle phenotype adaptations during training. During the training, our subjects muscle response was not affected by lifting technique, motivation, food, or any other psychological parameters. With these variables controlled, the purpose of this study was to test the hypothesis that a high-intensity resistance training protocol with insufficient recovery time, could influence the morphology and myosin heavy chain (MHC) expression in rat skeletal muscle. The major findings of this study were: (i) a reduction in IIA and IID plantaris muscle fibers CSA, and (ii) fiber type frequency change and MHC isoforms content transition from slow to fast.

Training programs can cause specific adjustments in the phenotype of muscle fibers in order to supply the body's needs and optimize physical performance (Siu et al. 2004). However, when recovery time, volume, and intensity are inadequate, it can cause a series of hormonal and physiological changes in the organism (Lehmann et al. 1999; Fry et al. 2005), such as alterations in muscle fibers, a decrease in strength and an increase in protein catabolism, leading to a state called overtraining (Fry et al. 1991; Lehmann et al. 1999). Petibois et al. (2003) observed that overtrained individuals presented higher amino acids and lower protein blood accumulation in response to exercise than well-trained individuals, suggesting that proteins were catabolised for amino acid supply during exercise. This increased requirement for amino acids during hypermetabolism is partly satisfied by an augmentation of muscle proteolysis, the major storage pool of amino acids, and by a concomitant reduction in muscle anabolism (Smith 1999). In addition, Seene et al. (2004) showed that during overtraining condition degradation increased and a decreased muscle protein synthesis rate lead to a decrease in muscle mass, particularly in fast twitch muscles.

These authors also observed in rat fast twitch muscles during overtraining, that the DNA content per muscle decreased due to a loss of myonuclei consequent to muscle atrophy (Seene et al. 2004). Collectively, the results of these studies suggest that during conditions of overtraining an increase in the catabolism/anabolism ratio could lead to muscle fiber atrophy. To test this hypothesis we used an animal model of high-intensity resistance training, designed to induce exhaustive overload on muscle. Although measurements of the muscle protein and DNA contents were not performed, we did show that high intensity/volume training with insufficient recovery time between bouts, promoted a reduction in muscle fiber CSA's. Direct statistical analysis supports this interpretation. Compared to the Co group, the Tr group exhibited a significant ($p<0.05$) decrease in pure fiber IIA and IID CSA in the DR portion of the plantaris muscle. However, there was a tendency for pure fiber I and IIB CSA to decrease in the DR portion ($p=0.08$ and $p=0.09$, respectively). Our results are consistent with several studies which show a predominance of catabolic condition (Seene et al. 1999; Petibois et al. 2000; Seene et al. 2004) in situations with a persistent combination of excessive overload plus inadequate recovery (Jamurtas et al. 2000; Fatouros et al. 2006). In our study a possible overtraining stimulus lasted too long in each exercise bout and was too frequent; this interrupted the recovery phase and the adaptation needed by the muscle did not occur and led to atrophy in the plantaris muscle. Although the molecular events that underlie our findings remain unknown, these observations raise questions as to what signals and cellular conditions initiate muscle mass changes during overtraining conditions. Furthermore, we observed a loss of body weight in the Tr group. Although some studies have reported loss of appetite, due to the arduous training schedule (Mackinnon 2000; Meeusen et al. 2006), our results showed no differences in food intake between groups (data not shown). Thus, the weight loss observed in the Tr group could also be related to the reduction in plantaris muscle fiber CSA's, although

there could other related factors, such as loss of motivation, apathy, irritability, and depression (Fry et al. 1991; Mackinnon 2000).

Animal experiments have shown that overtraining decreases the number of satellite cells (Seene et al. 1999), which are cells, located under the basal lamina of skeletal muscle fibers and when activated, proliferate, differentiate, and fuse with muscle fibers (Rosenblatt et al. 1994). A decrease in activated satellite cells means that new fibers are not forming and muscle atrophy develops. With skeletal muscle atrophy, myonucleus numbers decrease, decreasing DNA units in overtrained muscle, thereby decreasing synthesis and increasing degradation rate of muscle proteins; this promotes the development of overtraining myopathy (Seene et al. 1999). Although the mechanisms responsible for muscle atrophy are not completely defined, several factors seem to be involved; these include reduced neuromuscular activity, systemic activation of neurohormones and inflammatory cytokines (Dalla Libera et al. 2001; Filippatos et al. 2005), myostatin/follistatin imbalance (Lima et al. 2010), and ubiquitin-proteasome pathway activation (Schulze and Upäte 2005). The ubiquitin-proteasomal pathway, which includes the muscle-specific E3 ligases, atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING Finger 1 (MuRF1), is known to be a powerful contributor to muscle proteolysis (Bodine et al. 2001, Gomes et al. 2001). We did not evaluate the ubiquitin-proteasomal pathway in our study, but it is possible that this molecular pathway may be involved in the control of gene expression related to skeletal muscle atrophy that occurred in our high-intensity resistance training and insufficient recovery time model.

In addition, high resistance training has been shown to promote muscle fiber type modulation and myosin heavy chain (MHC) isoform changes (Campos et al. 2002; Ratamess et al. 2009). Several studies have reported the transition from fast-to-slow muscle fiber types

