UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

Erick Valentino

Efeitos do uso de Decanoato de Nandrolona sobre a junção neuromuscular de ratos em processo de envelhecimento submetidos ao exercício resistido

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestre em Bases Gerais da Cirurgia.

Orientador(a): Profa. Dra. Selma Maria Michelin Matheus

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Palavras-chave: Decanoato de Nandrolona; Junçao neuromuscular; envelhecimento; exercicio fisico; fibra muscular.

Dedicatória

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Ayrton Senna

Sumário

Lista de Abreviaturas

AAS- Anabolic androgenic steroids
ACH- Acetilcolina
AChE- acetilcolinesterase
ATP- Adenosina trifosfato
Ca++- Cálcio
DN- Decanoato de Nandrolona
EAA- Esteróide androgênicos anabólicos
EDN- Grupo exercício esteróide
EDNi- Grupo exercício esteróide idoso
END- Physical exercise + Nandrolone decanoate
EV- Grupo exercício veículo
EV- Physicalexercise + Vehicle
EVi- Grupo exercício veículo idoso
HE- Hematoxylin-Eosin
JNMs- Junção Neuromuscular
MET- Microscopia eletrônica de transmissão
nAChR- Acetylcholine receptors
nAChR- Receptores de acetilcolina
ND- Nandrolone Decanoate
NMJs- Neuromuscular junction
PP min- Potencial de placa em miniatura
PPt- Potencial de placa terminal
REL- Reticulo endoplasmático liso
RER- Reticulo endoplasmático rugoso
SDN- Sedentário esteróide
SDNi- Sedentário esteróide idoso
SND- Sedentary + Nandrolone decanoate
SOL- Musculo sóleo
SV- Grupo sedentário veículo
SV- Sedentary + Vehicle
SVi- Grupo sedentário veículo idoso
TEM- Transmission Electron Microscopy

Introdução

Os Esteróides Andrógenos Anabólicos (EAA) são derivados sintéticos da testosterona, hormônio sintetizado nos testículos que apresenta funções distintas em diferentes fases da vida, tendo assim efeitos androgênicos (CLARK et al., 1997;EVANS, 2004; BINAYI et al 2015).

Durante a vida embrionária, a ação dos androgênios é central para o desenvolvimento do fenótipo masculino, e na puberdade é responsável por estabelecer características secundárias (WILSON, 1996). Durante a vida adulta, o homem produz em média cerca de 40- 50 mg de testosterona por semana, cerca de 2500 mg de testosterona cada ano, e um total de 130 g até os 75 anos de idade (BARDIN, 1996; FRONCZAK et al., 2012).

A testosterona atua aumentando o número de células musculares progenitoras (SINHA-HIKIM et al., 2003), promovendo sua diferenciação miogênica (SINGH et al., 2003,2006). Estimula ainda a biogênese mitocondrial, aumentando o fornecimento de oxigênio ao tecido, o número de glóbulos vermelhos e a capilaridade do tecido (COVIELLO et al., 2008; GUPTA et al., 2008).

A atividade anabólica da testosterona e seus derivados se manifesta principalmente na sua ação miotrófica que resulta em maior massa muscular e força, isto, em conjunto com os efeitos estimuladores de androgênios sobre o cérebro, levou à utilização generalizada de EAA por atletas em todos os níveis (EVANS, 2004; DI LUIGI et al., 2005; RIEZZO, 2014).

Os EAA são farmacologicamente importantes no tratamento de uma variedade de disfunções, tais como osteoporose (CRANDALL et al., 2002; GORDON et al., 2010; DIMOPOULOU et al. 2016), deficiências no crescimento, algumas desordens sanguíneas (SHOKRI et al., 2009; WIT et al. 2015), angioedema hereditário, câncer de mama, desnutrição, anemia refratária (KARBALAY-DOUST & NOORAFASHAN, 2006), tratamento de disfunções renais (JOHNSON, 2000; DEICHER & HORL, 2005; JOHANSE et al., 2006), em portadores de HIV, e em pacientes hipogonadais limítrofes (MULLIGAN & SCHAMBELAN,2002; DUDGEON et al., 2006; KIETSIRIROJE, 2015) e na regeneração muscular (BEINER, 1999; PIOVESAN et al, 2013).

Levando-se em conta que os EAA estimulam a síntese de proteínas presentes nos músculos estriados esqueléticos (efeito anabólico), eles têm sido

utilizados por atletas e não atletas visando aumento de força e/ou desempenho na prática de atividade física e da massa muscular (DOHLE et al., 2003; BREUNER,2014). No entanto, eles vêm sendo utilizados sem indicação médica e em doses altas por homens e mulheres (SHOKRI et al., 2009) e especialmente, por fisiculturistas (REARDON & CREADO,2014). Seu uso envolve doses suprafisiológicas, de 10 a 100 vezes maiores que as doses terapêuticas (CLARK et al.,1997,SHAHRAKI, 2015).

Embora haja melhora no desempenho físico, doses elevadas destas drogas levam a vários efeitos colaterais. Entre eles, danos nos sistemas nervoso central, cardiovascular, endócrino e genital e no próprio músculo esquelético (SOCAS et al., 2005).

O Decanoato de Nandrolona (DN) é um derivado sintético da testosterona (JOHNSON, 2000; GORDON et al., 2010) e é o EAA mais utilizado entre os atletas, especialmente, do sexo masculino, por apresentar menores efeitos adversos (JOHANSE et al., 2006).

Esta substância é formada pela esterificação de um grupo 17 a –hidroxil com o ácido decanóico, um acido graxo de cadeia longa. Após a injeção intra-muscular a droga é liberada muito lentamente na corrente sanguínea, exercendo uma atividade anabólica excelente acima de seis a sete dias (MOTTRAM;GEORGE, 2000; SHAHIDI, 2001; CUNHA et al., 2004).

O DN é classificado como não aromatizante, pois a sua taxa de conversão em estrogênio é baixa, sendo assim reduz os efeitos feminilizantes recorrentes do uso a longo prazo das super dosagens da droga (MOTTRAM & GEORGE, 2000; KUHN, 2002; CUNHA, 2004).

Esta substância tem sido utilizada na recuperação de alguns nervos periféricos (GHIZONI et al.,2013), tendo ainda sido descrita como facilitadora da regeneração muscular pós lesão (PIOVESAN et al.,2013). É capaz também de modular a adesão e proliferação de mioblastos tendo um efeito positivo sobre a injúria muscular (OLIVEIRA et al., 2014).

No músculo, os anabolizantes tem ação anabólica, tendo ainda importante ação importante sobre a regulação e reabsorção óssea, e o fator de crescimento que se assemelha à insulina 1 (IGF-1). Eles agem regulando processos metabólicos como a modulação positiva da síntese protéica por influenciarem na expressão de fatores de transcrição miogênica, entre eles a MyoD, bem como regulam vias

metabólicas exercendo importante papel no controle da glicólise e gliconeogênese. A ação catabólica no músculo é mediada pela miostatina e glicocorticóides, permitindo que ele possa executar as respostas a um estímulo gerando adaptações locais, produzindo mudanças nas características do tecido (LABREE, 1991; BOFF, 2010).

O tecido muscular é constituído por células especializadas alongadas, multinucleadas, as fibras musculares, cujos núcleos estão localizados na periferia. O diâmetro das fibras pode variar de 10 a 100 µm e o comprimento pode chegar até 10 cm, dependendo da arquitetura do músculo (JUNQUEIRA & CARNEIRO, 2009). Morfologicamente as fibras musculares são constituídas por sarcômeros, que são estruturas repetidas, consideradas a unidade contrátil da fibra muscular (HUXLEY, 1969). Os sarcômeros são formados por proteínas contráteis: miosina (filamento grosso) e a actina (filamento fino), além de outras proteínas estruturais. O filamento fino formado pela Actina, apresenta ainda 2 proteínas reguladoras, a troponina e tropomiosina, já o filamento grosso é formado pela polimerização de 200 a 300 moléculas de miosina da classe II (MCCOMAS,1996).

A musculatura esquelética adulta é composta por diferentes tipos de fibras, que diferem em sua organização molecular e também em sua estrutura contrátil (SCHIAFFINO & REGGIANI, 2011).

A partir do final do século XVIII já se tem relatos sobre os tipos de fibras musculares esqueléticas, quando RANVIER (1873) baseado na coloração classificou os músculos em vermelhos e brancos.

De acordo com o padrão de reação para atividade da ATPase miofibrilar na porção globular da miosina, foram descritas 3 tipos principais de fibras musculares: I, IIA e tipo IIB (GUTH & SAMAHA,1969; BROOKE & KAISER,1970).

PETTE E STARON (2001) através da reação histoquímica para ATPase miofibrilar, utilizando anticorpos específicos para isoforma de miosina(MHC) e da eletroforese em gel de policrilamida, classificaram os tipos de fibras musculares em fibras de contração lenta-Tipo I(fibras Slow), expressando MHCI e fibras de contração rápida-Tipo II(fibras fast), subdivididas em tipo IIA, expressando MHCIIa; tipo IID, expressando MHCIId e tipo IIB, expressando MHCIIb. Essas fibras foram denominadas de fibras puras, por expressarem uma única forma de miosina de cadeia pesada. As fibras do tipo IID (MHCIId) se equivalem as fibras IIX(MHCIIx) descritas em ratos (SCHIAFFINO E REGGIANI, 1994).

As fibras híbridas expressam duas ou mais isoformas de miosina, desta forma, assume-se uma organização sequencial das fibras puras (I, IIA, IID, IIB), as quais são intermediadas por fibras híbridas (IC, IIC, IIAD, IIDB) (PETTE E STARON, 2000).

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). Contudo, as fibras musculares exibem uma alta plasticidade, o que habilita este tecido a alterar suas características morfológicas, metabólicas e funcionais, como resposta a estímulos específicos (PETTE & STARON, 2000; MAGAUDDA et al., 2004).

Entre os fatores extrínsecos que podem alterar o comportamento das fibras musculares, o exercício físico tem sido investigado como um importante fator no processo de adaptação do músculo esquelético (WILLIAMSON et al., 2000; PARCELL et al., 2003). O treinamento físico sistematizado pode provocar a modulação das fibras musculares, em resposta a uma supercompensação metabólica, para suprir as necessidades do organismo aos estímulos sucessivos, otimizando assim, o desempenho físico (BARBANTI, 2001; SIU et al., 2004).

O exercício pode aumentar a capacidade oxidativa muscular, levando a um aumento de massa muscular e reduzindo ainda o risco de doenças metabólicas (SHEPHERD et. al., 2014). Sendo que esse tipo de atividade favorece positivamente o potencial morfofuncional do músculo (MCCLUNG et al., 2005).

As fibras, do tipo I (contração lenta), e tipo II (contração rápida) são recrutadas gradualmente de acordo com o exercício realizado. As fibras do tipo I são recrutadas predominantemente nos exercícios de longa duração e baixa intensidade, pois possuem uma alta produção de ATP na presença de oxigênio sendo, portanto eficientes nos exercícios de alta resistência aeróbica (treinamento de resistência). As fibras do tipo II, ao contrário das do tipo I são melhores adaptadas aos exercícios na ausência de oxigênio (treinamento resistido), assim são predominantemente recrutadas na realização de exercícios anaeróbicos, tendo como produto final do seu metabolismo, o lactato intramuscular, que pode ocasionar uma fadiga precoce levando a uma redução da tolerância ao exercício (POWERS, 2000; WILMORE & COSTIL, 2001).

Durante a puberdade o aumento nos níveis de testosterona contribui para o crescimento, bem como para o acúmulo de massa muscular (BHASIN et al., 1996,

2001; BROWER, 2002; KUHN, 2002) através da indução de hipertrofia sem mudanças no número total de fibras tanto do tipo I,como do tipo II (SINHA-HIKIM et al, 2002). Segundo MACCLUNG et al.(2005),não existe aumento significativo na massa muscular após uso de DN- derivado sintético da testosterona, cujo efeito pode estar relacionado ao déficit de crescimento, decorrente do uso crônico deste anabolizante, havendo perda de gordura e massa magra, o que pode ser minimizado com associação da prática de exercícios físicos moderados (BINAYI et al.,2015).

Alterações que acometem a fibra muscular, bem como a manutenção, funcionamento e regeneração funcional dos músculos esqueléticos estão diretamente relacionadas com a integridade da junção neuromuscular (JNM) aumentando a importância do conhecimento da sua organização estrutural, arquitetura molecular e funções (SANES & LICHTMAN, 1999; KOIRALA et al, 2003).

Há várias décadas a junção neuromuscular (JNM) dos vertebrados vem sendo um modelo para estudo da estrutura, função, desenvolvimento e plasticidade das sinapses devido a sua morfologia simples e fácil acessibilidade (KOIRALA et al 2003).

As JNMs são passíveis de remodelações devido a sua alta capacidade de se adaptar aos diferentes estímulos intrínsecos e/ou extrínsecos, como é o caso da atividade física (MAGAUDDA et al., 2004; PETTE & STARON, 2000). Podendo sofrer alterações frente à compressões, esmagamentos e secções traumáticas de nervos periféricos (GATTUSO et al, 1988), bem como grau de atividade (DESCHENES, 1993; FAHIM, 1997; FAHIM & ROBBINS, 1986).

O grau de atividade pode levar a alterações na estrutura da junção neuromuscular como: tamanho (ANDONIAN, 1987; WAERHAUG, 1992; DESCHENES, 1993), comprimento (WAERHAUG, 1992; FAHIN, 1997), expressão das subunidades dos nAChRs (MA, et al, 2007, SOUZA, et al., 2011), dimensões do ramo terminal do nervo (ANDONIAN, 1987; DESCHENES,1993; FAHIN,1997) e dispersão das vesículas sinápticas e receptores (DESAULNIERS, 1998; DESCHENES, 2000).

Segundo DESCHENES et al. (2000) as JNMs devem ser mais sensíveis à respostas adaptativas induzidas por exercício que outros aspectos do músculo esquelético. Tanto o aumento como a diminuição de atividade é capaz de remodelar a estrutura da junção neuromuscular, sendo que o exercício afeta a ramificação

nervosa pré-sináptica aumentando seu comprimento e complexidade (DESCHENES et al, 2006).

A JNM consiste em uma estrutura especializada quanto à morfologia e funcionalidade, onde ocorre a transmissão de um sinal elétrico do neurônio motor para a fibra muscular promovendo assim a contração muscular (MARTIN et al. 2015).

Morfologicamente essa estrutura é organizada em três compartimentos (Figura 1), o compartimento pré-sináptico, onde estão presentes o terminal nervoso e a célula de Schwann terminal; o compartimento extracelular, preenchido pela lâmina basal juncional; e o compartimento pós-sináptico, que compreende o sarcolema pós juncional que apresenta as dobras juncionais e o sarcoplasma o qual proporciona suporte estrutural e metabólico para a região pós-sináptica (ENGEL, 2003; FREIRE, 2014).

No compartimento pré-sináptico, as células de Schwann, apresentam prolongamentos que recobrem este terminal protegendo-os de lesões químicas e mecânicas (SANES; LITCHMANN, 1999). O terminal nervoso contém mitocôndrias, microtúbulos, neurofilamentos, REL, glicogênio, lisossomos e vesículas sinápticas as quais contém ACh, ATP e uma alta concentração de íons Ca++ e Mg2+ (WHITTAKER, 1984). Muitas dessas vesículas estão agrupadas próximas a membrana pré-sináptica, formando placas densas, chamadas de zona ativa. Neste local ocorre a fusão das vesículas com a membrana pré-sináptica para liberar a ACh na fenda sináptica por exocitose (HUGHES, KUSNER, KAMINSKI, 2006).

As vesículas sinápticas apresentam em média 50-60 nm de diâmetro, são sintetizadas pelo corpo celular dos neurônios motores e transportadas ao longo do axônio para o terminal nervoso (BOOJ et al., 1986; SALTER, 2008).

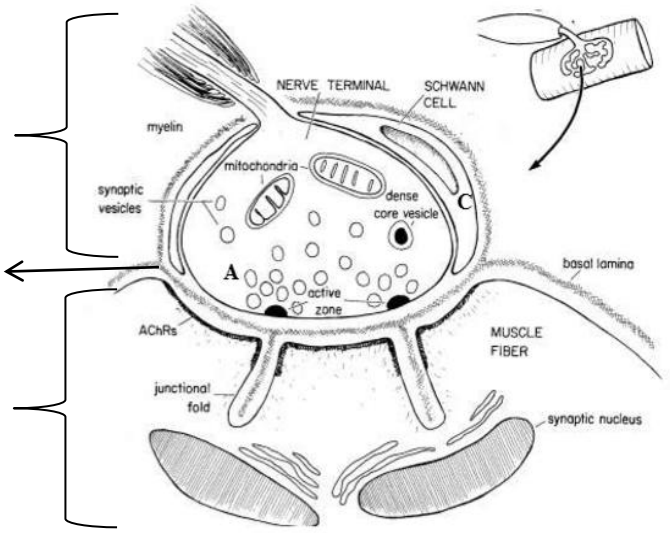

Figura 1. Esquema dos três compartimentos que compõe a junção neuromuscular: (A) Pré-sináptico (B) fenda sináptica (C) pós-sináptico. Fonte: Adaptado de Hall e Sanes (1993).

O compartimento extracelular está localizado entre a membrana pré-sináptica e pós-sináptica e contém a lâmina basal sináptica, a qual é contínua com a lâmina basal que envolve a fibra muscular (HALL; SANES, 1993). A lâmina basal sináptica contém proteínas distintas daquelas encontradas na lâmina basal extra sináptica, entre elas a Acetilcolinesterase (AChE), Agrin, laminina-4, laminina-9, laminina-11, colágeno IV que a tornam bioquimicamente especializada e importante para o desenvolvimento e função da JNM (SLATER, 1990; PATTON et al., 1997; PATTON, 2003; SANES, 2003). Outras proteínas como ARIA, colágeno III, proteoglicanos, heparan-sulfato, N-CAM presentes na fenda sináptica são responsáveis pela estabilidade e agregação dos nAChR no sarcolema da fibra muscular (ENGEL, 2003).

A acetilcolinesterase (AChE), é uma enzima presente na fenda sináptica responsável por hidrolisar a Acetilcolina, em AcetilCoA + Colina (SANES, 2003; XU et al., 2015).

O compartimento pós-sináptico é composto pelo sarcoplasma juncional e pelo sarcolema imediatamente justaposto ao terminal nervoso. O sarcoplasma juncional caracteriza-se pela presença de núcleos sinápticos, que são

morfologicamente distintos dos não sinápticos, pois são maiores e seus genes transcrevem proteínas específicas da membrana sarcoplasmática sináptica, sendo responsáveis pela transcrição do mRNA das subunidades dos nAChR; nesta região estão presentes mitocôndrias, REL, RER, Complexo de Golgi, estruturas lisossomais, microtúbulos e grânulos de glicogênio (HALL; SANES, 1993; ENGEL, 2003; RUFF, 2003).

