Karina Fontana

"Influência do Esteróide Anabólico - Androgênico Mesterolona em Camundongos Transgênicos Sedentários ou Exercitados"

Campinas

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Doutor em Farmacologia.

Orientadora: Prof. Dr. Maria Alice da Cruz-Höfling

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Quem estuda e não pratica o que aprendeu é como o homem que lavra e não semeia. (Provérbio árabe)

Sumário

Lista de Abreviaturas

AMP	Adenosina Monofosfato
Apo AII	Ácido Ribonucléico
Apo B	Adenosina Trifosfato
Apo I	Animal Transgênico
ATP	Apolipoproteína AI
CETP	Apolipoproteína AII
CETP/R1	Apolipoproteína B
cNOS	Atividade da Lecitina Colesterol Aciltransferase
EAA	Atividade da Lipoproteína Lípase
eNOS	Cadeia Leve de Miosina
FSH	Cadeia Pesada de Miosina
HDL	Esteróide Anabólico Androgênico
HIV	Folículo Estimulante
LCAT	Gonadotrofinas Hipofisárias Luteinizantes
LDL	Isoforma Constitutiva
LH	Lipoproteína
LPL	Lipoproteína de Alta Densidade
LPL	Lipoproteína de Baixa Densidade
MHC	Óxido Nítrico
MLC	Óxido Nítrico Sintase
nNOS	Óxido Nítrico Sintase Endotelial
NO	Óxido Nítrico Sintase Neuronal
NOS	Proteína de Transferência de Ester de Colesteril
RNA	Triglicérides
TG	Vírus da Imunodeficiência Adquirida

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Resumo

Resumo

O uso abusivo de esteróides anabólico androgênicos para melhorar a performance e aparência física tem sido associado a sérios efeitos adversos, alguns dos quais fatais, entretanto o seu uso controlado terapeuticamente pode trazer benefícios. O presente trabalho teve por objetivo avaliar no músculo sóleo (SOL), tibial anterior (TA) e gastrocnêmio (GAS), no músculo cardíaco e nos tendões, o efeito do uso oral do esteróide anabólico androgênico mesterolona (M, 17 beta-hydroxy-1 alpha methyl-5 alpha-androstan-3-one, $C_{20}H_{32}O_2$) em camundongos sedentários e submetidos a um intenso programa aeróbico de corrida em esteira, usando um modelo murino transgênico ($CETP^{+/-}LDLr^{-/+}$). Grupos experimentais de camundongos machos (8 semanas no início dos experimentos) sedentários e exercitados (6 semanas de corrida involuntária em esteira, média de 16 m/min, 50 min/dia, 5 dias/semana) foram tratados com mesterolona dissolvida em goma arábica (veículo), ou com o veículo (ambos na concentração de 2 µg/g corpóreo: dose supra-fisiológica) nas últimas 3 semanas. Os animais eram semanalmente pesados e a pressão arterial aferida através de coxim caudal com pletismógrafo e os seus músculos foram pesados no momento do sacrifício. Foram delineados os tipos de fibras musculares com base na reação de m-ATPase, bem como a ação miotrófica da mesterolona, do exercício ou de ambos combinados nos diferentes tipos de fibras musculares. Ao microscópio de luz e eletrônico de transmissão foram avaliados no SOL, o número de miofibras, a área seccional transversa das miofibras e das mitocôndrias, a densidade mitocondrial/área de miofibra, o número de capilares/miofibra, a presença de células satélites ativadas, de "split fibers" (fibras fendidas) e a morfologia dos fusos musculares, bem como possíveis alterações da ultraestrutura em geral. A

expressão da isoformas de óxido nítrico sintase neuronal (NOS I) e endotelial (NOS III) foi investigada através de imunohistoquímica e immunoblotting. Como parte, do sistema músculo esquelético, foi avaliada a resposta do tendão de Aquiles em termos de síntese de colágeno, concentração de hidroxiprolina, diâmetro e área (relativa) das fibras colágenas e aspecto ultraestrutural dos fibroblastos tendinosos (tenócitos). Finalmente, a investigação estendeu-se ao músculo cardíaco e métodos quantitativos, morfológicos e bioquímicos foram empregados para a avaliação da plasticidade cardíaca e do perfil lipídico desse modelo murino transgênico, o qual foi geneticamente modificado para estar mais próximo ao perfil lipídico humano.