and MHC isoforms after resistance training (Roy et al. 1997; Carroll et al. 1998; Sharman et al. 2001). Kesidis et al. (2008) observed a high percentage of fibers expressing MHCIIa and MHCI/IIa in bodybuilders, compared to non-trained individuals. Similarly, Otis et al. (2007) in a study on tumor-bearing rats submitted to functional overload, observed a reduction in MHCIIb isoform, associated with increased levels of MHCI isoform. Based on these studies, the modulation of fiber types and MHC isoforms toward the slowest contracting fibers, contributes to increase strength, muscle power and fatigue tolerance. In contrast, in our study, the high-intensity resistance training with insufficient recovery time between bouts changed MHC isoform content and fiber type frequency toward fast contracting activity in rat plantaris muscle. There was a significant decrease of MHCI and MHCIIa in the Tr group compared to controls. These data were corroborated with a decrease of Type I and IIA fiber frequencies. Moreover, there was a significant increase in MHCIIb in the Tr group which was associated with a significant increase in the Types IIBD fiber frequency, which express more IIb than IId isoforms (MHCIIb > MHCIId) (Pette and Staron 2000; Pette and Staron 2001). However, the reduction in Type IID fiber frequency in the SW portion in Tr was not associated with a significant ($p>0.05$) change in MHCIId content. Contrary to previous resistance-training studies that show a MHCIIb-to-MHCIIa transition within the fast fiber population (Campos et al. 2002; Harber et al. 2004), we observed a transition of MHCI and MHCIIa toward MHCIIb isoform content in plantaris muscle during high-intensity/volume training with insufficient recovery time between bouts. This might indicate that the activity patterns of fiber types I and IIA (MHCI and MHCIIa isoforms), which have relatively high oxidative potential, possibly were more susceptible to oxidative damage by reactive oxygen species during high volume exercise in rats plantaris muscle. In addition, Seene et al. (2004) also observed an increase of fast MHC isoforms after 6 weeks of endurance overtraining. These authors reported that

overtraining syndrome in skeletal muscle may decrease capillarization impairing oxygen exchange between capillaries and muscle tissue which contributes to exercise intolerance. In this sense, considering that muscle fiber type is also classified according to the type of energy metabolism used (glycolytic or oxidative) (Gunawan et al. 2007), our results together with those of Seene et al. (2004), suggest that overtraining situations can result in a decrease in muscle oxidative capacity.

In conclusion, high-intensity resistance training with insufficient recovery time between bouts promoted muscle atrophy and a shift from slow-to-fast contractile phenotype in rat plantaris muscle. While the molecular events that underlie our findings remain unknown, we think that the stimulation of a transcriptional pathway, e.g., the ubiquitin-proteasomal which promotes muscle proteolysis (Bodine et al. 2001, Gomes et al. 2001), might occur as a result of overtraining. Alternatively, the decrease in number of satellite cells during overtraining (Seene et al. 1999) could also be a determinant factor in preventing muscle fiber regeneration. Although these possible hypotheses need to be tested, understanding skeletal muscle morphological and biochemical changes following resistance training with appropriate recovery could help physiologists and coaches to improve the supervision of athletic performance.

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8. ARTIGO 2: Será submetido para publicação na Revista Anatomical Record, 2010

High-intensity resistance training with short recovery time alters MyoD and IGF-1 mRNA content in rats

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Abstract

Skeletal muscle is a dynamic tissue capable of alter its phenotype feature in response to specific stimule. Factors such as heavy resistance training can alter muscle fiber morphology and molecular features. Myogenic regulatory factors (MRFs) and the growth factor IGF-1 are important mediators of muscle mass during physical training. In this study we test the hypothesis that high intensity resistance training with short rest/recovery time, similar to overtraining (OT) conditions, may result in a decrease of muscle mass and alteration in (MRFs) MyoD, myogenin and IGF-I gene expression in rat plantaris muscle. Male *Wistar* rats were divided into 4 groups: trained 8 weeks (T8), control 8 weeks (C8), trained 12 weeks (T12) and control 12 weeks (C12). T8 and T12 groups were subjected to a high weight-lifting training program (5 days/week), involving jumps sessions into water, carrying progressive overload equivalent to percentage of body weight. At the end of each period the animals were sacrificed, the middle portion of plantaris muscle were collected and frozen. Histological cryo sections were submitted to HE stain for morphological fiber analysis and to myofibrillar adenosine triphosphatase (mATPase) histochemistry to classify the muscle fiber type and to measure fiber type cross-sectional area (CSA). MyoD, myogenin and IGF-1 mRNA gene expression were analised by qPCR. From beginning to end of the experiment all groups increased body weight, however, the T12 group was lower compared to the C12. Regarding the cross-sectional area, there was a significant reduction of the IIC fibers and IIAD in SW in T8 group and IIA and IID in DR in T12, group compared to their respective controls. The mRNA gene expression of myogenin was similar between trained and control groups. However, in the T12 group, there was a decreased in the expression of MyoD and an increased in IGF-1, compared with the group C12. In conclusion, the present study shows, for the first time that, long term of high intensity resistance training with short rest/recovery time, similar to overtraining conditions, induced plantaris muscle atrophy with decrease in MyoD mRNA levels, fact that could suggest a lower activity of satellite cells during conditions of inadequate muscle repair during OT. In addition, the increased mRNA content of IGF-1 may have occurred in an attempt to prevent the loss of muscle mass.

Key words: Skleletal muscle; Overtraining; Myogenic Regulatory Factor, IGF-1; Muscle atrophy

Introduction

Skeletal muscle is a highly dynamic tissue capable of alter its phenotypic features in response to specific stimuli (Pette and Staron 2001), such as resistance training. Classical response to resistance training included an increase of power, strength and muscle mass, beyond of alteration in biochemical and morphologic features of skeletal muscle (Campos et al. 2002). The primary determinant of muscle adaptations is the interaction of molecular pathways that affect the rate of synthesis and degradation of proteins. A higher rate of synthesis than degradation increase the muscle mass, while the opposite leads to decreased in muscle mass (Baar et al. 2006). In addition, the muscle phenotypic alterations in response to distinct models of resistance training are directly related to specific changes in the expression of muscle specific genes and proteins (Saltin and Gollnick 1983; Psilander et al. 2003).

In *vitro* and in *vivo* studies indicate that two primary processes are involved with the compensatory hypertrophy response in mammalian skeletal muscles. The first are associated with the accretion of protein to support myofiber enlargement. The second involves proliferation of satellite cells that appear to be necessary to provide myonuclei to the enlarging myofibers (Allen et al. 1995). The cellular and molecular mechanisms underlying these processes have been extensively characterized. In particular, the steps associated with the initiation of cellular proliferation are generally well established (e.g., can be found in cell biology textbooks). Based on these well-characterized processes, several studies used representative mRNAs as markers of potential myogenic responses of skeletal muscle during adaptation mechanism (Bickel et al. 2003).