O sarcolema é pregueado, contendo dobras juncionais, com cerca de 1 µm de profundidade que aumentam a superfície pós-sináptica e, portanto a eficácia da transmissão sináptica (SANES; LITCHMAN, 1999; TINTIGNAC;BRENNER;RÜEGG, 2015).

O compartimento pós-sináptico exibe dobras juncionais compostas por duas regiões distintas em forma e função: o ápice/crista e fundo. No ápice/crista encontram-se agrupados os receptores nicotínicos para acetilcolina (nAChRs) em uma densidade de aproximadamente $10^4/\mu m^2$ (SANES e LICHTMAN,1999) e no fundo estão dispostos canais de sódio responsáveis pela geração do potencial de ação (HALL e SANES, 1993).

Os nAChR são proteínas integrais de membrana compostas por cinco subunidades arranjadas em forma de rosácea ou canais iônicos (Figura 2) (CHANGEUX; EDELSTEIN, 1998; LINDSTROM, 2000; MA et al., 2007).

Apresentam-se distribuídos em duas formas: extrajuncional imatura, presente na fibra embrionária, constituída pelas subunidades α1(2), β1, δ e γ (alfa, beta, delta e gama); e juncional madura constituída pelas subunidades α1(2), β1, δ e ε (alfa, beta, delta e epsilon) (MISHINA et al., 1986; TINTIGNAC;BRENNER;RÜEGG, 2015).

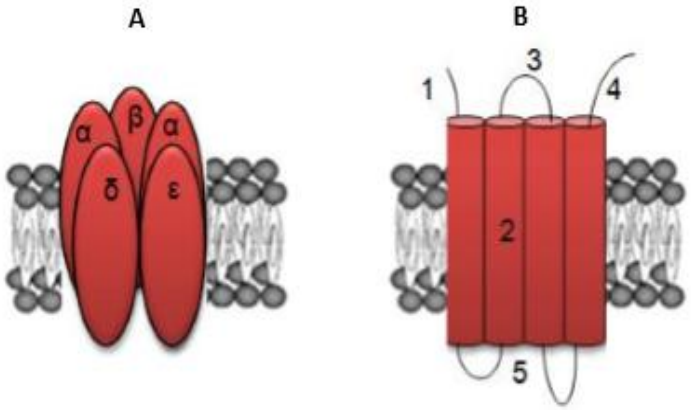

Figura 2- Representação esquemática do nAChR. A subunidades α1(2), β1, ε e δ. B – domínios presentes em cada subunidade: N-terminal hidrofílico (1), quatro segmentos hidrofóbicos transmembrana (2), domínio hidrofílico (3), C- terminal hidrofóbico (4) e alças formando sítios de fosforilação (5). Fonte: adaptado de Naguib et al. (2002) e Ventura et al. (2010).

Para a transmissão neuromuscular, necessita-se a chegada do impulso nervoso na membrana pré-sináptica, ativação e a abertura dos canais de Ca++ voltagem-dependente permitindo o influxo deste íon (TINTIGNAC;BRENNER;RÜEGG, 2015). Esse mecanismo permite a ativação de proteínas SNAREs (sinaptobrevina, SNAP-25 e sintaxina), comprometidas com a ancoragem e fusão das vesículas que contém o neurotransmissor ACh na membrana pré-sináptica (SEAGAR et al., 1999). A ACh é liberada na fenda sináptica e se liga aos canais iônicos presentes na membrana pós-sináptica da fibra muscular , há entrada de Na+ e saída de K+ concomitante a abertura de canais de Na+ voltagem-dependente no fundo das dobras juncionais gerando um potencial de placa em miniatura (PPmin). A somatória dos PPmin gera um potencial de placa terminal (PPt) com uma maior despolarização do sarcolema da fibra muscular, promovendo a liberação de Ca++ do retículo sarcoplasmático para o sarcoplasma da fibra muscular levando à contração muscular (HUGHES; KUSNER; KAMINSKI, 2006; FERRARO; MOLINARI; BERGHELLA, 2012; GONZALEZ-FREIRE et al., 2014).

Além da atividade física as JNMs podem sofrer também alterações decorrentes da idade (ANDONIAN & FAHIM, 1987; BARKER & IP, 1965; FAHIM , 1997; PRZYBYLA , 2006; GONZALES-FREIRE et al, 2014).

A idade é um fator que interfere comprovadamente junto às fibras musculares e JNMs (GONZALEZ-FREIRE et al., 2014; KEEVIL,. & ROMERO-ORTUNO, 2015). O envelhecimento do músculo esquelético é caracterizado por uma perda progressiva de massa muscular, bem como uma diminuição da sua função (NARICI E MAFFULLI, 2010). Este déficit muscular associado à idade é conhecido como sarcopenia, a qual contribui profundamente para a qualidade de vida dos idosos e predispõe os idosos a um risco aumentado de morbidade, mortalidade e invalidez (JANSSEN et al., 2004). A etiologia da sarcopenia é um processo multifatorial, que envolve tanto fatores intrínsecos como extrínsecos. No entanto, vários estudos realizados em animais e humanos, sugerem que a degeneração de neurônios motores, seguida de alterações na integridade estrutural e funcional da JNM, desnervação funcional, e a perda de unidades motoras contribuem significativamente para a progressão do envelhecimento do músculo esquelético.

IBEBUNJO et al (2013), verificaram que a sarcopenia tem inicio aos 21 meses de idade no rato. Análise proteômica de amostras com 6, 18 e 27 meses confirmou a depleção de proteínas mitocondriais, metabolismo de energia e proteínas da JNM. Juntos, estes resultados sugerem que as abordagens terapêuticas que, simultaneamente, estimulem a mitocondriogênese reduzam a proteólise muscular e inflamação tem potencial para tratar sarcopenia.

Poucos estudos na literatura foram encontrados sobre o efeito do DN na junção neuromuscular associada ou não com idade.

Considerando que o músculo esquelético associado à junção neuromuscular possui uma alta plasticidade frente a diferentes fatores, entre eles a atividade física e idade, o objetivo deste trabalho foi: avaliar o efeito de doses suprafisiológicas de decanoato de nandrolona associado a atividade física, sobre as fibras musculares e junções neuromusculares do músculo sóleo (SOL) de ratos e verificar se esse tipo de intervenção é capaz de prevenir os efeitos deletérios no envelhecimento.

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Capítulo 1

Supraphysiological doses of nandrolone decanoate change neuromuscular junctions of the soleus muscle of young rats subjected to physical exercise.

Erick Valentino[1], Ana Paula Silveira Leite[1], Paula Aiello Tome de Souza Castro[2], Selma Maria Matheus Michelin[3] *

[1]Graduate student of General Basis of Surgery, Botucatu Medical School, Uni Estadual Paulista, Botucatu, São Paulo, Brazil

[2]Post doctoral student at the CCBS / UFSCar / DFisio, muscle plasticity area, Universidade Federal de São Carlos, São Carlos, São Paulo, Brazil

[3] Department of Anatomy, Institute of Biosciences, UNESP, Botucatu, São Paulo, Brazil

Corresponding author: Selma Maria Michelin Matheus, Department of Anatomy, Institute of Biosciences, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil, CEP: 18618-689, Botucatu, São Paulo, Brazil. Telephone number: +55(14)3880-0025. E-mail: micmath@ibb.unesp.br

ABSTRACT

Anabolic androgenic steroids (AAS) are synthetic substances derived from testosterone that have anabolic properties. For this reason, they are used by athletes and non-athletes in order to increase muscle mass, strength and performance in physical activity. Nandrolone decanoate (ND) is the most widely used AAS among athletes. This study intends to analyze the effect of supraphysiological doses of nandrolone decanoate on the neuromuscular junction of young rats submitted to physical activity. Twenty (300-350g) male Sprague-Dawley rats were used, 130 days old, treated for 8 weeks and distributed according to the treatment in groups of sedentary (SV and SND) or exercised animals which received or not ND (EV and END). Physical training was conducted by sessions of water jumping three times per week. After eight weeks of the experiment, the soleus muscle was harvested, and the following analyzes were conducted: morphology and morphometry of neuromuscular junctions; ultrastructure of muscle fibers and associated neuromuscular junctions; immunohistochemistry and morphometric analyses of fast and slow-twitch fibers; HE staining for morphological analysis of fibers and quantitation of central nuclei; Red picrossirius staining for quantification of collagen fibers, distribution of nAChRs by confocal laser scanning microscopy. The morphology of the NMJs remained normal in the SV and SND groups; in the physically trained group there was scattering of acetylcholine receptors, consistent with increased diameter of the neuromuscular junctions proved by non-specific esterase. In the SND group there were alterations: in the muscle fibers, with regions of splitting fibers and clusters of small fibers; increased number of central nuclei, increase in the percentage of collagen, decreased number of peripheral nuclei, and increased number of type I fibers. The results point to the fact that the dose used did not promote gain in muscle mass, body or muscle weight, but induced morphological changes in NMJ structure and sol muscle toward a more oxidative pattern. The morphophysiological and neuromuscular changes to be further investigated in other doses associated with physical exercise Thus we can conclude that the use of supraphysiological doses of ND (without exercise), promoted change in skeletal muscle tissue, resulting from damage or regeneration, still promoted increase of muscle oxidative pattern.

Introduction

Anabolic androgenic steroids (AAS) are synthetic substances derived from testosterone that have anabolic properties. Their properties as well as their derivatives is mainly manifested in its myotrophic effect (DI LUIGI *et al*., 2005; RIEZZO, 2014). AAS, among them Decanoate Nandrolone, have long been used especially among bodybuilders to improve their performance and promote exponential increase in muscle mass and in the percentage of lean mass (TURILLAZZI *et al*., 2011; REARDON & CREADO, 2014; BREUNER, 2014). Nandrolone decanoate **(**ND) is the most widely used AAS among males, since it has less significant adverse effects (JOHANSE *et al*., 2006; GORDON *et al*., 2010; JOHNSON, 2000). Exercise has been investigated as a major factor in the adaptation process of skeletal muscle (WILLIAMSON *et al.*, 2000; PARCELL *et al*. 2003; MEAKIN *et al*., 2015; SCHILD *et al*., 2015). One important adaptation is the modulation of muscle fibers that may occur during systematic physical training (SEENE *et al*., 2007;GEHLERT *et al*., 2012), in response to metabolic overcompensation, in order to meet the needs of the body to repeated stimuli, therefore optimizing physical performance (BARBANTI, 2001; SIU *et al*., 2004; DUNN *et al*., 2014, BOOTH *et al*., 2015).

As a consequence, Type I muscle fibers (slow-twitch contraction) and Type II (fast-twitch contraction) are gradually recruited according to their specific characteristics in response to the exercise performed. In prolonged low-intensity exercise, type I fibers are recruited because of high ATP production in the presence of oxygen, being efficient in the high resistance aerobic exercises (POWERS, 2000; WILMORE, COSTIL, 2001). Type II fibers respond better to exercise performed in the absence of oxygen, the end product of their metabolism being intramuscular lactate, which can cause early fatigue, leading to reduction in exercise tolerance (POWERS, 2000; WILMORE, COSTIL, 2001).

The moderate practice of physical exercise can induce the modulation of intermediate fibers (IIa and IIb) into more (I) or less (IIx) oxidative fibers (WENFENG *et al*., 2014).This type of practice can also reduce collagen deposition, contributing to minimize muscle fibrosis, what can be established as a consequence of high fat diet as well as of obesity (PINCU *et al*., 2015). High intensity exercises promote modulation of intermediate fibers (IIx) to more oxidative fibers (IIA and I) (WENFENG *et al*., 2014).

SOUZA *et al*. (2015) studying aerobic training associated to heart failure in rats, found decreased catabolic activity through the decreased expression of the components involved in skeletal muscle atrophy, therefore minimizing muscle mass wasting.

Exercise also has beneficial effects on the nervous system, including the peripheral nervous system and neuromuscular junctions (NMJ) (BOOTH *et al*., 2002; HANDSCHIN *et al*., 2008).

According to DESCHENES *et al*. (2000), the NMJ are more sensitive to exercise-induced adaptive responses than other parts of the skeletal muscle. Both increase as well as decrease in activity may reshape the structure of neuromuscular junctions; exercise affects the presynaptic nerve branching, increasing its length and complexity (DESCHENES *et al*., 2006).

According to SOUZA *et al.*, 2015, aerobic training for 10 weeks in rats with heart failure was able to modify the structure of the NMJ changing the area classification (Class IV) in the exercised group, and attenuating the expression of the nAChRs subunits. Several chemical compounds and/or drugs have been used in association with physical exercise with the objective of enhancing its effects.

Considering that the skeletal muscles associated with the neuromuscular junctions have high plasticity in response to physical activity, and that the indiscriminate and excessive use of AAS among adults and adolescents with no treatment purposes has become a public health problem, the objective of this study was to analyze the effect of supraphysiological doses of nandrolone decanoate on the muscle fibers and associated junctions of the soleus muscle of young rats subjected to physical exercise.

Material and methods

For this study, 20 male Sprague-Dawley rats (300-350g), were used, acquired from the Multidisciplinary Center for Biological Research of the Universidade Estadual de Campinas (CEMIB/Unicamp). The animals were kept under appropriate conditions in the animal research laboratory of the Department of Anatomy, IBB-UNESP (CEUA-IBB protocol n 448).

Experimental groups

The animals were divided into four experimental groups (05 rats per group), and euthanized at 130 days of age (Figure 1).

SV: sedentary group, no steroid (n = 5).

SND: sedentary group, with steroid (n = 5).

EV: physical exercise group, no steroid (n = 5).

END: physical exercise group, with steroid (n = 5).

Experimental Protocol

The END and SND groups received intramuscular injections (IM) of Nandrolone Decanoate (ND), Deca Durabolin® (10mg/kg/week), twice per week, for 7 weeks (SHOKRI *et al*., 2009).

This dosage, according to POPE & Katz (1988) is equivalent to the abusive dose used by athletes, 10 to 100 times higher than the treatment dose. The SV and EV groups received IM injections of vehicle (0.2 ml/kg body weight of propylene glycol) twice per week for 7 weeks.

Resistance physical training by jumping in liquid medium

The animals of the exercise groups (EV and END) were subjected to jumping sessions into a PVC cylinder containing water at 30C (HARRI & KUUSELA, 1986), 38 cm deep. During the first week (first to fifth day) the rats of the exercise groups went through a period of adaptation to exercise in liquid medium. From the second week on, the animals were subjected to 4 series of 10 jumps, at 60-seconds intervals, three times per week with a progressive body weight overload of 50% (second and third weeks), 60% (fourth and fifth weeks), and 70% (sixth, seventh and eighth weeks) (Figure 2). The weight overload was placed on the ventral chest by means of a vest.

Physical exercise and ND injections were started after 56 days of age, respecting the time needed for spermatogenesis to happen (SHOKRI *et al*., 2009).

Throughout the entire physical training period, the sedentary animals (SV and SND), not subjected to the jumping sessions, were placed in a box with shallow water, also at 30° C, with no need to jump in order to have contact with the water.

The animals were dried with cotton towel and kept warm for 30 minutes after each physical training session.

Material processing

After 48 hours of the last jumping session, the rats from the experimental groups were euthanized in CO_2 gas chamber, and body weights were recorded (GRIEWE *et al*., 1999). The soleus muscles of five animals from each experimental group were subsequently dissected, removed, weighed and processed according to the protocols below.

1.Morphometry of neuromuscular junctions

After removing the muscles from the left antimeres, they were reduced to the medial region (containing the motor point) and cut longitudinally into 3 or 4 pieces.

The slides prepared for the morphologic study of the neuromuscular junctions were used for morphometric analysis, the material was then submitted to non-specific esterase (LEHRER, ORNSTEIN, 1959) in order to identify the neuromuscular junctions (NMJ).

1.1 Morphometric analysis

. The maximum diameter of 50 junctions was measured in 5 animals of each experimental group. The measurements were analyzed with the software Image J (http://rsbweb.nih.gov/ij/).

1.2 Transmission Electron Microscopy (TEM) and Confocal Microscopy

After anesthesia and shaving, the skin of the pectoral region was dissected and the sternal plastron retracted. The thoracic viscera were exposed and perfusion conducted via the left ventricle. The animals were first perfused with PBS and then fixed in 2% paraformaldehyde in 0.1M sodium phosphate buffer, pH 7.4. The caudal *Vena cava* was severed at the level of the right atrium in order to drain blood and the excess of infusion fluid.

1.3 Transmission Electron Microscopy (TEM)

The muscle portion containing the motor point was cut in fragments that were then submerged in Karnovsky fixative solution (50 ml of 8% paraformaldehyde - 10 ml of 25% glutaraldehyde, completed with 0.2M phosphate buffer, pH 7.3) and subjected to the routine technique for TEM. The tissues were included in such a

way that longitudinal muscle sections were obtained for the identification of the neuromuscular junctions.

The ultrathin sections obtained were examined and documented using a Philips transmission electron microscope (FEI CM100 model).

Confocal microscopy

The Soleus muscle of the left antimere was reduced to the region containing the motor point, fixed in 2% paraformaldehyde, and submitted to the protocol for acetylcholine receptors (nAChR).

After fixation for 15 min, the muscle fragments were flushed several times with PBS (14g of monobasic sodium phosphate, 4.3 g of potassium phosphate dibasic anhydrous and 72g of sodium chloride in 1 L of distilled water, pH 7.5) and, in order to inactivate the fixative, were incubated in 0.1M glycine for 20 min in an orbital shaker. After flushing with PBS they were incubated with 1% collagenase (Sigma Type I C-0130) for 20 min on the shaker to detach the connective tissue from the muscle. They were subsequently flushed with PBS and the nAChRs were labeled with rhodamine conjugated-alpha-bungarotoxin (Rh-BTX - Molecular Probes T1175,1: 1000 in PBS), placed in the shaker for 30 min. After this period, the muscles were flushed with PBS and incubated with Triton X-100 1% (Sigma T9284) for 1 hour in order to attain permeabilization of the muscle fibers. They were subsequently mounted on slides under cover slip in medium for fluorescence DABCO - (Sigma), and analyzed under the Confocal Laser Scanning microscope (Leica TCS-□-SPE).

Analysis of SOL muscle fibers: Hematoxylin-Eosin (HE), Picrosirius Red and Immunohistochemistry (fast and slow-twitch fibers)

After removing the muscles from the left antimeres they were reduced to the medial region (containing the motor point), and the pieces processed to non-specific esterase; the two remaining portions of each muscle (proximal and distal) from each animal were involved in neutral talc and frozen in liquid nitrogen (stored in a freezer at -80C). Sequential 6 µm histological sections were subsequently obtained on a cryostat (Leica CM 1800) at -25°C. Four slides were obtained, the first stained with HE and photographed on Olympus BX41 microscope (SC30 camera); the images obtained (20X) were used for the general morphological analysis of the muscles and to count the central and peripheral nuclei. For this analysis around 200 muscle fibers

selected from 3-4 random fields were used. This quantification was attained using the software Image J.