- Os resultados mostraram que os músculos soleo, tibial anterior e gastrocnêmio respondem diferentemente ao tratamento com mesterolona, ao exercício aeróbico ou a ambos associados. Igualmente, o mesmo se dá em relação à fibra tipo I ou aos vários tipos de fibras II, ou seja, dependendo do músculo em que estão inseridas, há variabilidade na sua resposta, isto é, elas podem ser recrutadas, não serem recrutadas, ou serem recrutadas diferencialmente. O mesmo pode ser dito em relação à sofrerem ou não hipertrofia e em que proporção ela ocorre. Igualmente, a expressão das óxido nítrico sintases, NOS I e NOS III, sofre modulação diferente dependendo do tratamento e músculo analisado. A associação do tratamento com mesterolona ao exercício físico intenso pode gerar efeitos sinérgicos ou antagônicos.
- Os resultados mostraram que a mesterolona, o exercício físico e a combinação de ambos foram capazes de aumentar o conteúdo de hidroxiprolina, o diâmetro e área das fibrilas colágenas (contidas em uma área teste) do tendão e ao microscópio

eletrônico os fibroblastos mostram-se hipertrofiados, com muitos prolongamentos e grande desenvolvimento da maquinaria sintética.

- Os resultados mostraram o indesejável remodelamento e dano cardíaco, a indução de perfil pró-aterogênico nos animais tratados com mesterolona, e que o exercício neutraliza alguns desses efeitos.

Abstract

Abstract

The abuse of anabolic-androgenic steroids (AAS) to improve physical performance is associated with serious adverse effects sometimes fatal, however benefits can be gain by its therapeutic controlled use. The present study aimed to evaluate the effects of the use of the anabolic androgenic steroid mesterolone (M, 17 beta-hydroxy-1 alpha methyl-5 alpha-androstan-3-one, $C_{20}H_{32}O_2$) in the *soleus* (SOL), *tibialis* anterior (TA) and *gastrocnemius* (GAS), in the Achilles tendon and in the cardiac muscle remodeling and lipemic profile of transgenic mice ($CETP^{+/-}LDLr^{-/+}$), sedentary or submitted to a treadmill running aerobic exercise program. Experimental groups of male mice (aged 8 weeks at the beginning of the experiments) sedentary or exercised (6 weeks involuntary treadmill running, average of 16 m/min, 50 min/day, 5 days/week) were treated with mesterolone dissolved in gum arabic (vehicle), or with the vehicle (both at 2 µg/g body weight administered orally – supra physiological dose) in the last three weeks. The animals were weighed every week and the muscles were weighed after sacrifice of the animals. The arterial blood pressure was measured by tail-cuff plethismographys weekly. The delineation of the fibers types was done in the SOL, TA and GAS muscles through the m-ATPase reaction. The myotrophic action of mesterolone, exercise, or both combined, were also determined in relation to fiber types. The number of myofibers, the cross-sectional area of the myofibers, and mitochondria, the density of mitochondria/area of myofiber, the number of capillaries/myofiber, the presence of activated satellite cells, split fibers and the morphology of the muscle spindles and of muscle tissue in general were evaluated by light and electron microscopy. The expression of the neuronal (NOS I) and endothelial (NOS III) nitric oxide synthase isoforms was investigated through

immunohistochemistry and immmunoblotting. As part of the musculoskeletal system, the Achilles tendon response was evaluated in regard to the collagen synthesis, quantification of hydroxyproline, collagen fibers diameter and area (relative) and ultrastructure of the tendon fibroblasts (tenocytes). Finally, the investigation focused on the cardiac muscle remodeling and quantitative, morphological and biochemical approaches were employed for evaluating the cardiac plasticity and the lipid profile from this transgenic murine model whose lipid profile was transgenically modified to be more close to the human lipid profile.

- The results showed that the *soleus*, *tibialis* anterior and *gastrocnemius* muscles respond differentially to the mesterolone treatment, to the aerobic exercise or both in association. Seemingly, fiber type I and the several types of II fibers showed variability. Depending on the muscle to which they belong to they can be recruited, not be recruited or be recruited differentially. Likewise, different degrees of hypertrophy, or absence of hypertrophy can be displayed by a given fiber type depending on the muscle. Similarly, the expression of the nitric oxide synthases, NOS I and NOS III, can be modulated differentially depending on the treatment and muscle analyzed. The association of mesterolone treatment and intense physical exercise can generate synergic or antagonistic effects.