Myogenic regulatory factors (MRF) MyoD, myogenin, MRF-4, and Myf-5 are traditionally thought markers of skeletal muscle growth and hypertrophy, because they can

modulate satellite cell activity. After activation, satellite cells may undergo one or several rounds of proliferation, during which MyoD and Myf-5 is expressed. Before and just after differentiation, myogenin and MRF-4 is upregulated in satellite cell nuclei (Megeney and Rudnick 1995). Differentiation occurs when the satellite cell exits the cell cycle and fuses to the parent fiber, with which it is associated, thereby adding to the nuclear population of the fiber, or fuses with other satellite cells to form a new fiber. Together, these markers are often used as an indication of myogenic cell differentiation in settings associated with muscle formation or muscle hypertrophy. Psilander et al. (2003) reported that myogenin, MyoD and MRF4 mRNA levels were elevated in human skeletal muscle after a single bout of heavy-resistance training, supporting the idea that the MRFs may be involved in regulating hypertrophy.

Additional to MRFs, the insulin-like growth factor-1 (IGF-1) has also received attention in recent years in studies examining exercise-induced muscle hypertrophy. IGF-1 increases muscular protein synthesis and stimulates satellite cell proliferation and differentiation in vitro (Dalla Libera et al. 2004) and also have been described as an important anabolic signal that stimulate muscle protein synthesis in several physiological conditions (Barton-Davis et al. 1999; Glass 2003; Goldspink 2005). Strong evidences suggest that locally produced IGF-1, rather than circulating IGF-1 plays an important role in muscle mass maintenance, repair and hypertrophy (Velloso and Harridge 2009). On the other hand, GH administration significantly increased serum and tissue levels of IGF-1, which has endocrine and autocrine/paracrine actions and regulates muscle growth (Lewis et al. 2000). IGF-1 has been shown to stimulate muscle fiber hypertrophy (Coleman et al. 1995) and prevent cellular death by apoptosis (Dalla Libera et al. 2004). Furthermore, IGF-1 can attenuate the loss of muscle mass by reducing ubiquitin-dependent proteasomal pathway activity (Dehoux et al.

2004) through the phosphatidylinositol 3 kinase (PI3K) route (Kandarian and Jackman 2006).

Although the evidence suggests that MRFs and IGF-1 are involved in skeletal muscle fiber maintenance and adaptations in several conditions, it remains unknown the alterations in gene expression of MRFs and IGF-1 in skeletal muscle during overtraining conditions. The purpose of this study was to test the hypothesis that high-intensity resistance training with insufficient rest/recovery time, which potentially could lead to overtraining, may result in a decrease of muscle mass and, consequently, alter the gene expression of MRFs and IGF-1 in skeletal muscle. Our model of overtraining was based on the overshoot phenomenon (healing process associated with an adaptation) (Clarkson and Trambley et al. 1988), which generally involves application of the progressive overload, beyond a comfortable level in order to optimize performance (Fry et al. 1991; Stone et al. 1991; Fry and Kraemer 1997). Training and competition are associated with a mild tissue trauma followed by recovery (Smith 2000). When adequate recovery is allowed, an adaptative process occur, being called "adaptive microtrauma" (AMT) (Smith 2000), fact that may contribute with the increased synthesis of skeletal muscle proteins, leading to muscle hypertrophy (Clarkson and Trambley 1988; Macintyre et al. 1995). However, when intensity/volume and rest/recovery are imbalance during the training program, can induce to a repetitive trauma in skeletal muscle, in a harmful state called overtraining (Kuipers and Keizer 1988). Our model ensures an intense workload and does not allow a complete muscular recovery between training sessions, fact that potentially leads to animals the overtraining condition. To our knowledge, this is the first study that investigated the changes in muscle fiber CSA and gene expression during conditions of overtraining in isolated muscle. We analyzed MyoD, myogenin and IGF-1 mRNA expression and cross-section area (CSA) in the plantaris muscle because it is highly recruited in our overtraining model.

Material and methods

Research Design

An animal model was used to test the hypothesis that high resistance training associated with insufficient recovery/rest time, could result in harmful effects in the skeletal muscle compared with non-training animals. Two days after the final day of training, morphometric and molecular analysis was performed. The animal model provided the only accurate method to isolate single muscles and perform analysis on whole muscle preparations, reflecting the total muscle response.

Animals and Experimental Groups

Male *Wistar* rats (250 to 300g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP, Campinas, São Paulo, Brazil). They were housed in collective polypropylene cages (four animals/cage) covered with metallic grids, in a temperature-controlled room (22-24ºC) under a 12-hour light-dark cycle and provided with unlimited access to standard rat chow and water. We used the independent variable high intensity resistance training time to examine the effects on the skeletal muscle parameters, CSA and mRNA expression (dependent variables). For this purpose, rats were randomly divided into four groups: Control 8 weeks (C8, n=9); Trained 8 weeks (T8, n=9); Control 12 weeks (T12, n=9) and Trained 12 weeks (T12, n=9). This experiment was approved by the Biosciences Institute Ethics Committee, UNESP, Botucatu, SP, Brazil (Protocol No. 018/08-CEEA) and was conducted in conformance with the policy statement of the American College of Sports Medicine on research with experimental animals.

Training protocol

T8 and T12 groups were submitted to a high-intensity resistance training program, 8 and 12 weeks, respectively, similar to that described by Cunha et al. 2005 (Table 1). Before the initial training program, animals performed a one-week pre-training (once a day) to familiarize them with the water and exercise. In this phase, the rats individually performed sessions of jumping into a 38cm deep vat of water at 28-32°C (Figure 1). Animals jumped to the water surface to breathe without needing any direct stimulus to complete the jumping sessions. The depth was appropriate to allow each animal to breathe on the surface of the water during successive jumps. A jump was counted when the animal reached the surface and returned to the bottom of the vat, a movement performed repeatedly. The adaptation protocol consisted of increasing the number of sets (two to four) and repetitions (five to ten), carrying an overload of 50% body weight strapped to a vest on the animal's chest (Figure 1). After the adaptation period, T8 and T12 groups began the progressive high intensity resistance training program for five consecutive days per week with 40 seconds rest between each set. At the end of the corresponding periods, the intensity was equivalent to 70 and 85% of body weight for T8 and T12 groups, respectively (Table 1). The protocol of physical training was designed to cause a skeletal muscle overload with reduced recovery time between sessions. Sessions were performed between 2 and 3pm.