Picrosirius Red staining and collagen quantification

The second slide was stained with Picrosirius red (SWEAT *et al*., 1964), the collagen stained red, and the muscle fibers, yellow. In order to quantify intramuscular collagen, about 6 random images (20 X) were obtained for each animal of each experimental group. The percentage of collagen/total area was obtained using Leica QWin software. Images with polarized light were also obtained from the same slide in order to complement the analysis of the morphology of collagen fibers (JUNQUEIRA and CARNEIRO, 2009).

Immunohistochemistry of fast-twitch and slow-twitch fibers

The third and fourth slides obtained were subjected to the immunoperoxidase method StreptABComplex/HRP using commercial primary antibodies specific for each protein of the study as follows:

Monoclonal antibody	Clone	Manufacturer	Dilution
Fast Anti-myosin	WB-MYHCf	Novocastra	1:160
Slow Anti-myosin	WB-MYHCs	Novocastra	1:120

After identifying the types of muscle fibers (in cross-sectional slices), 5-6 fields were photographed per slide, in order to obtain about 200 fibers; the types of fibers were then counted and their area measured, which was obtained with the aid of Image J software after marking their contour using a pen mouse.

Results

Animal weight and weight of the soleus muscle

There was no statistically significant difference in the weight of the animals or of the muscles between all experimental groups, regardless of the use of decanoate (Tables 1 and 2).

Analysis of neuromuscular junctions and Morphometric analysis of the NMJ

Non-specific Esterase technique enabled visualization of the NMJ distribution in the soleus muscle as shown in Figure 3. NMJ are distributed in the middle third, aligned in a transverse or oblique fashion to the long axis of the muscle fibers, next to the nerve branches that take axons to these endplates.

These junctions have intensely branched and wide synaptic gutters. Variations in form (open, irregular, compact) characterize the polymorphism of these structures. Those should not be considered as morphological changes relative to the groups studied (Figure 4).

Table 3 contains the average of the maximum diameters of the NMJ of the groups studied. This analysis demonstrated that there was an increase in the diameter of the NMJ in the exercise group, with ND (54.6 ± 5.9) or without ND (58.4 ± 3.9) as compared to the sedentary group with (47.4 ± 4.6) or without (51.6 ± 2.3) nandrolone decanoate ($p < 0.05$).

Transmission electron microscopy

The NMJ associated to the soleus muscles were homogeneous in the groups studied, with no signs of alterations. All the NMJ studied presented standard morphology, the axon terminals arranged in synaptic gutters sometimes shallow or deep with varying numbers of synaptic vesicles and mitochondria (Figure 5 A, B, C and D).

In the pre-synaptic membrane the presence of electronically denser regions that correspond to active areas that are opposite to the apex of the junctional folds of the postsynaptic membrane was evident (Figures 5 A, B, C and D).

Confocal Laser Scanning Microscopy

The acetylcholine receptors labeled by alpha-bungarotoxin conjugated to the rhodamine of the soleus muscles of the different groups studied were examined using the confocal laser scanning microscope (SP5 - Leica) integrated to an inverted fluorescence microscope (Electron Microscopy Center of Botucatu, Institute of Biosciences UNESP/Botucatu/SP). For confocal microscopy the 20X and 40X lenses with oil immersion and 2X zoom were used. The 568 nm line was used in order to exciter rhodamine. Brightness and contrast were kept constant for all groups. Images

from one single focal plane or from image groups were acquired with the assistance of the manufacturer's software and saved to an optic disc.

All groups were homogeneously stained by chromogen-rhodamine (Figure 6 A, B, C and D).

A mild scattering of acetylcholine receptors was noted in the physically exercised group (with or without decanoate) (Figures 6 C and D) (this result was confirmed by NMJ morphometry).

ANALYSIS OF MUSCLE FIBERS

Analysis of morphology: HE

Morphological analysis was carried out examining images obtained after HE staining. In the sedentary animals that received only vehicle (SV) the fibers had polygonal shape, peripheral nuclei, and preservation of the endomysium and perimysium (Figure 7A). In the sedentary animals that received steroid (SND), it was noted that some fibers lost their typical shape, and regions containing fibers of small diameter with peripheral nuclei were found (Figure 7B). Fibers with central nuclei were also noted, as well as other regions presenting splitting fibers (Figure 7B'). In the exercised animals, with or without the use of ND (END, EV) morphology was preserved, the fibers presented typical shape and size (Figures 7C and D). In the END group the presence of fibers with central nuclei was also detected (Figure 7D).

Central and peripheral nuclei count

Morphology was used in order to count the number of central and peripheral nuclei; the analysis was conducted using the software Image J. Increased number of central nuclei in the SND group (23) was noted in this analysis, as compared to the SV group (5) ($p < 0.05$), and decreased amount of peripheral nuclei in the SND group (181), as compared to the SV group (201) ($p < 0.05$) (Figure 8).

Picrossirius Red staining and collagen quantification

The collagen fibers present in the endomysium and perimysium were visualized by Picrossirius Red staining; collagen fibers were marked in red and muscle fibers in yellow. This analysis was conducted using conventional light optical microscopy and polarized light optical microscopy (Figure 9). Morphological analysis demonstrated increased red staining in the groups that made use of ND (SND and

END), suggesting increased collagen (Figures 9 B, D, D'). In sedentary animals that used decanoate (SND), increased deposition of collagen fibers was noted in the perimysium region (Figure 9B). Using polarized light, this was confirmed in the animals of the SND group where there was a greater deposition of collagen, its fibers clustered and strongly birefringent, occupying mainly the region of the perimysium. Their birefringence ranged from yellow to green, suggesting the presence of collagen fibers type III (newly synthesized) (Figure 9 B'). In the other groups, little deposition of collagen fibers was detected, and under the polarized light they shed red tones (suggestive of the presence of type I collagen - mature collagen) (Figures 9A, A', C, C').

Collagen Quantification

Quantification of collagen areas was obtained from morphological observation of the Picrossirius Red-stained slides. For this analysis 6 fields at 20x magnification were run through the Leica Q win software. This analysis demonstrated statistically the observations in morphology described above, where in the SND group there was an increase in the percentage of the collagen area (24.6%) as compared to the SV group (14.5%) ($p<0.05$) (Figure 10).

Immunohistochemistry analysis: Fast-twitch and Slow-twitch fibers

After immunohistochemistry staining of the muscle fibers for fast and slow MHC, it was noted that in the slides where the antibody for slow myosin was used, the type I fibers (slow-twitch) were strongly stained brown, whereas the type II fibers (fast-twitch) did not react to the chromogen (Figure 11). On the slides where fast myosin antibody was used, fibers type II (fast-twitch) stained, whereas type I fibers (slow-twitch) did not (Figure 11).

This analysis detected predominant expression of the slow myosin heavy chain isoform in relation to type II fibers, which express the fast myosin heavy chain isoform. This predominance was present in all experimental groups, being the standard for the soleus muscle - predominantly oxidative (Tables 4 and 5).

In the SND group a statistically significant increase in type I fibers (slow-twitch) (198) was found, as compared to the SV group (185) ($p <0.05$) (Table 4). The amount of type II fibers (fast-twitch) remained stable in relation to the parameters analyzed (Table 5).

Measurement of the area of fast-twitch and slow-twitch muscle fibers.

In order to better understand the modulation of fast-twitch and slow-twitch fibers as a response to the treatments (exercise and use of ND), a class representation of the fibers areas was created, facilitating the comparison between groups. Respecting the classification in slow-twitch and fast-twitch fibers, the frequency of the areas was sorted as: 1-2 and 2-3 thousand μm^2- smaller classes; 3-4 and 4-5 thousand μm^2- intermediate size classes; fibers with a diameter greater than 5000 μm^2–larger classes (Figures 12 and 13).

Regarding the slow-twitch fibers, in the SV group there was a predominance of fibers ranging in size from 2-3 and 3-4 thousand μm^2. In the sedentary animals that received ND (SND), the fibers with areas 3-4 and 4-5 thousand μm^2 were predominant. Among the exercised animals, both those who received ND as those receiving vehicle, slow-twitch fibers predominated with an area of 3-4 thousand μm^2 (Figure 12).

Observing the areas of fast-twitch fibers, there was a predominance of small class fibers with diameters of 2-3 thousand μm^2 in both groups of sedentary animals (SV and SND). In the exercised animals receiving vehicle, the predominant class was 3-4 thousand μm^2 (intermediate class). In the animals of the END group, fibers with areas of 2-3 and 3-4 thousand μm^2 predominated (Figure 13).

Analysis of transmission electron microscopy (TEM)

The myofibrils were organized in the muscle fibers forming well-defined sarcomeres with morphological features related to the different types of muscle fibers; the most frequent type found in the soleus muscle contained thick Z-lines, intermyofibrillar mitochondria, and T-tubules organized in triads (Figures 14 A, B, C and D).

The presence of central nuclei (Figure 15 D) in the sedentary group with steroid and a few in the exercise group with steroid is noteworthy, what was also confirmed by the count of central nuclei. In the other groups only peripheral nuclei were observed. No other changes in morphology were noted (Figures 15 A, B and C).

Discussion

The objective of this study was to analyze the effect of the supra physiological dose of 10 mg/kg/week of ND on the muscle fibers and associated neuromuscular junctions of young rats' soleus muscle subjected to physical exercise. The main results were that the isolated use of nandrolone decanoate in a supra physiological dose not associated to exercise resulted in modifications in the muscle fibers (focal lesions and splitting fibers), as well as increased amount of central nuclei, and increase in the percentage of collagen and type I fibers.

The investigations on the effect and risk of ND, especially in supra physiological doses without medical prescription, have become increasingly common across the scientific community (TURILLAZZI *et al.*, 2011; BREUNER, 2014; REARDON, CREADO, 2014; BINAYI *et al*., 2015).

DE MELO NETO *et al*. (2015), investigating the interaction of supra physiological doses (10 mg/kg/week) of nandrolone decanoate with exercise noted weight reduction in the animals that received the drug. Our results did not find difference in the weight of the animals or of the soleus muscle. Nevertheless, a trend towards weight reduction was noted in the animals of the groups that received ND. According to CARSON *et al*. (2002), the mechanism involved in the reduction of body weight would be related to decreased appetite and increased lipid oxidation caused by excessive doses of AAS.

The SND group had alterations in the morphology of the fibers. Regions containing clusters of small fibers with peripheral nuclei were present; in mammals there are descriptions of a type of muscle hyperplasia, characterized by longitudinal division of muscle fibers, resulting from adaptations to resistance training. This type of adaptation occurs mainly as a consequence of high intensity resistance training, what represents a stressful situation for the muscular apparatus. During this process, structural changes that provide significant improvement in functional capacity of skeletal muscle fibers will be triggered (KADI *et al*., 2000).

Splitting muscle fibers, which is described as the fiber sarcoplasm dividing into smaller fragments as a result of hypertrophy (HARRIMAN, 1990) were also found in the SND group.

This alteration has been demonstrated in aged animals (PICHAVANT & PAVLATH, 2014), animals subjected to various physical exercise protocols (ERIKSSON *et al.*, 2006; SOFFE *et al*., 2015), and associated with the use of

anabolic steroids (FONTANA *et al*., 2010). This process has been pointed as a possible consequence of hypertrophy (SOFFE *et al.*, 2015), and considered a form of muscle regeneration (ERIKSSON *et al.*, 2006).

In addition to the modifications mentioned, the presence of central nuclei was noted; the statistical analysis proved significant in the animals of the SND group. The presence of those central nuclei associated to testosterone derivatives is related to the process of muscle regeneration and tissue repair (AGGARWAL *et al*., 2014; OLIVEIRA *et al*., 2014; PIOVESAN *et al*., 2013).

One should also consider that those central nuclei maybe represent new myonuclei, as a consequence of the activation of the satellite cells; androgen receptors would be located in these myonuclei, thus enhancing the anabolic effect of the AAS (SAR *et al*., 1990; TAKEDA *et al*., 1990; HYYPPA *et al*., 1997).

That same group (SND), presented increased amount of collagen fibers (24.6%) as compared to the sedentary, control animals (14.5%) ($p< 0.05$).

This finding has been reported in heart muscle and kidney, where this increase in collagen deposition after the use of ND was considered one of the changes resulting from the abnormal hypertrophy of the organs studied (BRAZIL *et al*. 2015).

Under polarized light, the deposition of collagen fibers in the SND group seems to represent newly synthesized collagen-III fibers (green color), what indicates a regenerative process in the muscle tissue (DE CARVALHO, 2015).

In the groups submitted to the protocol of physical exercise, polarized light showed the presence of more reddish fibers suggesting an increase in type I collagen fibers, which were more evident in the END group.

LIMA *et al*. (2015) reported this finding in heart muscle with the use of anabolic steroids associated to exercise, pointed by the authors as a possible aspect of an abnormal heart hypertrophy.

Regarding immunohistochemistry and its quantification, there was an increase in Type I fibers (slow-twitch) in the sedentary animals that used ND (SND) as compared to control (SV). This result was also found by FONTANA *et al*. (2010), who demonstrated that the increase in oxidative fibers after the use of mesterolone also caused increased number of mitochondria, therefore increasing fatigue resistance.

DE SOUZA et al. (2011) after testing resistance training protocol with insufficient recovery time noted an increase in type I and type IIBD fibers in the

plantaris muscle.

As for the area of the fibers, there was an increase in the area of oxidative fibers in the groups that received ND (3-4/4-5 thousand μm^2) as compared to control (23/3-4 thousand μm^2). FONTANA *et al*. (2013) described that the increase in the area of these fibers was attributed to the anabolic profile of the AAS. In exercised animals there was no alteration in the area of type I fibers, which remained predominantly with area between 3-4 thousand μm^2.

Among the glycolytic fibers, there was no modification in the animals of the SND group, maintaining the same predominance of class present in control (SV) - 2-3 thousand μm^2. In animals that were submitted to the exercise protocol there was an increase in the area of type II fibers as compared to control 3-4 thousand μm^2; in the animals that were exercised and given nandrolone decanoate, the area classes ranged between 2-3 thousand μm^2 and 3-4 thousand μm^2. These results can also be attributed to the catabolic counterpoint consequent of resistance exercise in the face of the characteristic anabolic effect of AAS (FONTANA *et al*., 2013).

In relation to the NMJ, both under confocal microscopy as in the morphometric analysis, there was an increase in the maximum diameter of the NMJ in the animals that practiced physical exercise as compared to sedentary animals (with or without decanoate).

Similar results were reported in rats submitted to a program of seven weeks resistance exercise where the animals exhibited increased total area and perimeter of the NMJ (DESCHENES *et al*., 2000).

DESCHENES *et al*. (2006) reports that physical exercise can significantly reshape the structure of the NMJ, although the nature and site of these changes are specific to how the activity is performed.

Increase in NMJ diameter has been reported as a result of physical training in young rats. However, many methodological aspects such as age, skeletal muscle/type of fiber, exercise and the training protocol can influence the results (NETO *et al*., 2015).

It is important to emphasize that the results of this study refer to a specific type of physical training protocol, associated with the application of supra physiological doses of ND for 7 weeks. Variations in the exercise protocol, the anabolic steroid chosen, dose and duration of treatment could lead to different responses, and it is important to consider that further studies are needed in order to clarify the positive-

negative effects of the use of AAS associated or not with exercise.

Conclusion

The results point to the fact that the dose used did not promote gain in muscle mass, body or muscle weight, but induced morphological changes in NMJ structure and sol muscle toward a more oxidative pattern. The morphophysiological and neuromuscular changes to be further investigated in other doses associated with physical exercise. Thus we can conclude that the use of supraphysiological doses of ND (without exercise), promoted remodeling in skeletal muscle tissue, resulting from damage or regeneration, still promoted increase of muscle oxidative pattern.

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Tables and figures

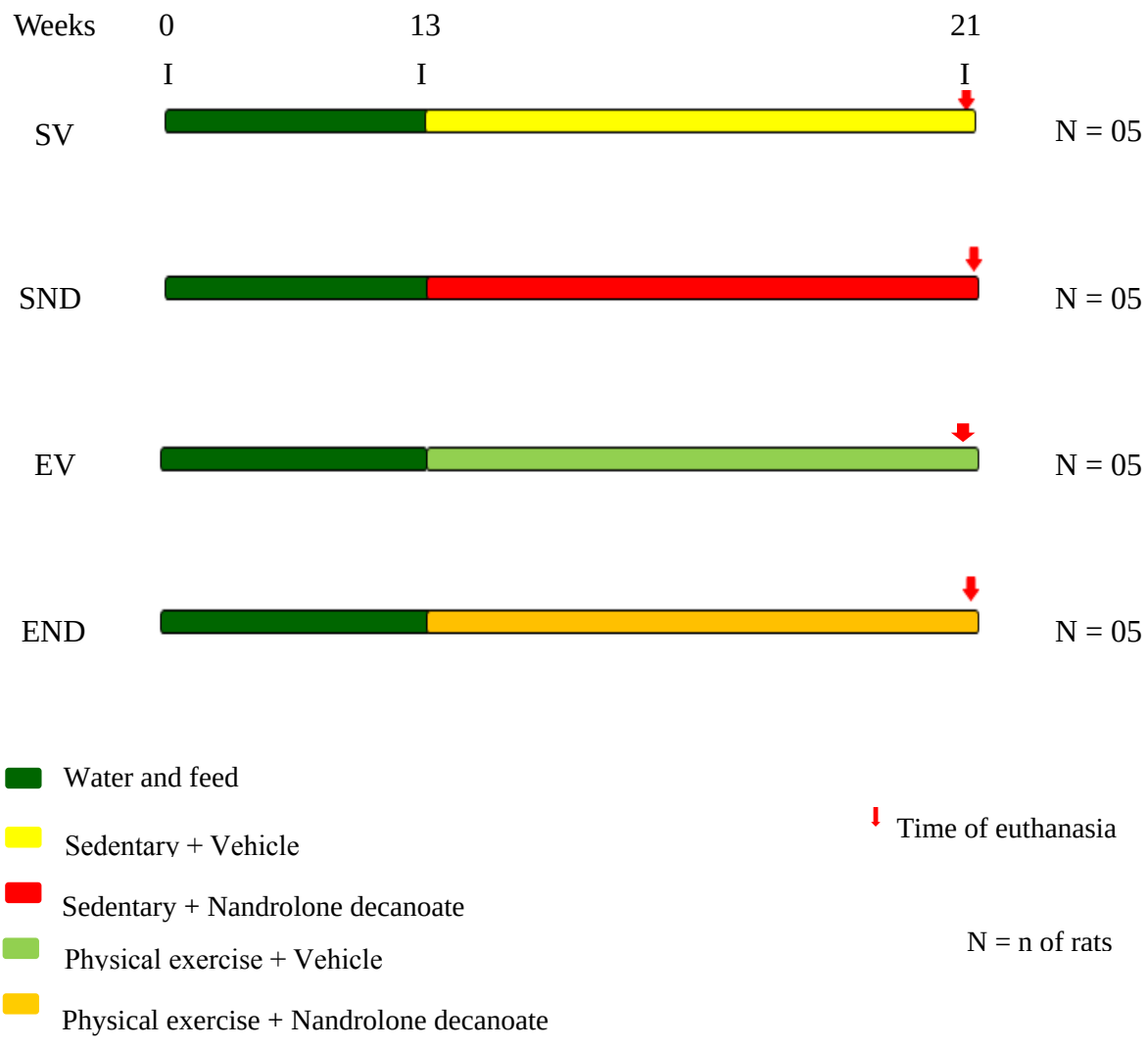

Figure 1- Experimental model SV: Sedentary group, with no steroid; SND: Sedentary group

with steroid; EV: Physical training group with steroid; END: Physical training group with steroid.