- The results showed that mesterolone, physical exercise or both combined were able to increase the hydroxyproline content, the diameter and area of tendon collagen fibrils (contained in an area probe), and to show evidences of increased synthesis of proteins by the fibroblasts, which besides showed several long and slender processes in

agreement with characteristics of hypertrophy and enhancement of the synthetic machinery.

- The results showed that mesterolone alone induced a pro-atherogenic profile and a pathogenic cardiac hypertrophy. The exercise counteracted these effects and modified favorably both the lipoprotein profile and the cardiac remodeling.

Introdução

1. INTRODUÇÃO

1.1. Esteróides Anabólico-Androgênicos (EAA)

Os esteróides anabólico-androgênicos (EAA) pertencem a um grupo de compostos naturais ou sintéticos formados pela testosterona e seus derivados (Thein et al., 1995). Os hormônios esteróides são produzidos pelo córtex da supra-renal e pelas gônadas (ovários e testículos). Os EAA são derivados do hormônio sexual masculino, a testosterona, a qual é promotora e mantenedora das características sexuais e do "status" anabólico dos tecidos somáticos (Handelsman, 2001). Os EAA podem ser naturais e sintéticos. Os últimos foram aperfeiçoados de forma a aumentar a atividade anabólica e minimizar o mais possível a indesejável atividade androgênica - caracterizada pela virilização refletida no espessamento das cordas vocais, aumento da libido, da secreção nas glândulas sebáceas, de pelos no corpo e da face (Hoberman & Yesalis, 1995; Bahrke & Yesalis, 2004) - como forma de driblar a evidência do "doping" no meio esportivo.

Os hormônios sexuais e os EAA agem em um único receptor que modula de forma indissociável os efeitos androgênicos e anabólicos (Thein, 1995; Ghaphery, 1995; Lukas 1996). Dentre os efeitos anabólicos, destacam-se o aumento da massa muscular, da concentração da hemoglobina, do hematócrito, da deposição de cálcio nos ossos e diminuição das reservas de gordura do corpo. Os EAA aumentam a retenção de nitrogênio, promovendo o crescimento e desenvolvimento de massa muscular através da uma melhor utilização da proteína ingerida. Dentre os mecanismos anabólicos desencadeados para aumento da massa muscular, incluem-se: aumento da síntese protéica, balanço nitrogenado positivo, inibição dos efeitos catabólicos na massa muscular esquelética, estimulação da osteogênese e da eritropoiese.

Nos esportes, o uso dos EAA vem sendo difundindo com o fim de aumentar a massa muscular, a força física, a agressividade e o desempenho em competições esportivas. Usuários de academias utilizam drogas anabólico- androgênicas para fins estéticos, concursos de fisiculturismo e retardo das conseqüências do envelhecimento.

1.2. Fisiologia dos Esteróides Anabólico-Androgênicos

Nos mamíferos, a secreção de testosterona se dá por pulsos, ou seja, é regulada por retroalimentação negativa. Assim, quando há deficiência de testosterona circulante, ocorre estímulo do hipotálamo que, através da secreção de hormônio liberador de gonadotrofinas, estimula a glândula pituitária a liberar LH e FSH, aumentando a síntese de testosterona (ver Wilson, 1996; Timon et al., 2007).

Em competições, após períodos de estresse agudo há aumento significativo da testosterona plasmática, entretanto este aumento não traz implicações para os testes de urina antidoping (Guezennec et al., 1995).

A testosterona dá origem a duas classes de esteróides: andrógenos 5-α-redutase (dihidrotestosterona), que são mediadores intracelulares da maioria das ações androgênicas, e estrógenos (estradiol), que potencializam alguns efeitos androgênicos, enquanto bloqueiam outros. A testosterona é convertida em vários outros metabólitos ativos como estradiol, androsterona, 3-α-hidroxi-5-β-androsta-17-ona e androstenediona (Wilson, 1996).

As substâncias ativas, inclusive metabólitos reduzidos (5-α-redutase) atravessam a membrana celular e ligam-se com alta especificidade e baixa afinidade a receptores citoplasmáticos para esteróides. O complexo droga-receptor é translocado para o núcleo e liga-se a regiões da cromatina, induzindo a transcrição do RNA e a produção de proteínas específicas e ocasionando seus efeitos anabólicos (Russel & Wilson, 1994; Wilson, 1996; Bahrke & Yesalis, 2004; Friedel et al., 2006).