Table 1 Program of high-intensity resistance training

Week	Sets x jumps	Overload (% Body weight)
1	4 x 5	60
2	4 x 7	60
3	4 x 12	60
4	5 x 6	65
5	5 x 8	65
6	5 x 10	65
7	4 x 10	70
8	4 x 10	70
9	4 x 8	80
10	4 x 8	80
11	4 x 6	85
12	4 x 6	85

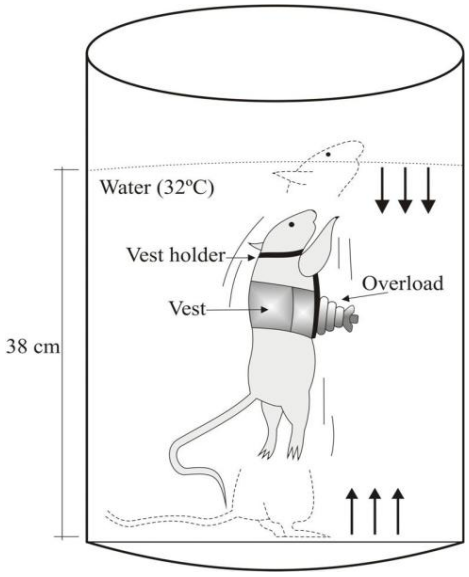

Figure 1. Sketch of the high intensity resistance training apparatus.

Morphological and histochemical procedures

At the end of the experiment animals were anesthetized with pentobarbital sodium (40mg/kg IP) and sacrificed by decapitation. Throughout the experiment was measured, weekly, body weight and feed intake of animals. Following, plantaris muscle was collected

and the middle portion frozen in liquid nitrogen at -156°C. Samples were kept at -80°C until use. Histological sections (12µm thick) were obtained in a cryostat (JUNG CM1800, Leica Germany) at –24°C and submitted to HE stain for morphological fiber analysis (Figure 2).

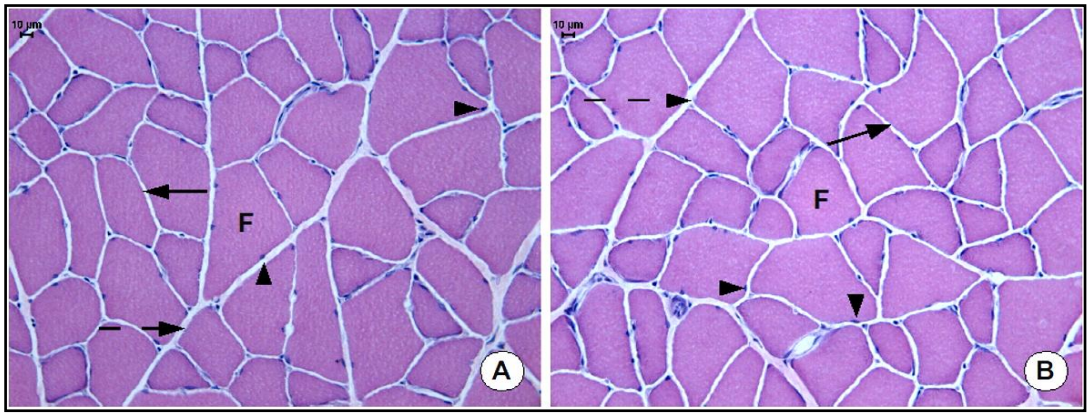

Figure 2. Histological section of plantaris muscle samples taken from a control (A) and trained (B) animal stained with hematoxylin and eosin. Muscle fibers (F), Perimysium (dotted arrow), Endomysium (continuous arrow) and Myonucleus (arrowhead).

To classify the muscle fiber type and obtained muscle fiber type cross-sectional area (CSA), myofibrillar adenosine triphosphatase (mATPase) histochemistry was performed using preincubation at pH 4.2, 4.5 and 10.6 (Guth and Samaha 1969; Brooke and Kaiser 1970). Pure muscle fibers (*I* Type I, *IIA* Type IIA, *IID* Type IID, *IIB* Type IIB) and hybrid muscle fibers (*IIC* Type IIC, *IIAD* Type IIAD and *IIBD* Type IIBD) were identified based on their staining intensities (Staron et al. 1999) (Figure 3). No attempt was made to delineate subtypes IIDA and IIDB (Staron and Pette 1993). Muscle fiber types CSA of approximately 200 fibers of each animal were determined using an Image Analysis System Software (Leica QWin Plus, Germany). Two regions of the plantaris muscle were analyzed. A superficial-white (SW) portion that contained the largest population of oxidative fibers (types I and IIA) and the deep-red (DR) portion that showed a higher percentage of glycolytic fibers (type IIB) (Staron et al. 1999).