Training day	Training - Overload (% Body Weight)
1st	2 series of 5 jumps (50%)
2nd	3 series of 5 jumps (50%)
3rd	4 series of 5 jumps (50%)
4th	4 series of 7 jumps (50%)
5th	4 series of 9 jumps (50%)
6th to 20th	4 series of 10 jumps (50%)
21th to 35th	4 series of 10 jumps (60%)
36th to 54th	4 series of 10 jumps(70%)

Figure 2 – Training protocol according to day and body weight %.

Table 1. Mean and standard deviation of animals' weight (g) according to the group and steroid use; S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p value
Animal Weight	S	627.0 (64.9)	559.0 (23.0)	p>0.05
	E	579.0 (72.8)	544.0 (38.9)	p>0.05
p Value		p>0.05	p>0.05	

Statistical results obtained by scheme-factor analysis of variance, complemented with Tukey's multiple comparison test (Zar, 2009).

Table 2. Mean and standard deviation of the weight of the soleus muscles (g) according to the group and steroid use; S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
Weight Soleus	S	0.257 (0.049)	0.199 (0.031)	p>0.05
	E	0.262 (0.035)	0.235 (0.044)	p>0.05
P Value		p>0.05	p>0.05	

Statistical results obtained by scheme-factor analysis of variance, complemented with Tukey's multiple comparison test (Zar, 2009).

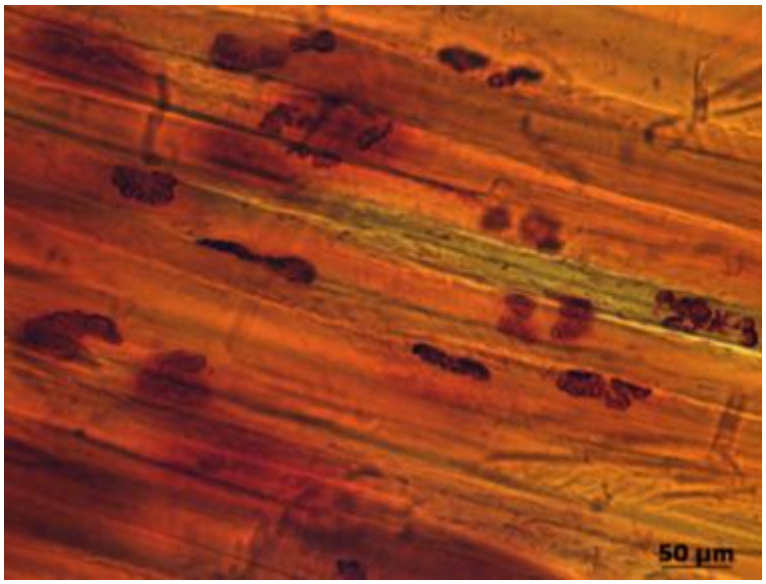

Figure 3- Light microscopy. Distribution of neuromuscular junctions (NMJ) in the soleus muscle. Full thickness preparation. Non-specific esterase reaction.

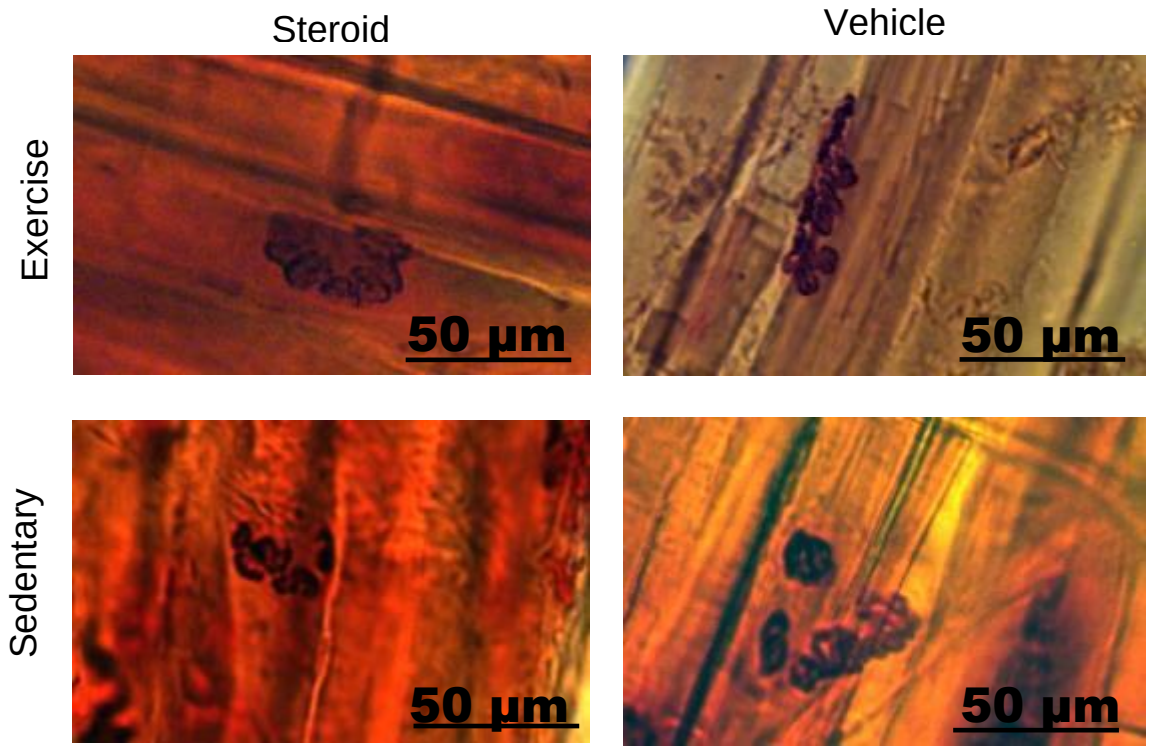

Figure 4- Light microscopy. Detail of the neuromuscular junctions (NMJ) in the soleus muscle of the groups studied. Full-thickness preparation. Non-specific esterase reaction.

Table 3. Mean and standard deviation of the maximum diameters (µm) of the NMJ present in the soleus muscles of the animals according to the group and steroid use; S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
Diameter Mean NMJ	S	51.6 (2.3)	47.4(4,6)	p>0.05
	E	58.4 (3.9)	54.6 (5.9)	p>0.05
p Value		p<0.05	p<0.05	

Statistical results obtained by two-factor analysis of variance, complemented with Dunn's multiple comparison test (Zar,2009),considering 5% significance level.* p < 0.05

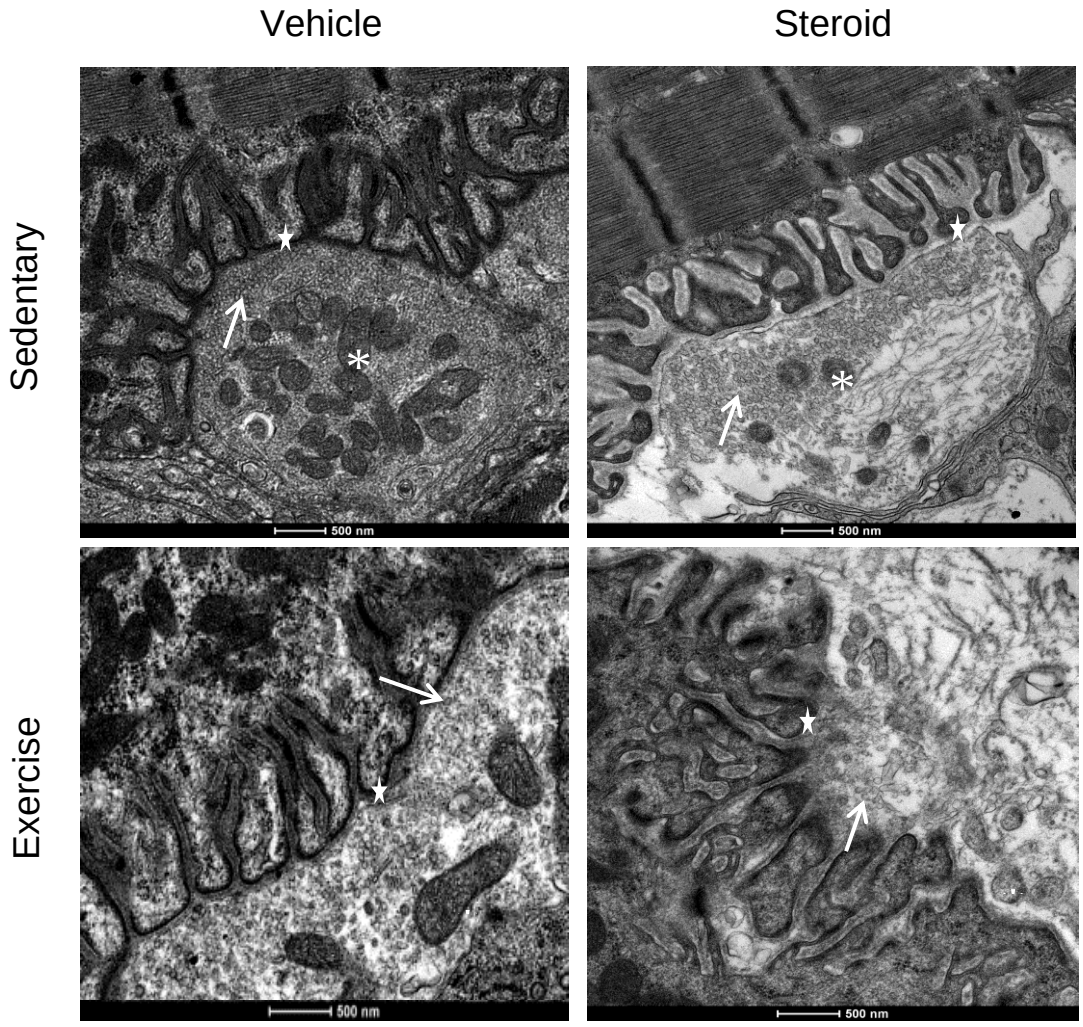

Figure 5- Transmission electron microscopy of neuromuscular junctions of the soleus muscle of all groups. Mitochondria (*), Synaptic vesicles (arrow), junctional folds (#), electronically dense pre synaptic region (★).

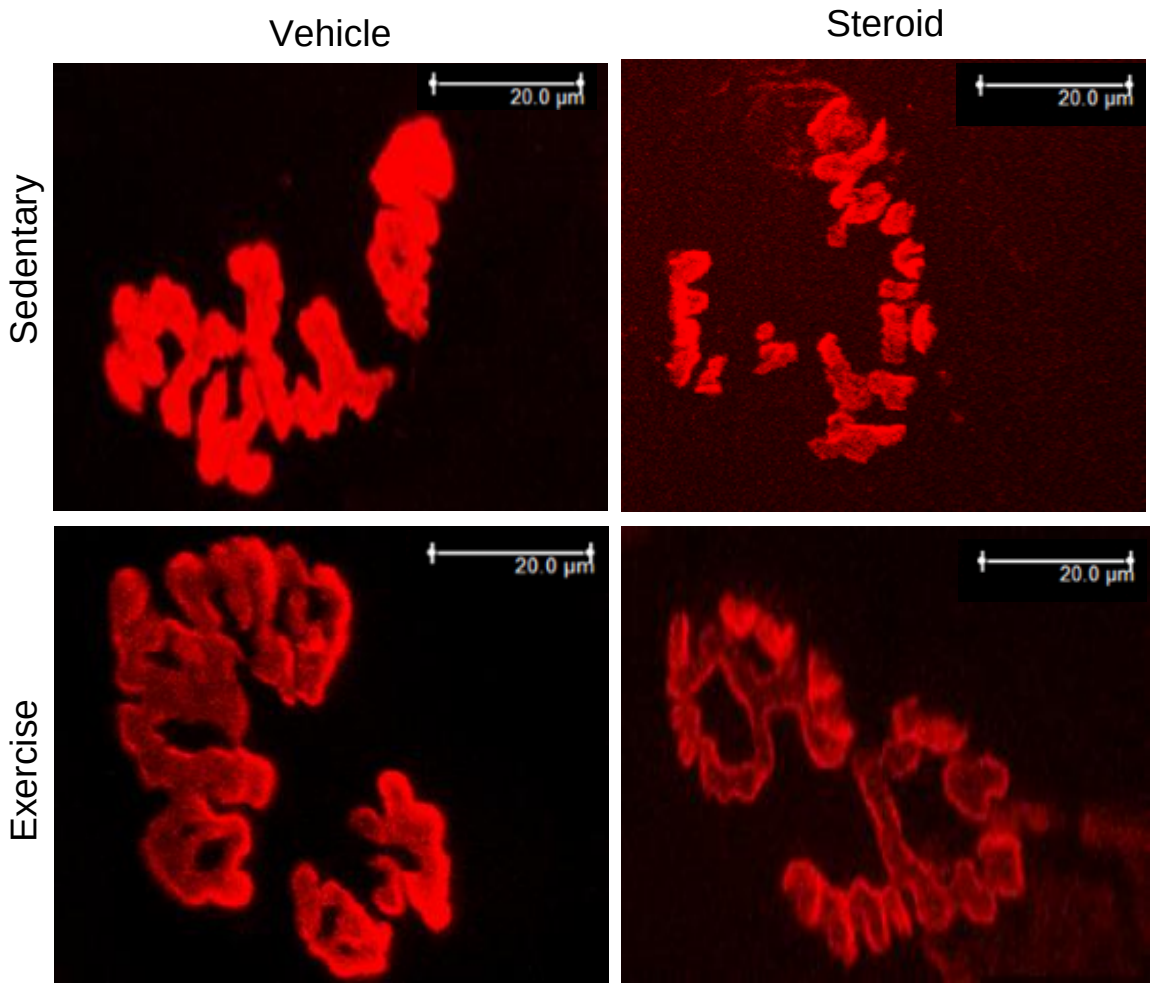

Figure 6- Confocal microscopy of acetylcholine receptors of the soleus muscles labeled with rhodamine-conjugated alpha-bungarotoxin.

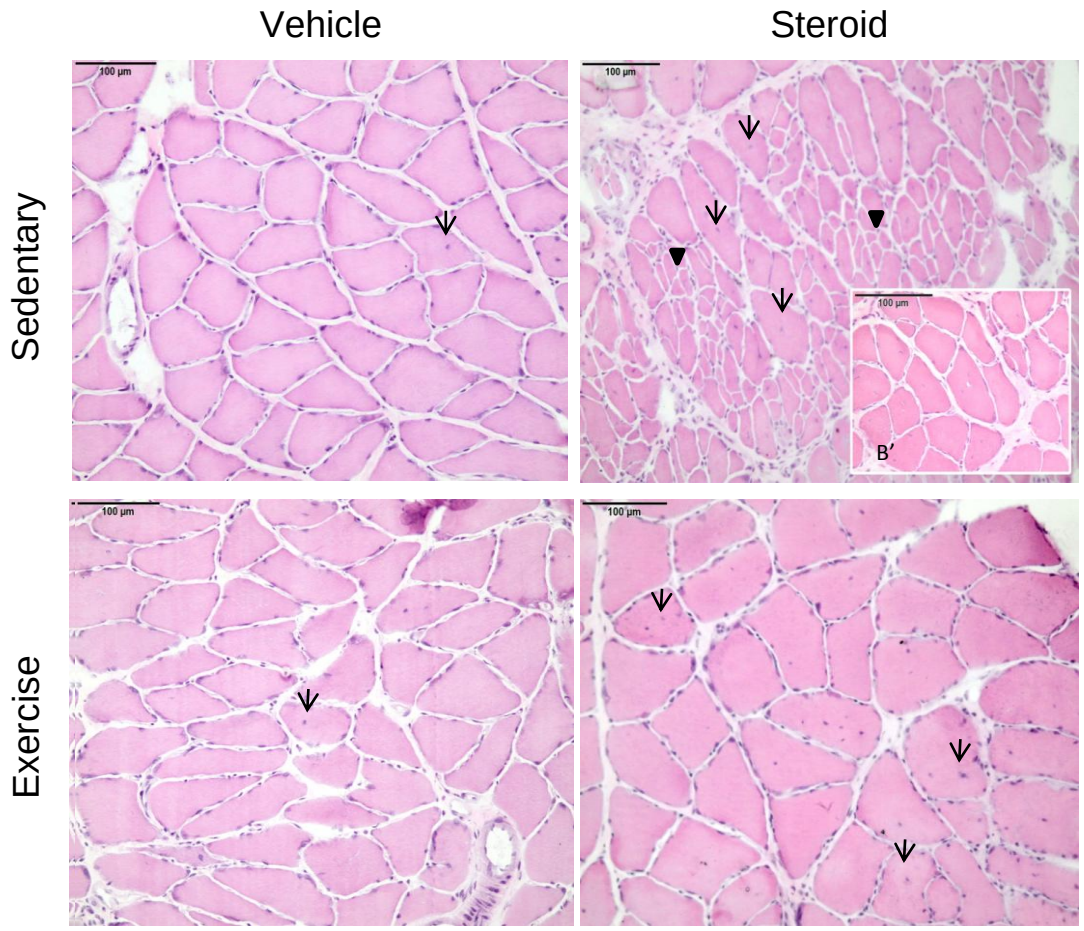

Figure 7- Photomicrographs of cross-sections of HE-stained soleus muscles of the experimental groups. A: SV-sedentary animals + vehicle; B and B': SND-sedentary animals treated with ND. C: EV –Animals that performed exercise + vehicle. D: END-Animals that performed exercise + ND; (arrow) fibers with the presence of central nuclei; (*) regions of splitting fibers. (arrowhead) regions with small fiber with peripheral nuclei.

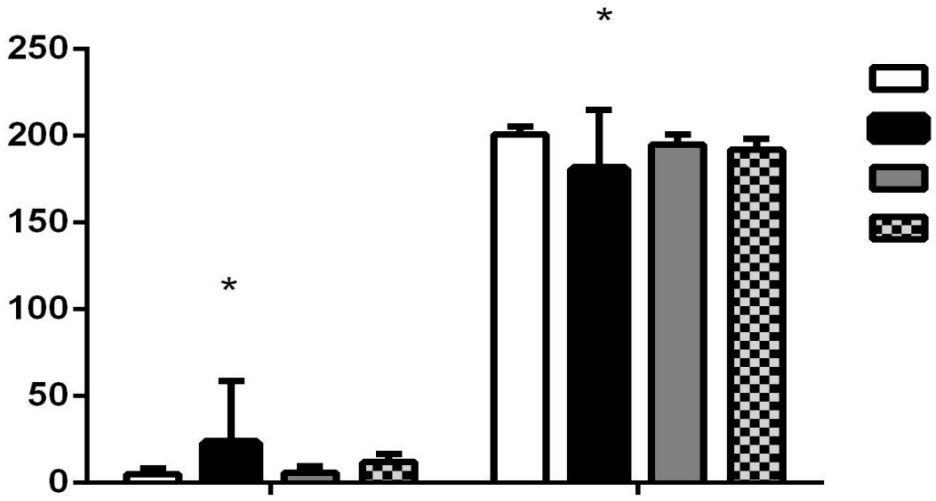

Figure 8- Graphic of mean and standard deviation of the central and peripheral nuclei present in each experimental group.