Geralmente, os EAA são usados por via oral ou parenteral. Entretanto, com o objetivo de evitar o metabolismo no fígado e a detecção pelo doping, os EAA podem ser administrados por via retal, implante de cápsulas, nasal, e transdérmica, (Bahrke & Yesalis, 2002, 2004). Além disso, a indústria farmacêutica também foi capaz de modificar a estrutura molecular da testosterona visando minimizar ou excluir o metabolismo hepático, originando três grupos de EAA derivados da testosterona (Thein et al., 1995):

- ésteres do grupo 17-β-hidroxil
- alquilados na posição 17-α
- com o anel esteróide alterado

A alquilação e a alteração do anel esteróide são utéis nas preparações via oral (etinilestradiol, fluoximeterona, metandrostenolona, oximetolona, metiltestosterona, stanozolol e mesterolona). A alquilação na posição 17-α retarda a metabolização hepática, favorecendo a efetividade oral, porém são mais tóxicos ao fígado (Lukas, 1993).

A esterificação é útil nas proporções parenterais (cipionato ou proprionato de testosterona, nandrolona). A esterificação do grupo 17-β-hidroxil favorece as preparações injetáveis com liberação lenta do esteróide na circulação e com menor toxicidade hepática que os orais.

Figura 1: Esquema da estrutura química da Mesterolona (Fonte: Ho et al., 2007).

1.3. Mesterolona (Proviron® - Schering Brasil)

Informações vide bula.

A Mesterolona compensa o déficit na formação de androgênio, a qual se reduz gradativamente com a idade. Desta forma, é possível tratar todos os estados de insuficiência provenientes de produção reduzida ou mesmo nula de androgênios endógenos. Na dose terapêutica recomendada, a mesterolona não prejudica a espermatogênese e é considerada satisfatória a tolerabilidade hepática. Após administração oral, a droga, na dose de 25 a 100 mg é rapidamente absorvida. O nível sérico máximo de 3,1 ± 1,1 ng/ml é alcançado em 1,6 ± 0,6 horas após a ingestão da droga. Posteriormente, o nível sérico diminui com meia vida de 12 a 13 horas. Aproximadamente 98% da droga liga-se às proteínas plasmáticas, sendo que 40% liga-se à

albumina e 58% às globulinas transportadoras de hormônios sexuais. É rapidamente metabolizada e sua taxa de depuração metabólica média é de 4,4 ± 1,6 $ml.min^{-1}.kg^{-1}$. A mesterolona não é excretada por via renal na forma inalterada, seu principal metabólito é a 1α-metil-androsterona, que na forma conjugada corresponde a 55-70% dos metabólitos excretados na urina. O metabólito principal conjuga-se com o glicuronato na razão aproximada de 12:1 em relação à sua conjugação com o sulfato. Outro metabólito identificado é o 1 α-metil-5 α- androstano-3 α, 17 β-diol, o qual corresponde a aproximadamente 3% dos metabólitos eliminados por via renal. Não foi observada conversão metabólica em estrogênios ou corticosteróides. Aproximadamente 80% são excretadas na forma de metabólitos na urina e 13% nas fezes. No intervalo de 7 dias, 93% da dose administrada são eliminados, sendo que metade desta é eliminada na urina dentro de 24 horas.

-Indicações: diminuição da capacidade física e mental em pacientes de meia-idade ou de idade avançada, distúrbios resultantes de deficiência androgênica, tais como redução de eficiência, fatigabilidade, diminuição da capacidade de concentração e memorização, irritabilidade, distúrbios do sono, estados depressivos, hipogonadismo e oligospermia.

- Contra-indicação: carcinoma de próstata e tumor hepático atual.

- Precauções: os androgênios não são adequados para estimular o desenvolvimento muscular em indivíduos sadios ou para aumentar a capacidade física. Quando indicado, seu uso é restrito ao sexo masculino, e em tratamentos prolongados, devem ser realizados exames periódicos da próstata com finalidade profilática. Em casos raros, podem surgir, desenvolvimento de tumor hepático benigno e hemorragia intra-abdominal.

- Reações adversas: em casos isolados ocorrem ereções freqüentes ou persistentes.

- Posologia: 1 comprimido (25 mg), 3 vezes ao dia.

1.4. Indicações Terapêuticas dos EAA

As indicações médicas aprovadas para o uso dos EAA relacionam-se ao ganho de peso por portadores de HIV (Vermeulen, 2001; Cuerda, 2005), diminuição da dor óssea na osteoporose, catabolismo induzido por corticosteróides, anemia grave, angioedema hereditário, câncer de mama ou deficiência hormonal masculina (Lukas, 1993; Ghaphery, 1995).