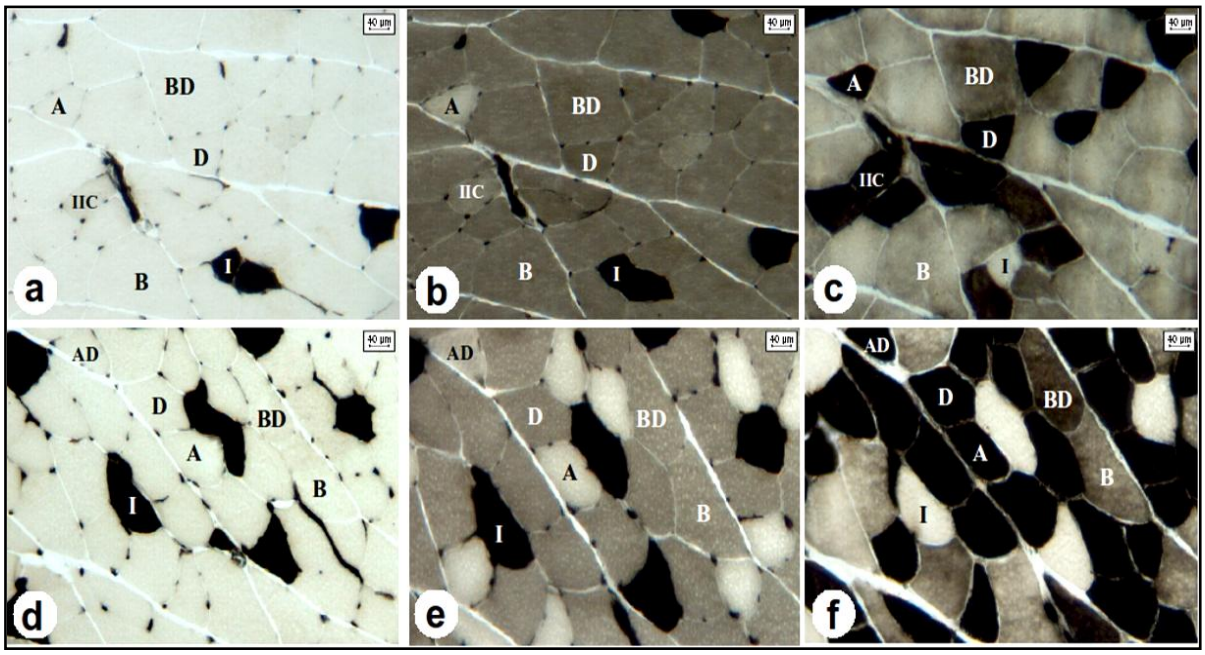

Figure 3. Cross sections of deep-red (a, b, c) and superficial-white (d, e, f) portions of plantaris muscle taken from a trained animal demonstrating fiber-type delineation using myofibrillar adenosine triphosphatase (mATPase) reaction after preincubation at pH 4.2 (a,d), 4.5 (b,e) e 10.6 (c,f). Pure (I, type I; A, type IIA; D, type IID and B, type IIB) and hybrid (IIC, type IIC; AD, type IIAD and BD, type IIBD) muscle fibers.

RNA isolation, reverse transcription, and qPCR

Total RNA was extracted from plantaris muscles with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), which is based on the guanidine thiocyanate-phenol-chloroform method. Frozen muscles were mechanically homogenized on ice in 1 mL ice-cold TRIzol reagent. Total RNA was solubilized in nuclease-free H_2O, incubated in DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA) to remove any DNA present in the sample, and quantified by measuring the optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio of ~2.0.

For each sample, cDNA was synthesized from 2 µg of total RNA by using components

from the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The reaction contained 10 µL 10X Reverse Transcription Buffer, 4 µL 25X dNTPs, 10 µL 10X random primers, 100 units of RNase inhibitor (Invitrogen Life Technologies, Carlsbad, CA, USA), 250 units of MultiScribe™ Reverse Transcriptase, and the final volume was adjusted to 100 µL with nuclease-free H_2O. The primers were allowed to anneal for 10 min at 25°C before the reaction proceeded for 2 h at 37°C. Control "No RT" reactions were performed by omitting the RT enzyme. These reactions were then PCR amplified to ensure that DNA did not contaminate the RNA. The resulting cDNA samples were aliquoted and stored at -20°C. Two microliters of cDNA, corresponding to 20 ng of total RNA, from the RT reaction was used as a template in the subsequent real-time PCR, performed in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the instrument's universal cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The reactions were run in duplicate using 0.4 µM of each primer and 2X Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 25 µL. Primer sequences were selected from the accession numbers in the National Center for Biotechnology Information database using the primer design function of the Primer Express v3.0 software (Applied Biosystems, Foster City, CA, USA) and are listed in (Table 2). Melting dissociation curves and agarose gel electrophoresis were performed to confirm that only a single product was amplified. Control reactions were run lacking cDNA template to check for reagent contamination. Baseline and threshold values were automatically determined for all plates using the Applied Biosystems 7300 SDS Software v1.4. The gene expression and the most stable reference genes were obtained using *geNorm* (version 3.5, written by Vandesompele et al. 2002).

Table 2. Oligonucleotide primers used for qPCR amplification of reverse transcribed RNA

Product	Accession N°	Sequence (5' – 3')
IGF–1	NM_178866.2	GCTATGGCTCCAGCATTCG
		TCCGGAAGCAACACTCATCC
MyoD1	NM_176079.1	TTTTTCATGCGACTCACAGC
		GAAGGCAGGGCTTAAGTGTG
Myogenin	M24393.1	GGAGTCCAGAGAGCGCCGTTGTTAA
		CGGTCGCGGCAGTCACTGTCTCT
Tata Box Binding Protein	NM_001004198	GCCACGAACAACTGCGTTGAT
		AGCCCAGCTTCTGCACAACTCTA
Hypoxanthine phosphoribosyl-transferase 1	NM_000194.1	TGACACTGGCAAAACAATGCA
		GGTCCTTTTCACCAGCAAGCT

Accession N°, GenBank accession number

Statistical Analysis

Fiber-type frequency data were analyzed using Goodman Test for contrasts inter-and intra-multinomial populations (Goodman 1964; Goodman 1965) to assess differences among all groups. Statistical comparisons among the groups were made using Analysis of Variance for the two-factor model (Zar 1999) for body weight. When significant main effects were revealed, specific differences were assessed using Tukey's post hoc comparisons. Data are expressed as Mean ± SD. Differences were considered significant with a p value < 0.05.

RESULTS

Body Weight

The initial and the final average body weight of the groups are shown in Table 3, respectively. Confirming that animals initiated the experiment with health status and physical activity level similar, no statistical difference was observed in the initial body weight among all groups. After 8 and 12 weeks of experiment a significant increase ($p < 0.05$) of the body weight were observed in all groups (Table 3). However, in the T12 group compared to C12 group, a significant ($p<0.05$) decrease of body weight was observed (Table 3).

Table 3. Initial and final body weight in experimental groups.

Group	Initial body weight (g)	Final body weight (g)
C8 (n=9)	275.4 ± 21.4	424.6 ± 30.2 *
T8 (n=9)	294.3 ± 47.8	400.2 ± 44.8 *
C12 (n=9)	286.7 ± 18.9	498.6 ± 30.5 *
T12 (n=9)	276.4 ± 15.5	440.9 ± 30.3 * #

Values are means ± SD. *C8* non-trained during 8 weeks, *T8* trained during 8 weeks, *C12* non-trained during 12 weeks and *T12* trained during 12 weeks (n=9 per group). * $p<0.05$ compared to initial body weight; # $p<0.05$ compared to C12 group (ANOVA, Tukey-test).