Data obtained through technical analysis of variance model with two factors complemented by multiple comparison test of Tukey (Zar , 2009) considering the 5% level of significance. * P <0.05.

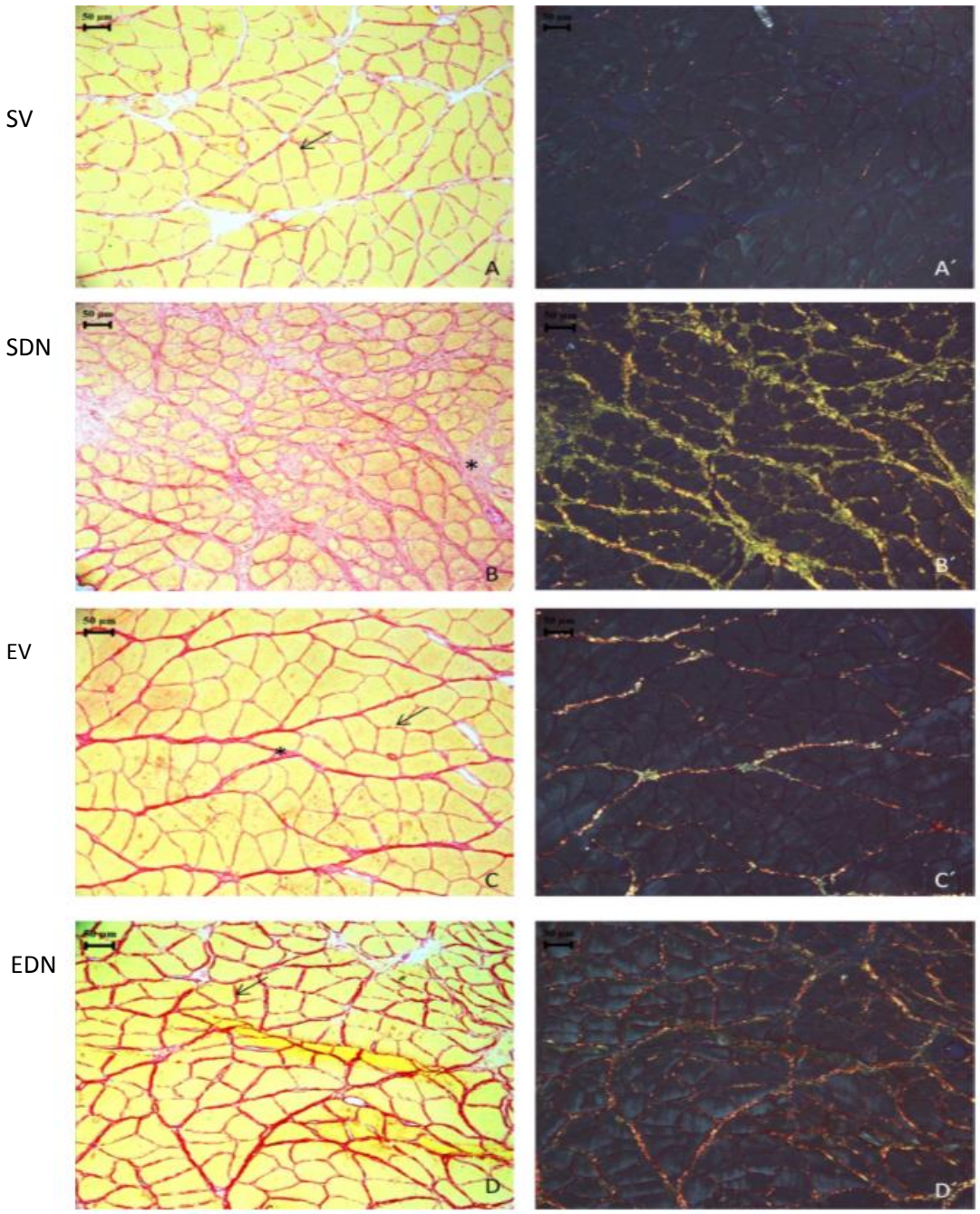

Figure 9- Photomicrographs of cross-sections of picrossirius red-stained soleus muscle from all experimental groups under conventional light microscopy (AD), and polarized light microscopy (A'-D'). A and A': SV-sedentary animals; B and B': SND -sedentary animals + ND; C and C': EV –exercised animals + vehicle; D and D': END –exercised animals + nandrolone decanoate. (*) endomysium; (arrow) perimysium.

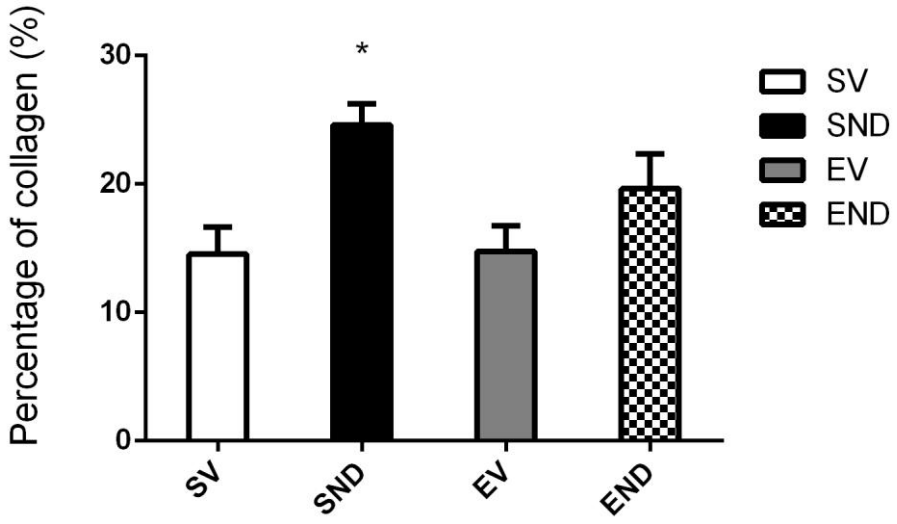

Figure 10- Graphic of percentage and standard deviation of the collagen areas in the groups studied.

Data obtained through technical analysis of variance model with two factors complemented by multiple comparison test of Tukey (Zar , 2009) considering the 5% level of significance. * P <0.05.

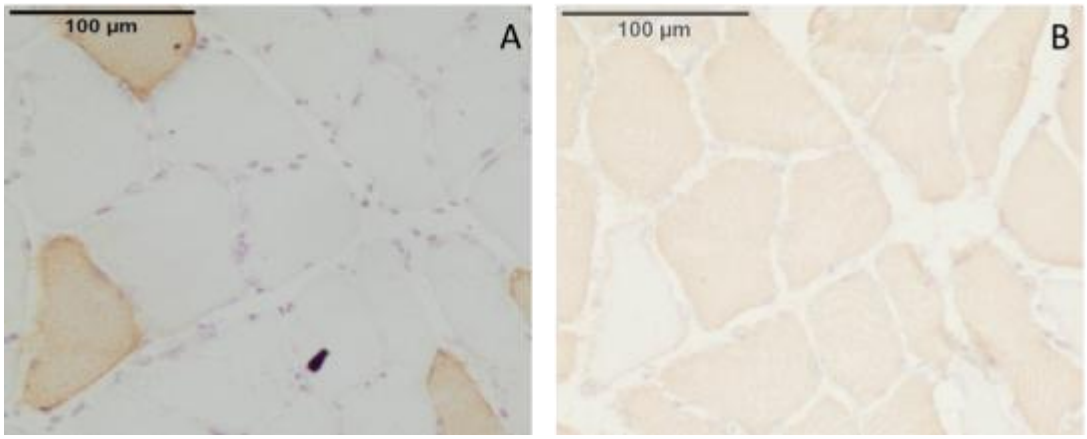

Figure 11- Histological cross-sections of soleus muscle fibers - Immunohistochemistry for fast-twitch (A) and slow-twitch (B) fibers, respectively.

Table 4. Mean, minimum and maximum values of the number of slow-twitch muscle fibers of the soleus muscle according to the group and use of steroids. S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
Slow-twitch fibers	S	185(163; 187)	198(187; 226)	p<0.05
	E	181(154;196)	82 (166;199)	p>0.05
p Value		p>0.05	p>0.05	

Statistical results obtained by the non-parametric two-factor analysis of variance, complemented with Dunn's multiple comparison test (Zar,2009), considering 5% significance level.* $p < 0.05$.

Table 5. Mean, minimum and maximum values of the number of fast-twitch muscle fibers of the soleus muscle according to the group and use of steroids. S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
Fast-twitch fibers	S	20(14;43)	19(2; 46)	p>0.05
	E	19(8;51)	22(2; 43)	p>0.05
p Value		p>0.05	p>0.05	

Statistical results obtained by the non-parametric two-factor analysis of variance, complemented with Dunn's multiple comparison test (Zar,2009), considering 5% significance level.* $p < 0.05$.

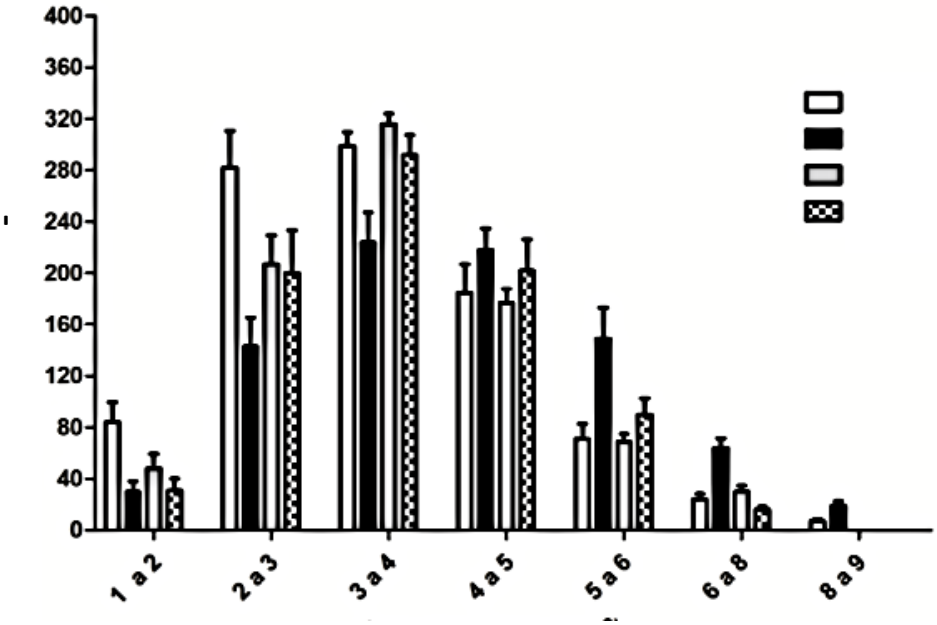

Figure 12– Graphic of frequency and standard deviation of the areas of slow-twitch fibers according to classes (thousand- μm²).

Statistical data obtained by the Goodman's association test complemented by multiple comparisons between and in the multinomial populations (GOODMAN, 1964, 1965).

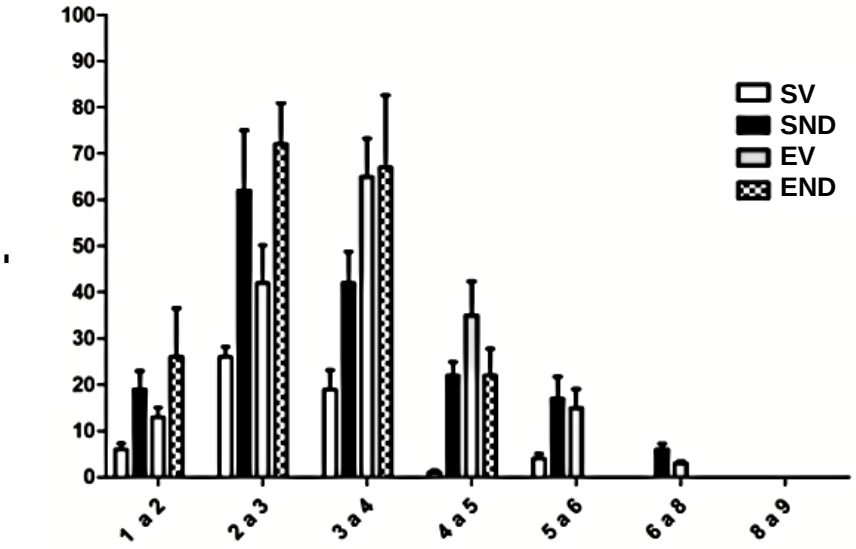

Figure 13 -Graphic of frequency and standard deviation of the areas of fast-twitch fibers according to classes (thousand- μm²).

Statistical data obtained by the Goodman's association test complemented by multiple comparisons between and in the multinomial populations (GOODMAN, 1964,1965).

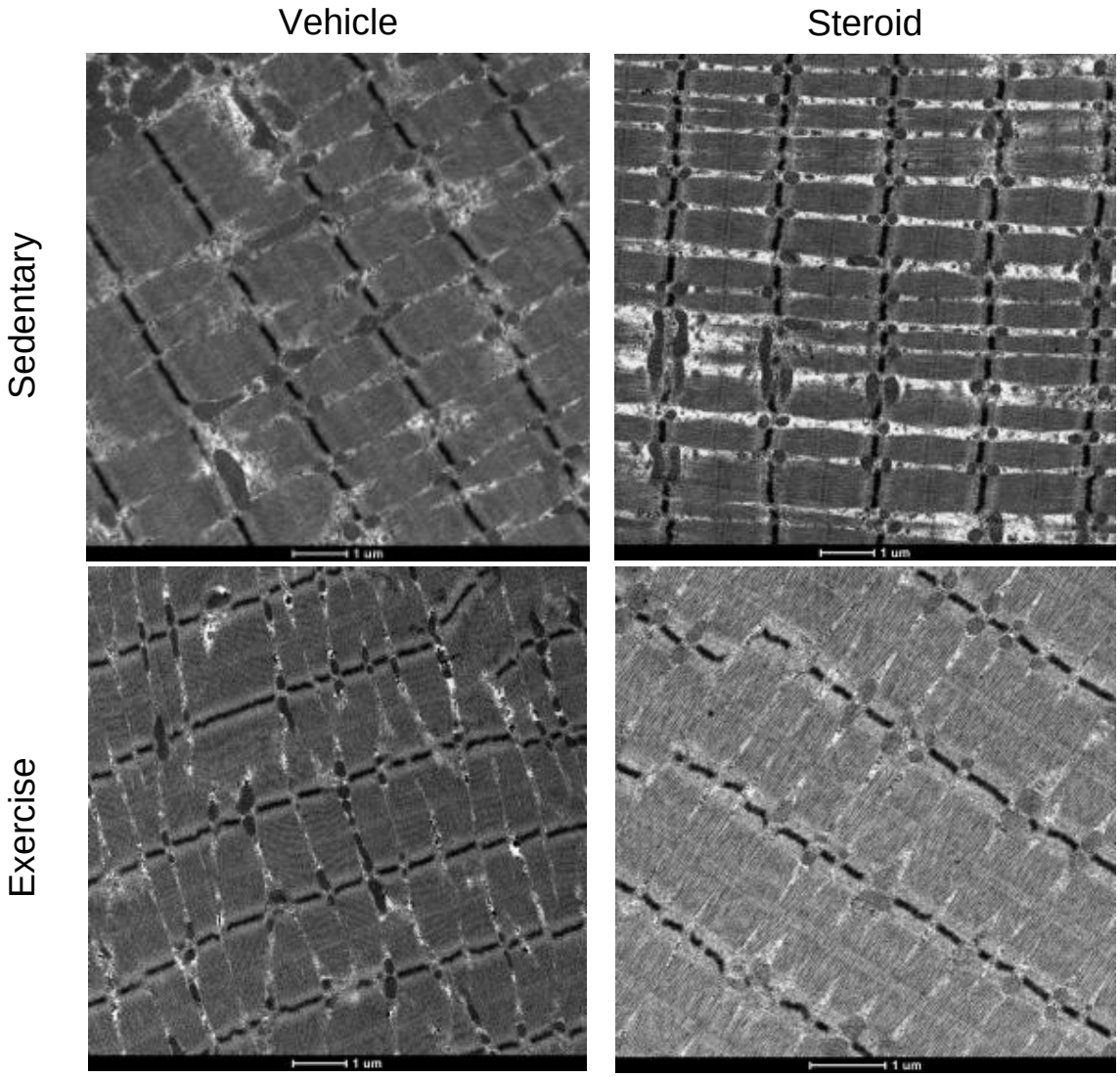

Figure 14- Transmission electron microscopy of the muscle fibers of the soleus muscle of all groups.

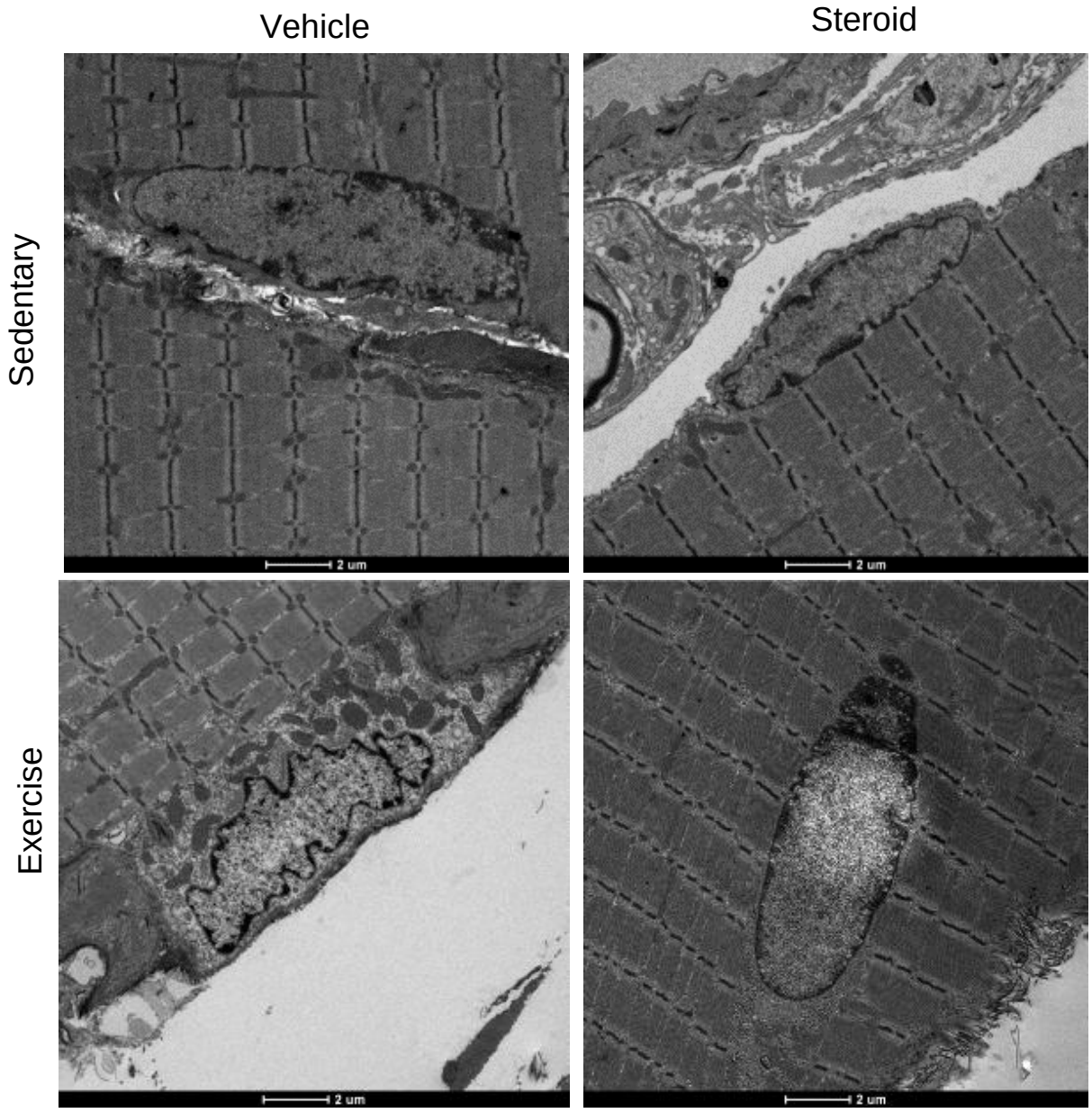

Figure 15- Transmission electron microscopy of muscle fibers, central (*) and peripheral nuclei (**) of the soleus muscle of all groups.