Os EAA são indicados em indivíduos do sexo masculino com deficiência androgênica para o desenvolvimento e manutenção das características sexuais secundárias masculinas (Matsumoto,

1996), em crianças com retardo de puberdade e adultos com insuficiência testicular (Thein, 1995; Lukas, 1996; Matsumoto, 1996; Miner, et al., 2007). Quando há necessidade de reposição androgênica, as preparações parenterais são as mais efetivas. Também podem ser utilizados sob forma de filmes de testosterona aplicados sobre a pele do escroto, permitindo a manutenção da concentração plasmática em níveis normais (Wilson, 1996).

Os EAA também possuem o papel de aumentar a eritropoiese, podendo ser utilizados nos tratamentos de anemias refratárias Além disso, os EAA também são utilizados no tratamento de anemia por falência de medula óssea, insuficiência renal e anemia aplástica (Wilson, 1996; Matsumoto, 1996).

1.5. Efeitos Adversos dos EAA

Vários são os efeitos adversos dos EAA, tais como: acne severa, retenção de líquidos, dores articulares, aumento da pressão arterial sanguínea, diminuição dos níveis de HDL-colesterol, favorecimento do aparecimento de doenças cardiovasculares, tumores no fígado, entre outras (Quadro 1). Nos homens podemos encontrar testículos com tamanho reduzidos, redução do número de espermatozóides, impotência sexual, infertilidade, calvície, ginecomastia (Pope, 1994), dificuldade ou dor para urinar como conseqüência do aumento da próstata (Wilson, 1996, Goldberg et al. 1996). Por outro lado, os efeitos adversos nas mulheres consistem do crescimento de pelos faciais, alterações ou ausência de ciclo menstrual, aumento do clítoris, engrossamento da voz, diminuição de seios (Lukas, 1996). Maturação esquelética prematura, puberdade acelerada e consequente crescimento raquítico são alguns dos efeitos adversos encontrados nos adolescentes (Matsumoto, 1996). Além disso, os efeitos dos EAA sobre o comportamento dos usuários também têm sido amplamente pesquisados. Estas alterações apontam atos de agressividade, irritabilidade, raiva e outros sintomas cognitivos como distração, esquecimento, confusão mental, e outros (Cowart, 1987; Bahrke & Yesalis, 2004).

Quadro 1 - Efeitos adversos dos EAA – Fonte: Lise et al., 1999.

Cardiovascular/Hematológico

* Aumento do colesterol total
* Diminuição do colesterol HDL
* Aumento do colesterol LDL
* Hipertensão (retenção de sódio e água)
* Aumento da agregação plaquetária
* Aumento das proteínas de coagulação facilitando a possibilidade de trombose
* Infarto miocárdico
* Hipertrofia de ventrículo esquerdo
* Acidente cerebrovascular

Hepático

* Lesão hepática
* Testes de função hepática alterados
* Icterícia colestática
* Carcinoma hepatocelular
* Hepatoma, adenoma hepático
* Hepatite
* Sangramento de varizes por hipertrofia porta secundária à hiperplasia nodular regenerativa

Psicológicos

* Comportamento agressivo
* Aumento/diminuição da libido
* Flutuações repentinas do humor
* Dependência
* Psicose
* Episódios maníacos e/ou depressivos
* Tentativa de suicídio
* Depressão quando da retirada
* Ansiedade
* Euforia

Dermatológico

* Acne
* Alopecia

Subjetivo

* Edema
* Espasmos musculares
* Aumento do débito urinário
* Uretrite
* Dor escrotal
* Cefaléia
* Tontura
* Náusea

Miscelânea

* Transmissão de HIV

Renal

* Elevação da creatinina
* Tumor de Wilms

Endócrino/reprodutivo (Homens)

* Menor produção de hormônios
* Atrofia testicular
* Oligo/azoospermia
* Ginecomastia
* Hipertrofia prostática
* Carcinoma prostático
* Priapismo
* Alteração do perfil tireoídeo
* Impotência
* Acne

1.6. Tipos de Fibras Musculares

Os vários músculos esqueléticos, embora possuam fibras cujas proteínas contráteis se organizam de forma idêntica e repetitiva dentro das várias miofibrilas e geram em cadeia várias unidades morfo-funcionais, os sarcômeros, eles não são iguais quanto às suas características metabólicas e contráteis. Nos sarcômeros predominam dois filamentos protéicos: um filamento fino, formado pelo complexo troponina, tropomiosina e actina, e um filamento grosso, formado pela miosina.