Morphological and histochemical analysis

In line with a normal tissue structure, 12 weeks after the beginning of high intensity resistance training, plantaris muscles from all groups exhibited polygonal fibers with peripheral nuclei. Involving each muscle fiber, were observed the endomysium and fibers fascicles surrounded by the perimysium (Figure 2).

A representative mATPase histochemistry reaction used to classify the muscle fiber type is shown in the Table 4. An atrophic effect was observed after high resistance training in

both T8 and T12 groups. The muscle fibers cross-sectional area (CSA) analysis showed a significant ($p<0.05$) decrease in the type IIAD fiber CSA in both SW and DR portions of the T8 group, compared to C8 group. In addition, the T8 group presented a decrease in the type IIC fiber CSA of the SW region, in relation to C8 group. After 12 weeks of resistance training, there was a significant ($p<0.05$) reduction in the types IIA and IID pure fiber CSA of the DR region, in the T12 group compared to C12.

Table 4. Cross-sectional area (μm^2) in superficial-white (SW) and deep-red (DR) portions of plantaris muscle. Control (C, n=9) and trained (T, n=9) groups.

Group	Muscle fiber type						
	I	IIC	IIA	IIAD	IID	IIBD	IIB
C8							
SW	1291.2 ± 330.1	1812.6 ± 234.0	1661.1 ± 253.9	2095.2 ± 529.7	1946.4 ± 420.3	2676.2 ± 628.3	4947.4 ± 1278.7
DR	2224.3 ± 401.0	2029.4 ± 298.9	2306.8± 484.2	2646.8 ± 582.5	2783.4 ± 635.1	3281.2 ± 571.4	4439.1 ± 1001.1
T8							
SW	1346.1 ± 152.4	1345.3 ± 125.1*	1614.1 ± 183.0	1741.7 ± 130.0 *	1889.5 ± 203.9	2314.8 ± 419.9	4505.0 ± 545.6
DR	2037.3 ± 349.8	1797.8 ± 363.8	2205.4 ± 235.7	2210.2 ± 380.9 *	2525.7 ± 496.3	2957.3 ± 446.5	4188.1 ± 347.1
C12							
SW	1364.4 ± 206.1	1332.7 ± 183.8	1616.3 ± 152.8	1629.7 ± 213.9	1980.8 ± 251.7	2775.7 ± 548.3	5561.3 ± 1143.1
DR	2088.1 ± 428.4	1946.5 ± 385.6	2303.2 ± 390.9	2498.5 ± 490.9	2611.4 ± 587.0	3269.7 ± 451.1	4723.5 ± 883.1
T12							
SW	1213.3 ± 191.5	1302.4 ± 295.8	1565.4 ± 198.0	1541.8 ± 324.9	1887.8 ± 291.0	2340.4 ± 221.0	4720.0 ± 692.6
DR	1780.8 ± 265.2	1649.5 ± 284.7	1944.8 ± 202.3 *	2095.2 ± 322.1	2109.0 ± 456.0 *	2867.8 ± 395.9	4099.2 ± 547.9

Values are means ± SD. * Significantly different from control group; $p < 0.05$ (ANOVA + Tukey-test)

MyoD, Myogenin e IGF-I mRNA levels estimated by qPCR

In our study, the mRNA levels of MyoD, myogenin and IGF-1 of T8 group was not changed compared to the control group (C8). On the other hand, with the development of training the T12 group showed a decrease in mRNA expression levels of MyoD and an increase in IGF-1 expression levels, compared to C12 group. Myogenin mRNA expression

levels was not changed compared to the control group (C12) (Figure 4).