Capítulo 2

Supraphysiological doses of nandrolone decanoate and exercise- does the combination during youth prevent neuromuscular changes in aging?

Erick Valentino[1], Ana Paula Silveira Leite[1], Paula Aiello Tome de Souza Castro[2], Selma Maria Matheus Michelin[3] *

[1]Graduate student of General Basis of Surgery, Botucatu Medical School, Univ Estadual Paulista, Botucatu, São Paulo, Brazil

[2]Post doctoral student at the CCBS / UFSCar / DFisio, muscle plasticity area, Universidade Federal de São Carlos, São Carlos, São Paulo, Brazil

[3] Department of Anatomy, Institute of Biosciences, UNESP, Botucatu, São Paulo, Brazil

Corresponding author: Selma Maria Michelin Matheus, Department of Anatomy, Institute of Biosciences, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil, CEP: 18618-689, Botucatu, São Paulo, Brasil.Telefone: (014) 3880-0025.E-mail: micmath@ibb.unesp.br

ABSTRACT

Population aging has been considered one of the greatest challenges of contemporary public health; among other factors there is decreased hormone release, progressive loss of muscle mass, and decreased muscle strength. Physical exercise has a beneficial effect on that system, as well as synthetic testosterone derivatives, which have anabolic effect. This study had the objective of evaluating whether the association of physical exercise enhanced or not by supraphysiological dose of nandrolone decanoate prevents age-related alterations in the soleus muscle of rats and in the associated neuromuscular junctions. 40 male Sprague-Dawley rats, 90 days old, were treated for 8 weeks and distributed according to the treatment in sedentary or exercised group, with or without the use of ND. Physical training was conducted by jumps in water three times per week. At the age of 300 days, the soleus muscles were collected and analyzed as follows: morphologic and morphometric analysis of neuromuscular junctions; ultrastructural analysis of muscle fibers and associated neuromuscular junctions; immunohistochemistry and morphometric analysis of fast and slow-twitch fibers; analysis of the distribution of nAChRs by confocal microscopy. The results demonstrated that there was a decreased weight in the animals that used ND, although the weight of the soleus muscle did not change. The general morphology and morphometry of the NMJ remained constant in the groups studied, and in regard to ultrastructure the junctional folds were scarce. The animals that performed exercise had a pattern of nAChRs in continuous branches, in all the other animals the "island" pattern was present. The morphometric and quantitative pattern of slow and fast-twitch fibers remained stable in all groups. Central nuclei and focal areas of injury, as well as myofibrillar disorganization were observed in the animals that used ND. The results enable the conclusion that the alterations observed in this study were consequent to aging, and that physical exercise performed in youth maintained the structural pattern of nAChR present in the young animals. ND did not prevent morphological changes in the neuromuscular system consequent to aging.

Introduction

Population aging has been considered one of the greatest challenges of contemporary public health. This phenomenon began in developed countries, but has recently been more intense in developing countries (VERAS, 2008).

Age is a factor that has been proved to act over the neuromuscular system. Aging of skeletal muscle is characterized by a progressive loss of muscle mass and decreased function (NARICI and MAFULLI, 2010). This muscular deficit associated with age is known as sarcopenia, which intensely affects the quality of life of the elderly and predisposes them to an increased risk of morbidity, mortality and disability (JANSSEN *et al*., 2004).

The etiology of sarcopenia is multifactorial, involving both intrinsic and extrinsic factors. However, several studies conducted in animals and human beings suggest that the degeneration of motor neurons, followed by alterations in the structural and functional integrity of the neuromuscular junction (NMJ), functional denervation, and loss of motor units contribute significantly to the progression of skeletal muscle aging (IBEBUNJO *et al*., 2013).

Exercise has been regarded as an important factor in the adaptation process of skeletal muscle (WILLIAMSON *et al*., 2000; PARCELL *et al*., 2003).

Systematic physical training can cause modulation of muscle fibers in response to a metabolic overcompensation to meet the needs of the body to repeated stimuli, thus optimizing physical performance (BARBANTI, 2001; SIU et al., 2004).

Muscle activity may influence both pre and postsynaptic elements of the NMJ over time. Voluntary exercise, started in middle age, is able to inhibit the loss of nerve terminals that occurs in elderly NMJ (CHENG *et al*., 2013).

The muscle metabolic capacity can be further affected by changes in the levels of systemic hormones (ASMANN *et al*., 2006). Testosterone and its synthetic derivatives can increase lean body mass, muscle strength and the synthesis of muscle protein (BHASIN *et al*., 1996; FERRANDO *et al*., 2002).

Although several hormones are known as effectors of the metabolism of skeletal muscle (SHAKOOR and SHALET, 2003), the effect of testosterone on the capacity of the metabolism of skeletal muscle is not as widely investigated (ROGOZKIN, 1979; EGGINTON, 1987; RAMAMANI *et al*., 1999).

Testosterone acts by increasing the number of progenitor muscle cells (HIKIM-

SINHA *et al*., 2003), promoting their myogenic differentiation (SINGH *et al*., 2003, 2006). It also stimulates mitochondrial biogenesis, increasing the oxygen supply to the tissue, red cell number, and capillarity (COVIELLO *et al*., 2008; GUPTA *et al*., 2008).

Nandrolone Decanoate (ND) is a synthetic testosterone derivative (JOHNSON, 2000; GORDON *et al*., 2010); it is the most used anabolic androgen steroid (AAS) among athletes, especially males, since it has less adverse effects (JOHANSE *et al*., 2006).

The objective of this study was evaluate whether the association of physical exercise enhanced or not by supraphysiological doses of ND prevents age-related changes in the soleus muscle of rats and the associated neuromuscular junctions.

Material and methods

Twenty male rats were used (Sprague-Dawley), obtained from the Multidisciplinary Center for Biological Research of the Universidade Estadual de Campinas (CEMIB/Unicamp). These animals were kept during the trial period in the animal research laboratory of the Department of Anatomy, IBB-UNESP, under appropriate conditions (CEUA -IBB Protocol n ° 448).

Experimental groups

The animals in this study were divided into 4 experimental groups (Figure 1):

SVi: sedentary group, no steroid (n = 5).

SNDi: sedentary group with steroid (n = 5).

EVi: exercise group, no steroid (n = 5).

ENDi: exercise group with steroid (n = 5).

After being submitted to this experimental protocol the animals were kept under the regular conditions of the animal research laboratory mentioned above until they reached 300 days of age, in order to characterize aging.

Experimental Protocol

The ND groups (ENDi, SNDi) received intramuscular injections (IM) of Deca Durabolin® containing 10 mg/kg/week (5 mg/kg body weight twice per week),

according to the protocol developed by SHOKRI *et al*. (2009), for the course of 7 weeks. This dosage corresponds to 10 to 100x the normal, therefore being considered supraphysiological (POPE & KATZ, 1988). The SVi and EVi groups received injections (IM) containing only the vehicle - propylene glycol (0.2 mL/kg body weight), which was applied following the same procedure.

Resistance physical training through jumps in liquid medium

The animals of the EVi and ENDi groups were submitted to a training program of jumping sessions in a PVC cylinder, containing water at 30C (HARRI & KUUSELA, 1986), at a depth of 38 cm. In the first five days prior to the 1st injection, the animals from the exercise groups went through an adaptation period to exercise in liquid medium. During this period, they initially performed 2 series of 5 jumps gradually increased until reaching 4 series of 10 jumps. Overload weight was placed on the anterior area of the animal's chest through a vest. Physical training lasted for 08 consecutive weeks. The training program of jumps in liquid medium with overload weight was conducted three days per week, between 1pm and 3 pm. Each session consisted of four series of ten jumps each, with progressive increase in weight: 50% of body weight (second and third weeks), 60% (fourth and fifth weeks) and 70% (sixth, seventh week and eighth weeks). Rest intervals lasting 60 seconds were respected between the series, where the animal was removed from the water and kept at rest.

Throughout the whole physical training period, the sedentary animals (SVi and SNDi) who did not undergo the jump sessions, were placed in a box with shallow water, also at 30°C, with no need to jump in order to have contact with water.

The animals were dried with cotton towel and kept under warm temperature for 30 minutes after each training session.

Material processing

After the training period and respecting the aging period, all animals were weighed at 300 days of age and euthanized using CO_2 chamber. The soleus muscles of five animals from each experimental group were then dissected, removed, weighed and processed according to the following protocols.

ANALYSIS OF THE NEUROMUSCULAR JUNCTION

Morphology and morphometry

After dissection of the middle third of the soleus muscle of the left antimere, it was sectioned longitudinally with the use of razor and identification for NMJ was carried out (LEHRER & ORNSTEIN, 1959).

After identification of the NMJ, 50 junctions were used in order to measure the maximum diameter, done by means of 5 slides corresponding to each animal of each experimental group, using the Image J software (http://rsbweb.nih.gov/ij/).

Transmission Electron Microscopy (TEM) and Confocal Laser Scanning Microscopy

For these preparations the animals were perfused with PBS, followed by 2% paraformaldehyde solution in 0.1M sodium phosphate buffer, pH 7.4. In order to drain the blood and the excess perfusion fluid, the caudal vena cava was severed at the level of the right atrium.

For ultrastructural analysis the right antimere muscles were reduced in the middle third into fragments and kept in fixative solution, then processed according to the routine for TEM. In order to identify the neuromuscular junctions, the fragments were included longitudinally, ultra-thin sections were prepared and photographed using TEM Philips (FEI CM100 model).

For the preparation of the analysis in confocal microscope, the middle third of the soleus muscle of the left antimere was used. After perfusion, they were left in 2% paraformaldehyde for 15 minutes, and then subjected to the protocol in order to identify acetylcholine receptors. That consisted of several PBS flushes (14 g of monobasic sodium phosphate, 4.3 g of potassium phosphate dibasic anhydrous and 72 g of sodium chloride in 1 l of distilled water, pH 7.5), and incubation with 0.1 M glycine for 20 min in an orbital shaker. New PBS flush and incubation with 1% collagenase (Sigma Type I C-0130) for 20 min in the shaker were done. New PBS flush and 30 min incubation in rhodamine-conjugated alpha-bungarotoxin shaker (Rh-BTX - Molecular Probes T1175,1: 1000 in PBS). New PBS flushes and incubation for 1 hour with Triton X-100 1% (Sigma T9284). Finally the slides were mounted for analysis and photo documentation using the Confocal Laser Scanning Microscope (Leica TCS-SPE).

ANALYSIS OF MUSCLE FIBERS

Hematoxylin and eosin (HE), and Immunohistochemistry of the types of muscle fibers (fast MHC and slow MHC)

The proximal and distal thirds of the soleus muscle of the left antimere of each animal from each experimental group were cryopreserved, frozen in liquid nitrogen and stored in a freezer at -80°C. Using the Leica CM 1800 cryostat, the material was cut with a thickness of 6 µm. Three slides were obtained for each animal of each experimental group. The first was stained with Hematoxylin-Eosin (HE), and photographed with the use of Olympus BX41 image analyzer (SC30 camera); the images obtained (200X) were used for general morphological analysis of the muscle and in order to count the central and peripheral nuclei. For this analysis around 200 muscle fibers selected from 3-4 random fields were used. This quantification was obtained using the Image J software.

The other two slides were subjected to immunohistochemistry in order to identify fast and slow-twitch fibers. For immunohistochemistry the immunoperoxidase method StreptABComplex/HRP was carried out, using primary commercial antibodies specific for each protein of the study as follows:

Monoclonal antibody	Clone	Manufacturer	Dilution
Fast Anti-myosin	WB-MYHCf	Novocastra	1:160
Slow Anti-myosin	WB-MYHCs	Novocastra	1:120

After identifying the types of muscle fibers (in cross-sections), 5-6 fields were photographed per slide, in order to obtain about 200 fibers; the fiber types were counted and the area measured after marking their contour using pen mouse and Image J software.

Results

Animal weight and weight of the soleus muscle

Table 1 depicts the means and standard deviation of the weight of the animals of all groups. This analysis found weight decrease in the animals that received steroid, associated (443 ± 30.94) or not (485 ± 34.10) to physical exercise ($p < 0.05$). There was no statistical difference in the weight of the soleus muscle in all

experimental groups, considering the use or not of decanoate (Table 2).

ANALYSIS OF NEUROMUSCULAR JUNCTIONS

The identification of NMJ revealed homogeneous distribution in the middle third of the soleus muscle, mostly aligned in a cross-sectional fashion in relation to the long axis of the muscle fibers. The junctions had highly branched synaptic gutters. No morphological changes were seen in the groups studied (Figure 2), what was statistically proven by the morphometric analysis of the NMJ.

Morphometric analysis of the NMJ

Table 3 depicts the means of the maximum diameters of the NMJ of the groups studied. This analysis found no significant differences in the diameters of the NMJ with or without exercise, and associated or not to the use of nandrolone decanoate.

Transmission electron microscopy

The ultrastructure of the NMJ associated with the soleus muscles demonstrated the presence of myelin figures located in the axon terminals in the groups that received nandrolone decanoate (SNDi and ENDi) (Figures 3 B and D).

The postsynaptic membrane, limit of the depth of the synaptic cleft, had few, ill-defined junctional folds, with variable arrangement and dimensions containing no alterations or spaces between them.

The remaining morphology of the NMJ had no changes in the groups, the axon terminals arranged in synaptic gutters sometimes shallow or deep with varied amounts of synaptic vesicles and mitochondria. The presynaptic membrane had electronically denser regions that correspond to the active areas opposite to the apex of the junctional folds of the postsynaptic membrane.

Confocal Laser Scanning Microscopy

In all groups studied there was homogeneous fluorophore response, by which acetylcholine receptors were highlighted.

This analysis demonstrated that the postsynaptic region of the NMJ were intact; in the sedentary groups (SVi and SNDi) they were grouped in small islands (Figure 4 A and B); in the exercise group, the acetylcholine receptors had a

distribution in continuous branches (Figures 4 C and D).

Analysis of muscle fibers

Morphological analysis: HE

Morphological analysis was conducted observing images obtained after HE-staining. In the groups studied the fibers presented polygonal shape and preserved endomysium and perimysium (Figure 5). In the animals of the groups that received steroid (SNDi and ENDi), some fibers presented central nuclei, what was confirmed by electron microscopy and statistical analysis.

Central and peripheral nuclei count

Based on morphologic analysis was realized the quantification of central and peripheral nuclei using Image J software. There was increased number of central nuclei in the SNDi group (20) as compared to the ENDi group (4), $p < 0.05$, and decreased peripheral nuclei in the SNDi group (186) as compared to the ENDi group (200), $p < 0.05$ (Tables 4 and 5).

Immunohistochemistry analysis: fast and slow-twitch fibers

All groups stained positive by immunohistochemistry for fast and slow MHC (Figure 6).

In the slides where the antibody against slow myosin was used, type I fibers (slow-twitch) were strongly stained brown, whereas type II fibers (fast-twitch) did not react to chromogen. In the slides where fast-myosin antibody was used, type II fibers (fast-twitch) were stained, while type I fibers (slow-twitch) were not.

In all experimental groups in relation to the use or not of decanoate, there was no change in the number of slow-twitch (Table 6) or fast-twitch fibers (Table 7).

Measurement of the area of fast and slow-twitch muscle fibers.

Data on morphometry of fast and slow-twitch muscle fibers of sedentary (S) and exercised (E) animals, associated or not to the use of ND are depicted in Table 8.

The data demonstrate that there was no statistical difference both in the areas of slow-twitch fibers as in the areas of fast-twitch fibers of the soleus muscles in all experimental groups considering the use or not of decanoate

Transmission electron microscopy analysis (TEM)

Through the longitudinal preparation of the muscle fibers, in most groups a normal sarcomere pattern containing organized myofibrils with preserved organelles was noted. In animal groups that received ND (SNDi and ENDi), focal areas of injury were present, and disorganized sarcomeres were common (Figures 7 B and D), as well as central nuclei (Figures 8 B and D).

Discussion

Aging is associated with progressive loss of muscle mass, of strength, and decline in neurophysiological functions. The relationship between age and NMJ has a key role in the musculoskeletal impairment that occurs with aging. However, whether alterations in the NMJ precede or follow the decline in muscle mass and strength remains to be clarified. Many factors, such as mitochondrial dysfunction, oxidative stress, inflammation, changes in the innervation of muscle fibers, and mechanical properties of the motor units, most likely play an important role in the degeneration of the NMJ, and in the modifications in muscle mass, promoting decreased strength with aging (GONZALES-FREIRE *et al.*, 2014).

This study had the objective of investigating whether the association of exercise with the supraphysiological use of anabolic steroids in youth prevented neuromuscular age-related alterations. Our main findings were: decreased body weight in the animals that received ND; focal lesions in myofibrils, disorganized sarcomeres, and increased central nuclei at ultrastructural level; the junctional folds of the NMJ were altered, had myelin figures in the axon terminals, and the nAChRs were organized in islands. In animals that performed exercise during youth, the normal pattern of receptors as continuous branches was maintained.

Most of the changes observed refer to sarcopenia and muscle changes present in elderly muscles and NMJ (GONZALEZ-FREIRE *et al*., 2014).