A miosina pode ser clivada em dois fragmentos: miosina de cadeia leve (myosin light chain, MLC) e miosina da cadeia pesada (myosin heavy chain, MHC), sendo que o segmento mais curto, globular é dotado de atividade ATPásica (Vemuri et al., 1999; Wahrmann et al., 2001). A MHC entra em contato com o sítio ativo da actina, hidrolisa as moléculas de ATP promovendo o deslizamento da actina, gerando o encurtamento dos sarcômeros e imediatamente culmina na contração das fibras musculares. As MHCs são formadas por isoformas de proteínas que hidrolisam o ATP em diferentes velocidades de contração, isto é, fibras de contração lenta e rápida. As fibras de contração lenta expressam MHCI, enquanto as MHCIIa, IId/x e IIb estão presentes nas fibras de contração rápida (Schiaffino & Reggiani, 1994; Pette & Staron, 1997; Staron et al., 1999). Em roedores encontram-se as isoformas de miosona I, IIa, IId/x e IIb, já em humanos são encontradas as miosinas I, IIa e IIb. As isoformas IIb de humanos tendem a ser classificadas como IId/x, por estas apresentarem velocidade de contração semelhante à dos roedores (Bottinelli & Reggiani, 2000; Williamson et al., 2001; Parcel et al., 2003).

Além das fibras puras (I, IIA, IID/X, IIB) existem fibras que são formadas por duas ou mais isoformas de miosina, sendo classificadas como fibras híbridas (IC = MHCI > MHCIIa, IIC = MHCIIa = MHCI, IIAC = MHCIIa > MHCI , IIAD = MHCIIa > MHCIId, IIDB = MHCIId > MHCIIb) (Pette & Staron, 2000). Desta forma, podemos delinear 10 tipos de fibras musculares em ratos: I, IC, IIC, IIAC, IIA, IIAD, IIDA, IID, IIDB e IIB (Pette & Staron, 2000), já em humanos são encontrados 7 tipos de fibras: I, IC, IIC, IIAC, IIA, IIAB, IIB (Pette & Staron, 2000; Fry, 2004).

Esta diversidade de isoformas de miosina é responsável pela diversidade de tipos de fibras musculares quanto à sua atividade contrátil/metabólica. É também responsável pela plasticidade do músculo permitindo-lhe adaptações no sistema miofibrilar para ajustarem-se continuamente as

necessidades funcionais. É também devido à diversidade e possibilidade de combinação entre as isoformas que o músculo é um dos tecidos que mais responde a estímulos tanto exógenos como endógenos. Essa capacidade do músculo de adaptar-se a um determinado estímulo é o que se denomina plasticidade muscular, é o que ocorre, por exemplo, na resposta do músculo esquelético ao exercício físico (Goldspink, 1998; Pette & Staron, 2001; Williamson et al., 2001; Parcel et al., 2003).

O exercício físico pode promover a transição das fibras tanto no sentido de lento para rápido (I ≥ IIA ≥ IID ≥ IIB), quanto de rápido para lento (IIB ≥ IID ≥ IIA ≥ I). Essas transições se dão através da modulação da intensidade e duração do treinamento (Bompa & Cornacchia, 1998; Andersen et al., 2000). A aplicação de um treinamento físico de baixa intensidade e longa duração pode induzir a conversão de fibras rápidas em lentas, por outro lado, um treinamento de alta intensidade e curta duração pode converter fibras lentas em rápidas (Caiozzo et al., 2000; Campos et al., 2002).

Além do exercício físico, o envelhecimento é outro fator relacionado a alterações no sistema muscular esquelético, normalmente, acompanhado por pronunciada perda da massa muscular, diminuição da força e aumento da fatigabilidade dos músculos (Forbes & Reina, 1970; Dutta & Handley, 1995; Balagopal et al., 1997; Morley et al., 2001).