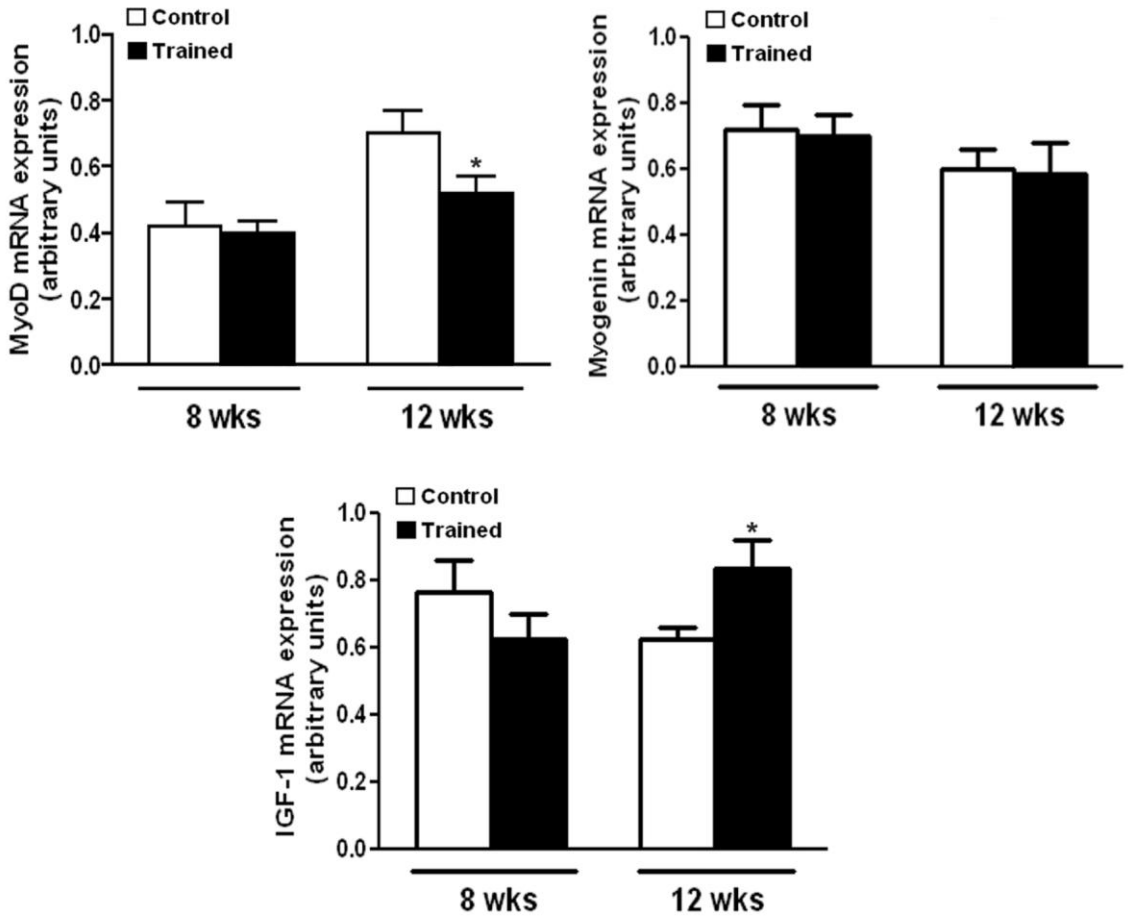

Figure 4. MyoD, Myogenin and IGF-I mRNA expression in the experimental groups. Control groups (n=9 per group) and Trained groups (n=9 per group). Values are means ± SD. *p<0,05 compared to Control group.

Discussion

The main findings of the present study were that the high intensity resistance training associated with short rest/recovery time promoted *1)* alteration in the expression of MyoD and IGF-I mRNA in plantaris muscle, *2)* decrease in the pure and hybrid fibers CSA, and *3)* reduction in the body weight. Resistance training programs, designed to induce skeletal

muscle hypertrophy, is the optimal activation of the anabolic and myogenic mechanisms that result in the accretion of the proteins necessary to support an increase in myofiber size. Because these cellular-level mechanisms must be activated to achieve meaningful adaptations, their activation must precede these adaptations. In human, training regimens generally consist of intermittent bouts of low frequency repetitions with high loads and long recovery. Under this regime of conventional resistance training has been well reported the molecular alterations necessary to promote increased protein synthesis to support the increase in myofiber size (Campos et al. 2002). However, the characterization of the expression of genes that regulate muscle mass in conditions of overtraining (OT) remains unknown.

In this study, we have used previously established molecular markers, representative of several cellular-level myogenic processes, to elucidate the molecular events that mediated the muscle mass alterations during OT condition. For this, we standardized a model of OT based on the model of conventional resistance training for rats, proposed by Tamaki et al. (1992). As our model promote a imbalance between intensity/volume and rest/recovery during training program, we tested the hypothesis that high-intensity resistance training with short rest/recovery time could influence the MyoD, myogenin and IGF-I mRNA expression in conjunct with change in the muscle mass. This hypothesis contradicts the beneficial effects of physical training reported in the literature, i.e. the gain in strength and performance (Kraemer et al. 2002; Galvão and Taaffe 2004; Trappe et al. 2006).

In our study, the OT program induced significant atrophy of hybrid and pure muscle fibers in T8 and T12 groups, respectively. In the T12 group, fibers atrophy was accompanied by significant reduction of body weight. After 8 wks OT, the atrophy was more pronounced in the IIC and IIAD muscle fiber types. As body weight did not decrease in this group, we think that this could be related to the low frequency of the atrophic hybrid fiber in the plantaris

muscle (Roy et al. 1997). However, with a longer period of OT in the T12 group, significant atrophy was observed in pure IIA and IID muscle fiber types, compared to C12 group. The results demonstrated that IIA and IID pure muscle fibers, that present more strength and fast contractile properties compared to type I fibers, are more recruited with the increase of intensity/volume of training. The data suggest that longer periods of OT may promote atrophy in hybrids and pure muscle fibers, more pronounced in fast-twitch pure fibers. For our knowledge, this is the first study that demonstrates the differential pattern of muscle fibers atrophy in an OT model. Moreover, muscle fiber atrophy in T12 group, was accompanied by body weight decrease. Although some studies have reported loss of appetite, due to the arduous training schedule (Mackinnon 2000; Meeusen et al. 2006), our results showed no differences in food intake between the groups (data not shown). Thus, the weight loss observed in T12 group, could be related to the atrophy in the pure IIA and IID muscle fiber types, considering that these fiber types represent approximately 40% of plantaris muscle fibers (Roy et al. 1997).

The possible mechanism to explain the atrophy resulting from our overtraining model could be related to a change in balance rate of synthesis and degradation of muscle proteins (Philips et al. 1997). Numerous studies involving the effects of heavy resistance training on the protein balance in skeletal muscle, showed a predominance of catabolic condition (Petibois et al. 2000; Wolfe 2006), in situations of persistent combination of excessive overload plus inadequate recovery (Jamurtas et al. 2000; Fatouros et al. 2006; Margonis et al. 2007). In addition, Petibois et al. (2003) observed that overtrained individuals presented higher amino acids and lower protein blood accumulation in response to exercise than for well-trained individuals, suggesting that proteins were catabolized for amino acid supply during exercise. This increased requirement for amino acids during hypermetabolism is partly satisfied by an

augmentation of muscle proteolysis, the major storage pool of amino acids, and by a concomitant reduction in muscle anabolism (Smith and Miles 1999). In our study, although measurements of the muscle protein content have not been performed, it is possible that high intensity/volume training, associated with insufficient rest/recovery time promoted a reduction in the muscle fibers CSA as a result of muscle protein catabolism.