The decrease in muscle mass has been described as the pattern for aged animals, and has been related to sarcopenia (KEEVIL & ROMERO-ORTUNO, 2015), decreased physical activity (SAVINAINEN, *et al*., 2004; WOO *et al*., 2006), and decreased activity of metabolic enzymes (GONZALES-FREIRE *et al*. 2014). BINAYI *et al*. (2015), studying Wistar rats, also found weight loss in the groups using ND associated with exercise as compared to controls.

The NMJ had similar morphometry in all the groups studied, what can be

partly explained by the fact that although there are important age-related changes in the characteristics of postsynaptic components, there are differences between the types of muscles analyzed; the NMJ alterations also vary depending on the age of the animal studied (JANG *et al*., 2011).

According to DESCHENES *et al*. (2013) along the aging process, pre and postsynaptic components of the NMJ remain unchanged, even when the NMJ of muscles with different patterns of neuromuscular activity are examined, therefore, even resistance training does not seem to change any presynaptic component of the NMJ of aged animals (DESCHENES *et al*., 2015).

NISHIMUNE (2014) describes that physical exercise has a positive effect on the maintenance and regeneration of the NMJ, even in regard to age-related changes, where the molecular mechanisms involved have potential to provide therapeutic possibilities, leading to a decrease of the effects caused by several factors including age.

Under TEM, myelin figures were present in the axon terminals of the NMJ associated to the soleus muscle, in the groups that received ND. The myelin figures are entangled membranous structures, consisting of phospholipids, which may be replacing groups of dead cells (PITON *et al*., 2010).

OZAKI *et al*. (2001), studying the changes caused by diabetes associated with aging, found myelin figures in the fibers; however, the NMJ and capillaries were intact.

In all experimental groups, limiting the depth of the synaptic cleft, the postsynaptic membrane had few and ill-defined junctional folds. Similar results were found by JANGET *et al*., (2011) after reviewing studies on age-associated changes in the NMJ, demonstrating that with age the length postsynaptic folds decrease in size. BANKER *et al*. (1983) found that in nerve terminals of 30 and 34 months old mice, additional to the reduction of junctional folds, there was decreased area of nerve terminals and loss of synaptic vesicles.

DESCHENES *et al*. (2011) noted that with increase in age, the NMJ present branches in the presynaptic nerve terminals, affecting the distribution and reception of neurotransmitters in the post-synaptic sites, this remodeling been added to neurophysiological changes.

Under confocal microscopy, an island-like pattern was observed in the sedentary animals; likewise, VALDEZET *et al*. (2010) in evaluating the structural

changes of aged mice as compared to young rats using α-bungarotoxin noted that nAChRs of aged rats were often fragmented into small islands (COURTNEY 1981; BALICE-GORDON 1997; VALDEZ *et al*., 2010). VALDEZ *et al*. (2010) noted that nAChRs of aged rats were often fragmented into small islands too, when studying the structural changes of aged mice as compared to young rats using α-bungarotoxin.

FERRETI *et al*. (2011) found the same pattern of nAChR when analyzing dystrophic laryngeal muscles of aged rats, which was associated with a decrease of utrophin, protein that acts on the anchoring of nAChR in the cytoskeleton (ZHANG *et al*., 2007).

LI *et al*. (2011) using a longitudinal analysis time series, found that the main structure of the NMJ remains stable for many months but may change suddenly with remodeling/fragmentation of the postsynaptic receptors in several small nAChR aggregates. That would explain the NMJ homogeneous morphologic and morphometric pattern found in this study by non-specific esterase, although confocal microscopy showed island-like pattern of the receptors.

In the animals in this study that were exercised with or without anabolic steroids use, the pattern of receptors remained as continuous branches, therefore maintaining the normal distribution pattern of the nAChRs present in adulthood (STEINBACH, 1981; BALICE-GORDON & LICHTMAN, 1993).

The ultrastructural analysis of the muscle fibers of the SNDi animals confirmed the morphological findings of HE in relation to the central nuclei. In addition, focal lesions were present, as well as signs of discontinuation in the sarcomeres. OZAKI *et al*. (2001) found similar morphology, described as myofibrillar disorientation and increased number of central nuclei in the soleus muscle of aged rats with or without diabetes, thereby suggesting that the alterations found refer to age-related changes in the fibers.

Conclusion

We therefore conclude that the changes observed in this study were related to the age factor, and that physical exercise performed in youth maintained the structural pattern of nAChR present in young subjects; ND, on its side, did not prevent age-related morphological changes in the neuromuscular system.

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Figures and Tables

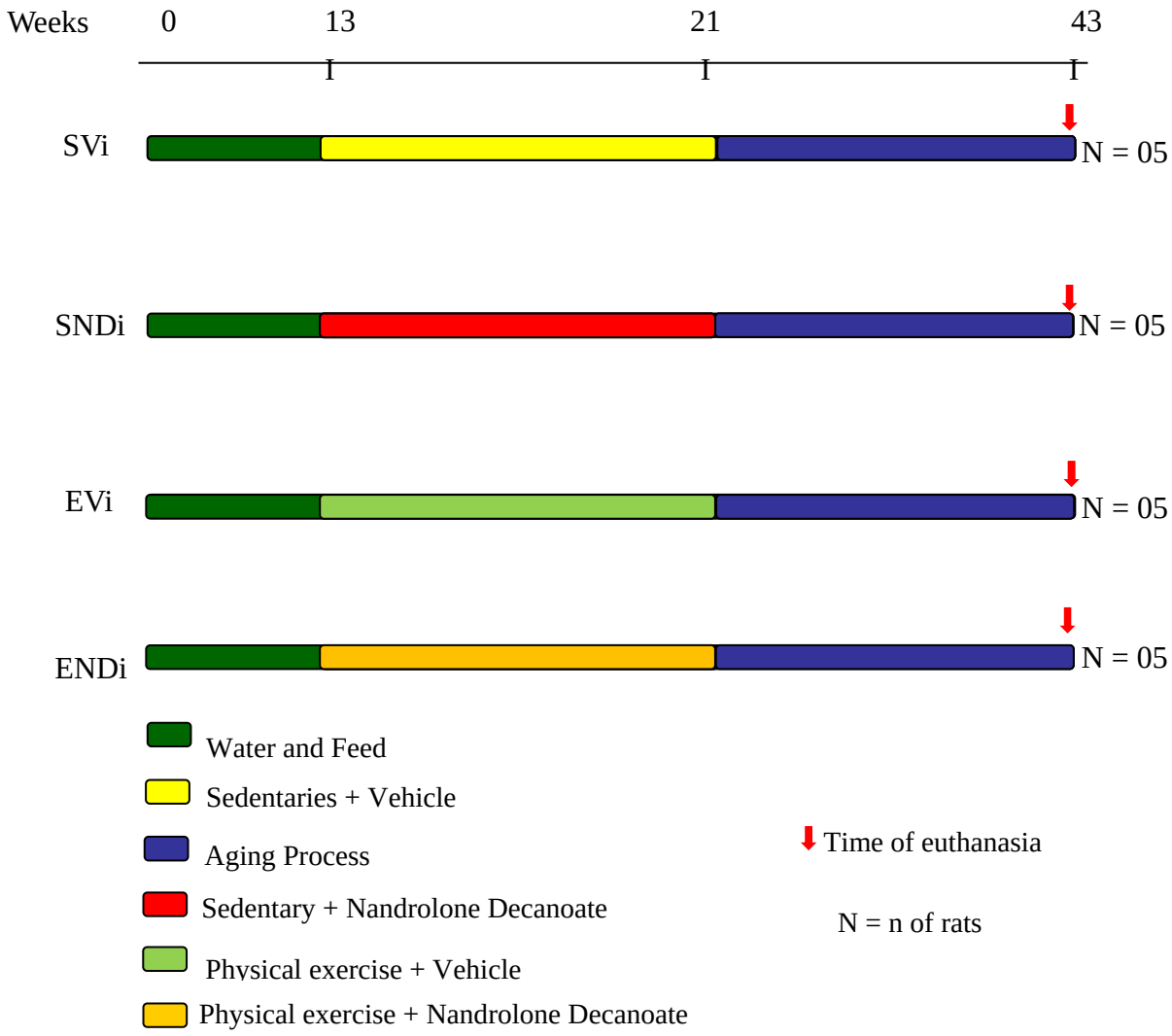

Figure 1- Representation of the experiment design where the animals are represented according to their groups: SVi: sedentary, no steroid; SNDi: sedentary, with steroid; EVi: exercise, no steroid; ENDi: exercise, with steroid.

Table 1. Means and standard deviation of body weight (g) according to group and steroid use; S- sedentary, E- exercise, No- no steroid, Yes– with steroid

		Steroids		
Variable	Group	No	Yes	p Value
Body weight	S	539.00 (24.08)	485 (34.10)	p < 0.05
	E	498.00 (38.83)	443 (30.94)	p < 0.05
p Value		p > 0.05	p > 0.05	

Statistical results obtained by scheme-factor analysis of variance, complemented with Tukey's multiple comparison test (Zar,2009).

Table 2. Means and standard deviation of weight of the soleus muscles (g) according to group and steroid use; S- sedentary, E- exercise, No- no steroid, Yes– with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
Soleus weight	S	0.249 (0.011)	0.232 (0.023)	p > 0.05
	E	0.246 (0.035)	0.219 (0.022)	p > 0.05
p Value		p > 0.05	p > 0.05	

Statistical results obtained by scheme-factor analysis of variance, complemented with Tukey's multiple comparison test (Zar, 2009).

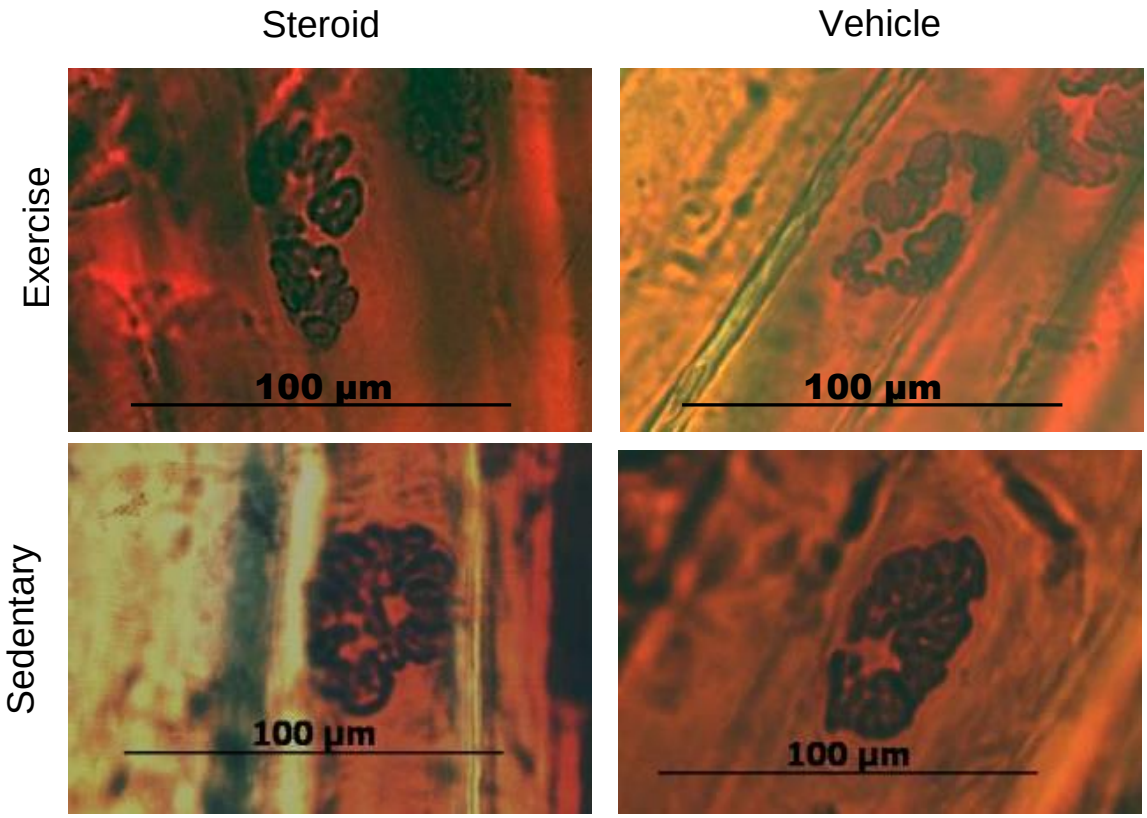

Figure 2- Light microscopy. Detail of neuromuscular junctions (NMJ) in the soleus muscle of the groups studied. Full preparation. Non-specific Esterase Reaction.

Table 3. Means and standard deviation of the maximum diameters (µm) of the soleus muscles NMJ according to group and steroid use; S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
NMJ diameter means	S	47.80 (1.92)	49.80 (2.17)	p > 0.05
	E	47.80 (3.49)	47.00 (2.12)	p > 0.05
p Value		p > 0.05	p > 0.05	

Statistical results obtained by two-factor analysis of variance, complemented with Dunn's multiple comparison test (Zar, 2009), considering significance level of 5%.* $p < 0.05$

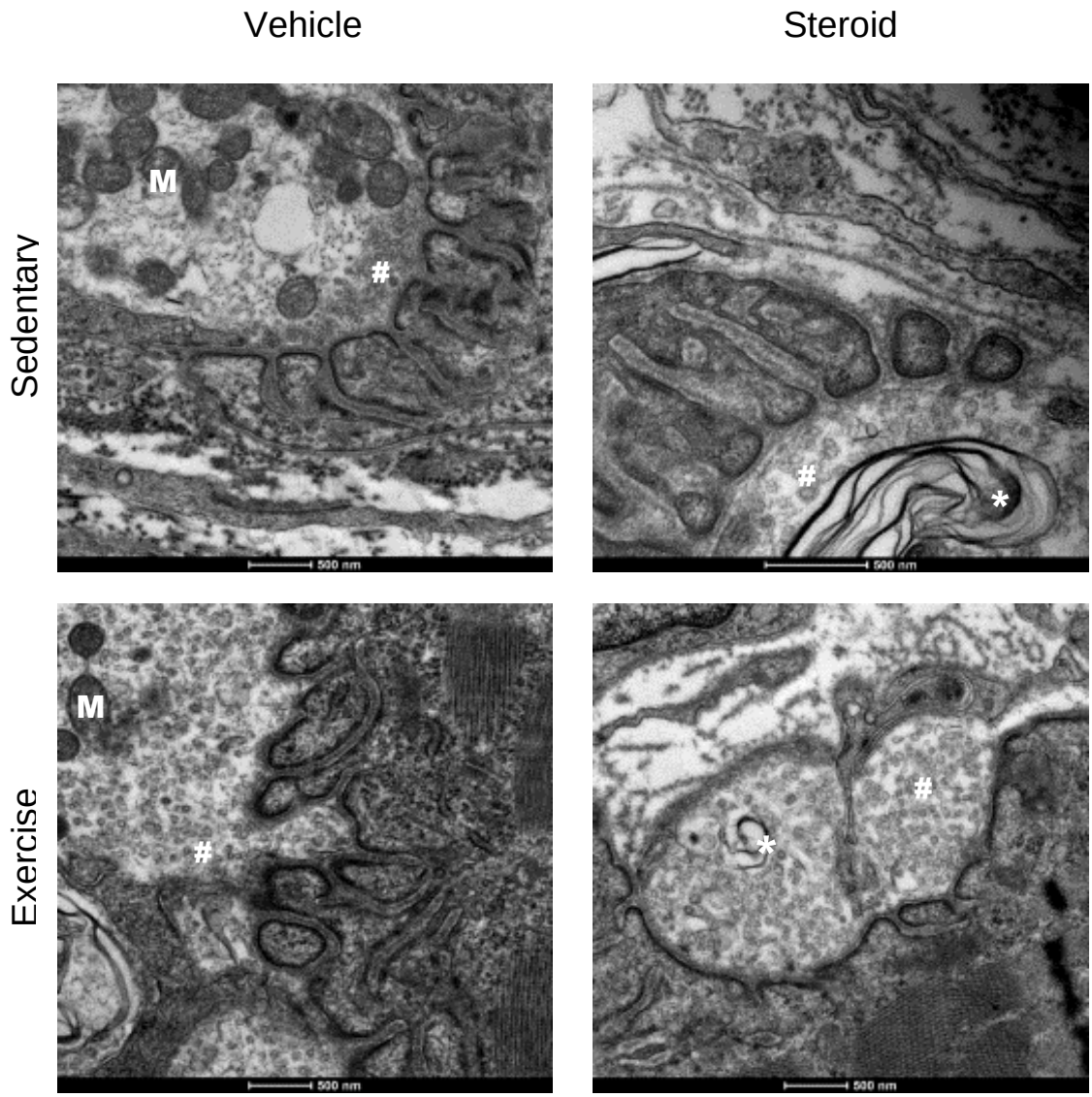

Figure 3. Transmission electron microscopy of neuromuscular junctions of soleus muscles of all the groups studied. A: SVi- sedentary animals + vehicle; B: SNDi sedentary animals treated with ND; C: EVi exercised animals + vehicle; D- ENDi exercised animals + ND. Mitochondria (**M**), Synaptic vesicles (#), Junctional folds (******), Myelin figures (*****)

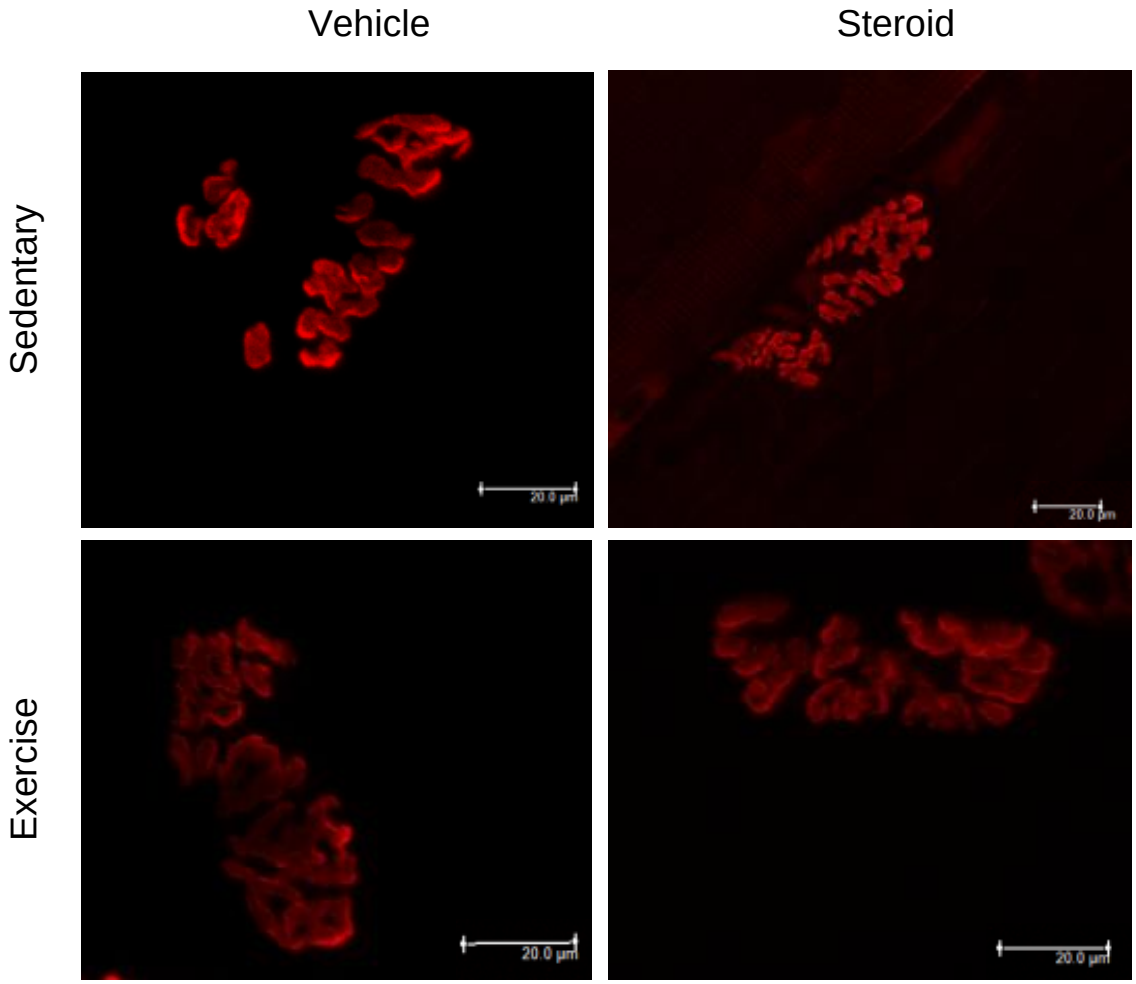

Figure 4. Confocal microscopy of the acetylcholine receptors of the soleus muscle identified by rhodamine-conjugated alpha-bungarotoxin. A: SVi- sedentary animals + vehicle; B: SNDi animals treated with ND; C: EVi exercised animals + vehicle; D- ENDI exercised animals + ND.