Estudos descrevem que a administração de EAA em atletas pode aumentar a força e a massa muscular, contribuindo para aumento da massa magra (Hartgens & Kuipers, 2004; Miner et al., 2007). Outros estudos comprovam que a testosterona aumenta a massa muscular (Bardin, 1996; Sinha-Hikim et al., 2003; Miner, et al., 2007), a força muscular (Arnold et al., 1996; Bhasin et al., 1996; Ternover, 1997; Urban, 1999) e a performance muscular, o que tem levado atletas de elite a utilizarem-na em regimes de treinamento físico (Hoberman & Yesalis, 1995). Alguns autores relatam ação da testosterona sobre o músculo levantador do ânus, um músculo altamente responsivo ao uso da testosterona (Bass et al., 1969; Gutmann et al., 1970; Boissonneault et al., 1990; D' albis et al., 1993; Sidor & Blackburn, 1998; Nnodim, 2001; Friedel et al., 2006). Relatos de exercícios de natação com alta intensidade de treinamento, associado ao uso dos EAA, reportam hipertrofia das fibras musculares de contração lenta (Ustunel, et al., 2002; Isayama, et al., 2006) e elevação da proporção das fibras de contração rápida (Dimauro, et al., 1992; Bhasin et al., 2006).

1.7. Óxido Nítrico (NO) e Óxido Nítrico Sintase Neuronal (NOS I) e Endotelial (NOS III)

O óxido nítrico (NO) é um mediador químico que tem papel nos mecanismos de sinalização dos sistemas cardiovascular e nervoso. Atua na defesa do organismo, e na ativação endógena da forma solúvel da guanilato ciclase. A função fisiológica do NO foi primeiro descoberta nos vasos, quando se mostrou que o fator de relaxamento derivado do endotélio podia ser quantitativamente determinado pela formação de NO pelas células endoteliais (Moncada et al., 1991).

O NO é um radical livre, pouco solúvel em água, que tende a existir como gás. É sintetizado a partir da oxidação do átomo de nitrogênio do grupo guanidino terminal do aminoácido L-arginina, através de enzimas específicas, as NOS, resultando na formação do aminoácido L-citrulina e do gás NO (Vladutiu, 1995). Por ser um gás, é facilmente difusível e possui uma vida curta (entre 3 a 20 segundos).

As NOSs são fundamentais no controle do processo biossintético do NO. São dioxigenases e existem em muitas formas moleculares, normalmente referidas como isoformas ou isoenzimas (Vladutiu, 1995).

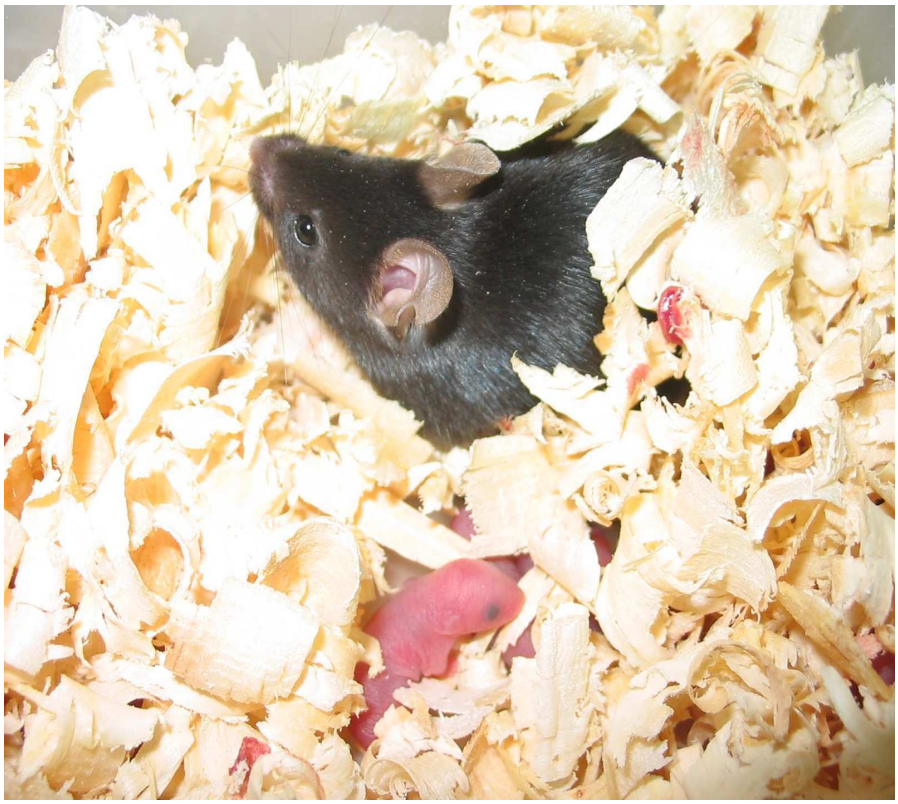
Estão muito bem documentadas na literatura as três isoformas de NOS, entretanto, apenas a isoforma neuronal e endotelial serão abordadas nesta tese.