The control of muscle mass is mediated by intracellular pathways that affect the rate of synthesis and degradation of proteins. During OT conditions a series of hormonal and physiological changes in the organism (Lehmann et al. 1999; Small 2002; Fry et al. 2005), such as decrease in strength and catabolism of proteins (Fry et al. 1991; Urhausen et al. 1998). In the present study, muscle fibers atrophy was associated with a decreased significant ($p<0.05$) of MyoD mRNA content and increased IGF-I mRNA content in T12 group, compared to C12 group. No change significant was observed in the myogenin mRNA content in T12 and MyoD, myogenin and IGF-1 mRNA levels in T8, compared to respective controls. MyoD is a myogenic transcription factor belonging to a MRFs family with four members: MyoD, myogenin, Myf5 and MRF4. MRFs form dimers with ubiquitous E proteins (e.g., E12 or E47), resulting in heterodimeric complexes that bind to the E-box consensus DNA sequence (5´-CANNTG-3´) that is found in the regulatory region of many muscle-specific genes (Murre et al. 1989), including Myosin Light Chain, troponin, desmin (Lin et al. 1991; Li and Capetanaki 1993) and Myosin Heavy Chain (Wheeler et al. 1999). Thus, the MRFs may contribute to regulating muscle mass controlling the rate of proteins synthesis and degradation. In our study, muscle fibers atrophy observed in T12 group was associated with a decrease significant ($p<0.05$) of MyoD mRNA content and increased IGF-I mRNA content, indicating that these genes may be involved in regulating muscle mass. The MRFs are known to be involved with the proliferation and differentiation of myoblasts (Murre et al. 1989). This

transcription factors are also expressed by muscle satellite cells that when activated, proliferate, differentiate and fuse with muscle fibers to promote hypertrophy (Rosenblatt et al. 1994; Lowe and Alway 1999). Several studies have shown that muscle injury induces satellite cell activation and MRF expression (Marsh et al. 1997). In our study, the decrease in MyoD mRNA levels during OT condition could be related to the decrease in satellite cell proliferation that, in turn, promoted muscle atrophy. The myogenin mRNA content was not changed in our experiment. Myogenin is a MRF that induces terminal differentiation of myoblasts, fusion given rise to multinucleated myotubes and mature (Megeney and Rudnicki 1995; Rudnicki and Jaenisch 1995). Several studies have also suggested that myogenin is mainly found in slow-oxidative muscle whereas MyoD is prevalent in fast glycolytic muscle (Hughes et al. 1993). The lack of changes in the expression of myogenin mRNA content could be explained by the plantaris muscle fibers composition, which is composed predominantly of fast-twitch glycolytic fibers (Roy et al. 1997). Moreover, myogenin could not influence the changes in muscle mass during OT conditions.

Additional to decrease in MyoD mRNA expression, the IGF-1 mRNA content significantly ($p<0.05$) increase in T12 group, compared to C12 group. No change was observed in T8 group. Previous studies provided evidence that insulin-like growth factor I (IGF-I) is a strong anabolic signal in muscle tissue and it acts as a potent positive key regulator of muscle growth (Florini et al. 1996); Moreover, IGF-I is involved in blocked the catabolic response in skeletal muscle following injuries by stimulating protein synthesis and inhibiting protein breakdown (Biolo et al. 1995). The hypertrophic effects of IGF-I are attributed to the satellite cell activation, proliferation and differentiation providing more myonuclei, increasing protein synthesis within existing myofibers (Bark et al. 1998; Barton-Davis et al. 1999). The increase of IGF-I mRNA levels within muscle cells by various in *vivo*

and in *vitro* methods leads to hypertrophy due to an augmentation of muscle protein and DNA content (Coleman et al. 1995; Adams and McCue 1998). In our study, the atrophic response following OT-12 wks was associated with an increase in IGF-1 mRNA levels, demonstrating that the molecular pathway mediated by IGF-I could influence the changes in muscle mass during OT conditions. One proposed mechanism to try explain our results could be related to the activation of PI3-K pathway (phosphatidylinositol 3-kinase), mediated by IGF-1, that in addition to be mediating muscle hypertrophy is also involved in controling the survival of the muscle, inducing anti-apoptotic proteins of the Bcl-2 family, so implicating IGF-1 in muscle cell survival (Mourkioti and Rosenthal 2005).

In conclusion, the present study shows, for the first time that, long term of high intensity resistance training with short rest/recovery time, similar to OT conditions, induced plantaris muscle atrophy with decrease in MyoD and increase in IGF-1 mRNA levels. Although there are many studies about the beneficial effects of exercise on skeletal muscle, scant informations exists regarding the adverse influence of OT on muscle phenotype. Then, further studies are required to understand the molecular pathways involved in muscle response during overtraining conditions.

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9. CONCLUSÕES FINAIS

A utilização de um treinamento resistido de alta intensidade, por um longo período, associado com um tempo insuficiente de recuperação, similar às condições de overtraining, promoveu perda de peso corporal, atrofia do músculo plantar e a transição do músculo para uma característica mais rápida, em ratos.

O treinamento utilizado resultou na redução da expressão da MyoD, sugerindo uma menor atividade das células satélites durante condições inadequadas de reparo muscular. Além disso, o aumento da expressão do IGF-1 pode ter ocorrido na tentativa de prevenir o processo de perda de massa muscular.

Nossos resultados evidenciaram alterações morfológicas e moleculares que ocorrem no músculo esquelético durante condições excessivas de treinamento. Investigações futuras são necessárias para melhor abordar os mecanismos celulares e moleculares que mediam as adaptações musculares durante a transição entre o treinamento e sobretreinamento.

10. COMITÊ DE ÉTICA

Declaração de autorização da Comissão de Ética na Experimentação Animal, Instituto de Biologia – UNICAMP.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha Dissertação de Mestrado intitulada "Morfologia e expressão dos fatores de regulação miogênica (MRFs) e IGF-1 no músculo esquelético de ratos submetidos ao treinamento resistido":

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

(X) tem autorização da seguinte Comissão de Bioética ou Biossegurança: Comissão de Ética na Experimentação Animal (CEEA), sob Protocolo nº 18/08.

Aluno: Rodrigo Wagner Alves de Souza

Orientadora: Maeli Dal-Pai-Silva

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Nome:
Função:

Profa. Dra. ANA MARIA A. GUARALDO
Presidente
Comissão de Ética na Experimentação Animal
CEEA/IB - UNICAMP

Certificado da Comissão de Ética na Experimentação Animal, Intituto de Biociências - UNESP – Botucatu.

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

CERTIFICADO

Certificamos que o Protocolo nº **18/08-CEEA**, sobre "Morfologia, expressão dos fatores de regulação miogênica (MRFs) e do IGF-1 no músculo esquelético de ratos submetidos aos treinamento resistido", sob a responsabilidade de **MAELI DAL PAI SILVA**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de **08/08/2008**.

Botucatu, 8 de agosto de 2008.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

NADIA JOVÊNCIO COTRIM
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