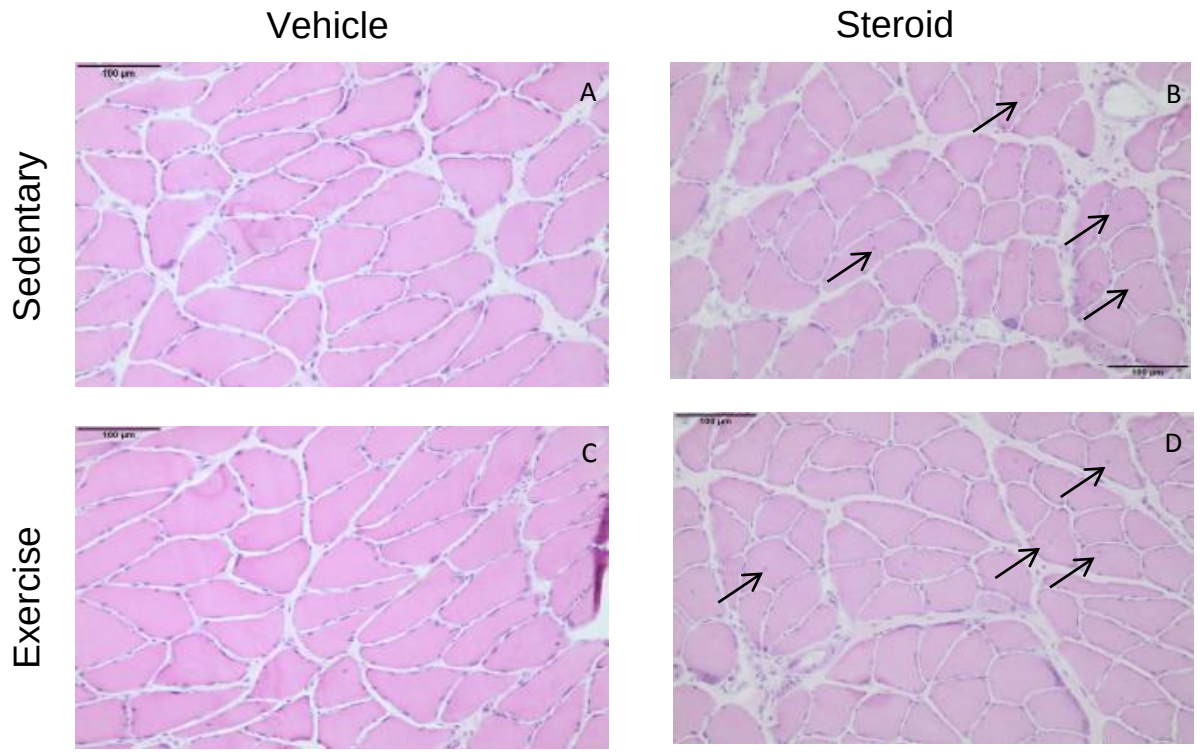

Figure 5- Photomicrographs of cross-sections of the soleus muscle of the experimental groups stained with HE. A: SVi- sedentary animals + vehicle; B: SNDi animals treated with ND; C: EVi exercised animals + vehicle; D- ENDI exercised animals + ND; (arrow) fibers with the presence of central nuclei.

Table 4. Means and standard deviation of the number of central nuclei of the soleus muscles according to group and steroid use, S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variables	Group	No	Yes	p Value
Central Nuclei	S	4 (0.34)	20.0 (12.89)	p > 0.05
	E	1(0.9)	4.0 (0.10)	p > 0.05
p Value		p > 0.05	p < 0.05	

Statistical results obtained by two-factor analysis of variance, complemented with Dunn's multiple comparison test (Zar, 2009), considering significance level of 5%.* p < 0.05.

Table 5. Means and standard deviation of the number of peripheral nuclei of the soleus muscles according to group and steroid use, S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
Peripheral Nuclei	S	200 (186.208)	186 (117.190)	p > 0.05
	E	203 (192.207)	200 (194.209)	p > 0.05
p Value		p > 0.05	p < 0.05	

Statistical results obtained by two-factor analysis of variance, complemented with Dunn's multiple comparison test (Zar, 2009), considering significance level of 5%.* p < 0.05.

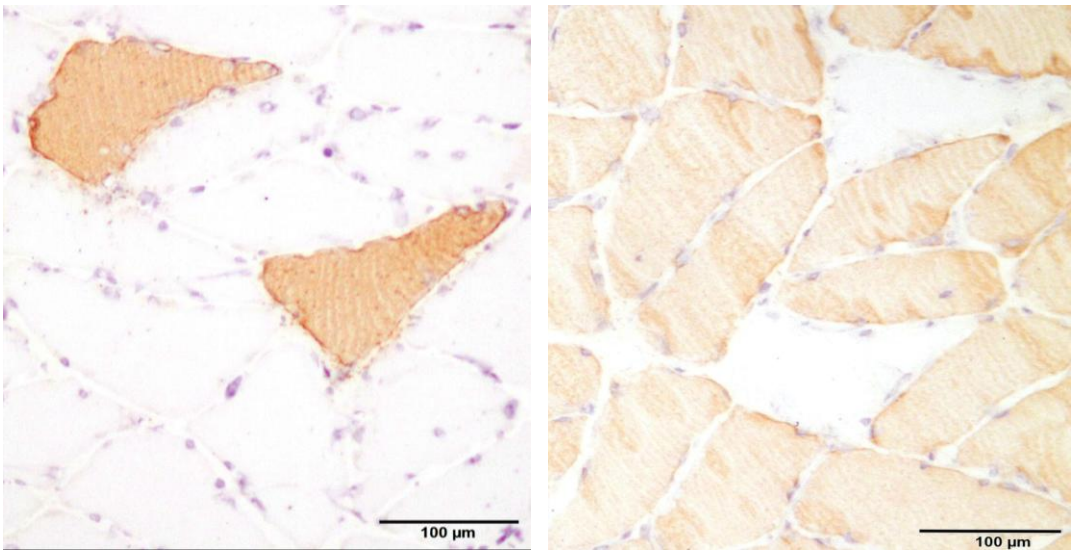

Figure 6- Histological cross-sections of soleus muscle fibers - Immunohistochemistry for fast-twitch (A) and slow-twitch (B) fibers, respectively.

Table 6. Means and standard deviation of the number of slow-twitch fibers of the soleus muscles, according to the groups and use of steroid; S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

Steroids				
Varible	Group	No	Yes	p Value
Slow-twitch fibers	S	192 (187;194)	175 (145;184)	p > 0.05
	E	165 (144;198)	159 (135;194)	p > 0.05
p Value		p > 0.05	p > 0.05	

Statistical results obtained by two-factor analysis of non parametric variance, complemented with Dunn's multiple comparison test (Zar, 2009), considering significance level of 5%.* p < 0.05.

Table 7. Means and standard deviation of the number of fast-twitch fibers of the soleus muscle, according to the groups and use of steroid; S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

		Steroids		
Variable	Group	No	Yes	p Value
Fast-twitch fibers	S	12 (7;16)	30 (16;60)	p > 0.05
	E	35 (5;58)	45 (5;67)	p > 0.05
p Value		p > 0.05	p > 0.05	

Statistical results obtained by two-factor analysis of non parametric variance, complemented with Dunn's multiple comparison test (Zar, 2009), considering significance level of 5%.* p < 0.05.

Table 8. Means and standard deviation of the area (μm²) of fast and slow-twitch fibers, according to the groups and steroid use; S- sedentary, E- exercise, No- no steroid, Yes- with steroid.

Steroids				
Variable	Group	No	Yes	p Value
Area Slow-twitch fibers	S	3,501.10 (667.88)	4,215.65 (1,051.68)	p > 0.05
	E	3,848.95 (859.43)	3,710.78 (603.80)	p > 0.05
p Value		p > 0.05	p > 0.05	
Area Fast-twitch fibers	S	3,019.45 (609.44)	3,533.68 (1,050.13)	p > 0.05
	E	3,245.55 (971.87)	2,863.90 (526.24)	p>0,05
p Value		p > 0.05	p > 0.05	

Statistical results obtained by two-factor analysis of non parametric variance, complemented with Dunn's multiple comparison test (Zar, 2009), considering significance level of 5%.* p < 0.05.

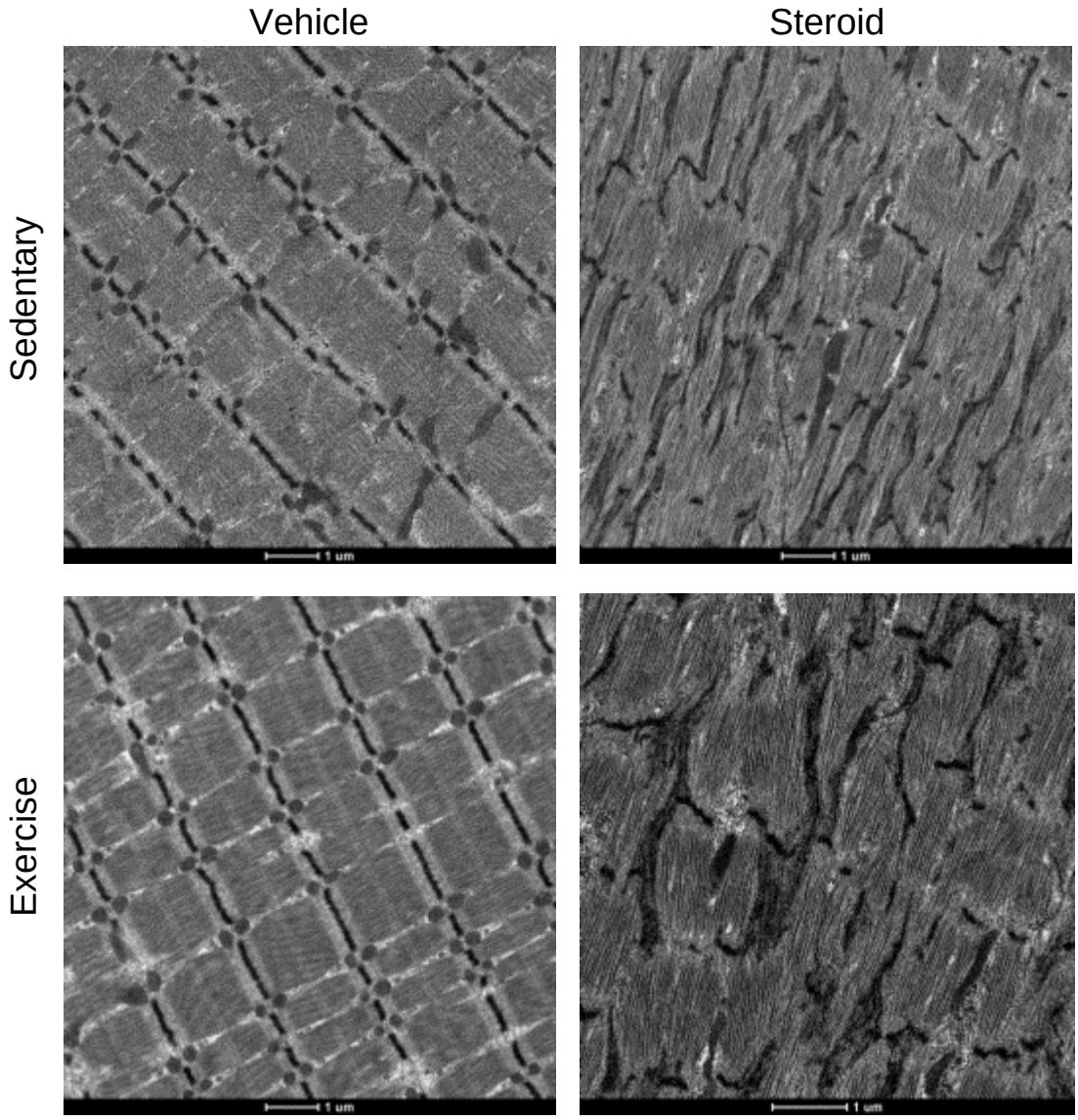

Figure 7. Transmission electron microscopy of the muscle fibers of the soleus muscles of all the groups. A: SVi- sedentary animals + vehicle; B: SNDi animals treated with ND; C: EVi exercised animals + vehicle; D- ENDI exercised animals + ND; sarcomeres disorganized (**).

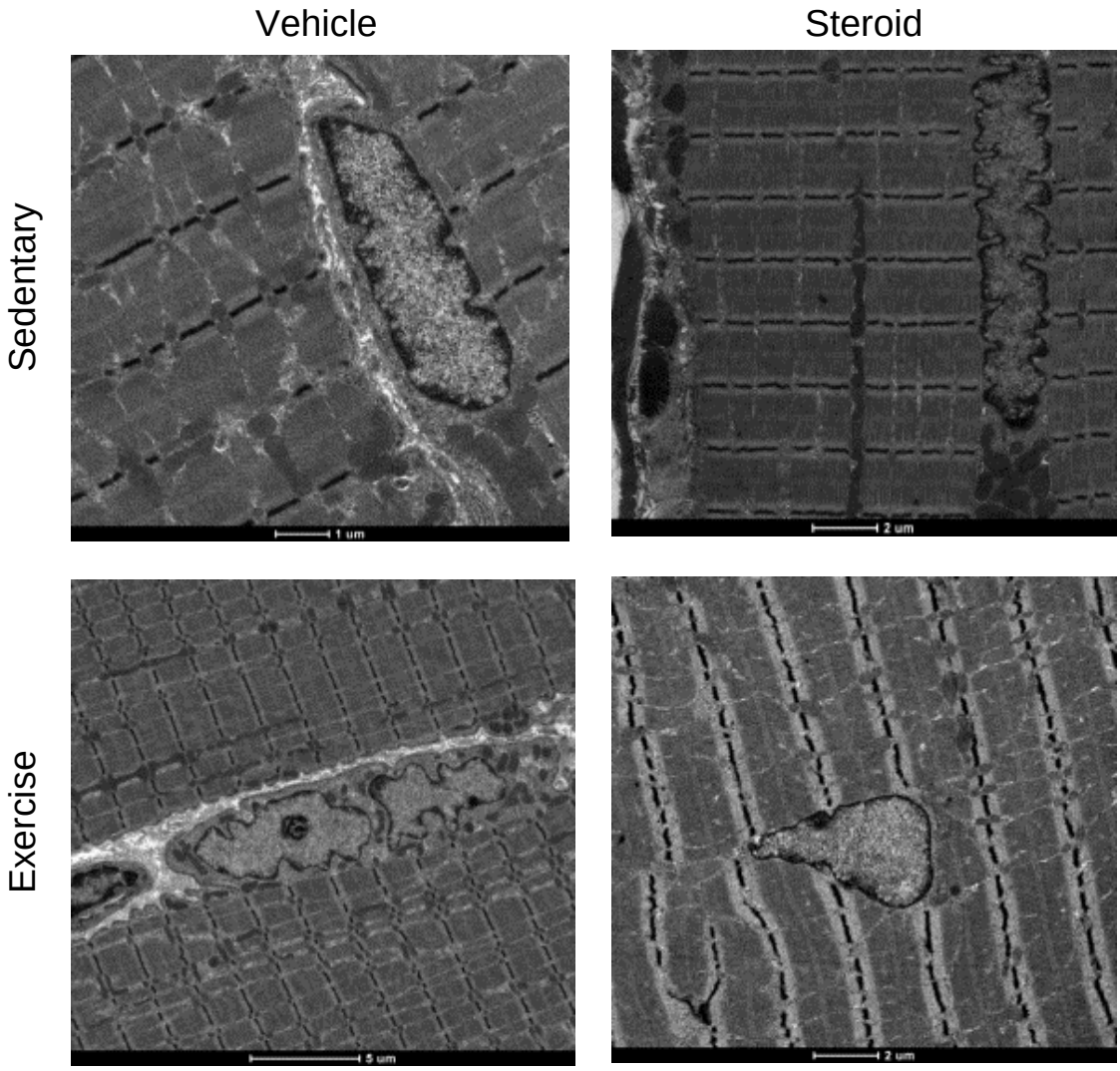

Figure 8. Transmission electron microscopy of the muscle fibers of all groups studied; A: SVi- sedentary animals + vehicle; B: SNDi animals treated with ND; C: EVi exercised animals + vehicle; D- ENDI exercised animals + ND. Central nuclei (*); Peripheral nuclei (**) of the soleus muscles.

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

Certificado

Certificamos que o Protocolo nº **448-CEUA**, sobre "Efeitos do uso de Decanoato de Nandrolona sobre a junção neuromuscular de ratos em processo de envelhecimento submetidos ao exercício resistido", sob a responsabilidade de **Selma Maria Michelin Matheus**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 30 de novembro de 2012.

Profª Drª Patrícia Fernanda Felipe Pinheiro
Presidente da CEUA

Instituto de Biociências - Diretoria Técnico Acadêmica
Distrito de Rubião Júnior s/n CEP 18618-970 Botucatu SP Brasil
Tel 14 3811 6013/6014 Fax 14 3815 3744 e-mail: dta@ibb.unesp.br