Isoforma I (nNOS ou **NOS tipo I):** é uma isoforma constitutiva (cNOS) que se liga à calmodulina de forma reversível, é Ca^{2+}- e calmodulina–dependente. É encontrada nos neurônios, em certas células epiteliais e fibras musculares (Chao et al., 1997). A nNOS provavelmente tem importante papel não somente nas funções fisiológicas neuronais, tais como liberação dos neurotransmissores, desenvolvimento neural, regeneração, plasticidade sináptica e regulação da expressão do gene, como também em uma variedade de doenças neurológicas (Yang et al., 1997). Nas fibras musculares a nNOS é encontrada no sarcolema extra-juncional, interagindo com outras proteínas associadas à distrofina. Alguns autores demonstram que, a expressão da nNOS está ausente ou reduzida no sarcolema de pacientes com distrofia muscular de Duchenne (Grozdanovic et al., 1996; Marques, 2004).

- **Isoforma III (eNOS** ou **NOS tipo III):** é uma isoforma constitutiva, Ca^{2+}- e calmodulina–dependente, em sua maior parte encontrada no endotélio de vasos sangüíneos. É uma proteína de membrana periférica, que tem como alvo a cavéola plasmática endotelial através da interação com a caveolina-1, proteína estrutural da cavéola endotelial e sarcolemal. É

também encontrada no sarcoplasma e mitocôndrias das fibras musculares, sendo sugerido que o NO produzido por ela provoca redução da taxa de liberação de cálcio e diminuição da força contráctil na musculatura esquelética e na musculatura lisa vascular (Kobzik et al., 1995; Tews et al., 1997). Em situações de estresse sua expressão pode estar aumentada.

1.9. Modelo Animal



Com o advento da alta tecnologia, a utilização de animais transgênicos tem sido uma ótima ferramenta para o estudo de diversos fenômenos fisiológicos e patológicos. Nosso modelo experimental difere dos demais pelo fato de os camundongos apresentarem perfil lipídico semelhante ao do ser humano. Esses camundongos transgênicos da linhagem C57/BL6 são resultantes do cruzamento de animais que não expressam o receptor para LDL-colesterol com animais que expressam a (CETP) proteína de transferência de ester de colesteril (normalmente não expressada por esses roedores) resultando na redução de HDL e aumento de LDL quando comparados aos *wild type*. Esse modelo foi muito bem descrito por Cazita et al., (2003) e Casquero et al., (2006) ao nível do metabolismo lipídico. Esses animais transgênicos ($CETP^{+/-}LDLr^{-/+}$) são

ferramentas úteis para o estudo da ação do exercício físico e de drogas que afetam o metabolismo lipídico. Este modelo foi utilizado em pesquisas do Departamento de Fisiologia e Biofísica do Instituto de Biologia (Unicamp) utilizando protocolo de exercício (natação) e alterações do perfil lipídico. Foi a primeira vez que esses trangenes foram utilizados em outros tipos de experimentos na nossa unidade. Aqui, um protocolo de corrida em esteira, simulando exercício aeróbico de alta intensidade e a administração de EAA foi aplicado em camundongos $CETP^{+/-}$ $LDLr^{-/+}$ para avaliação do perfil lipídico, remodelação cardíaca, plasticidade muscular esquelética, expressão das NOS (I e III) no músculo esquelético e remodelação do tendão de Achilles.

2. OBJETIVOS

- Gerais:

Investigar os efeitos causados pela administração de EAA combinado ou não com exercício físico em animais transgênicos, cujo perfil lipídico é semelhante ao humano. Uma vez que, tanto os EAA como o exercício físico possui "alvos biológicos" comuns, é nosso objetivo averiguar as interações entre ambos no nosso modelo murino avaliando alguns sistemas biológicos.

- Específicos:

- Investigar o efeito de exercício aeróbico intenso, da administração de mesterolona e de ambos associados em camundongos transgênicos que expressam CETP (cholesteryl ester transfer protein) e possuem deficiência parcial de receptores de LDL ($CETP^{+/-}$/$LDL^{-/+}$), através de técnicas histoquímicas, imunohistoquímicas, bioquímicas, clínico-laboratoriais, microscopia de luz, microscopia eletrônica de transmissão, estereologia e morfometria nos seguintes tópicos:

1) Efeitos no fenótipo do músculo (plasticidade): verificar alterações na composição de fibras em tres músculos que tem características metabólicas e contráteis diferentes: sóleo, com predominância de fibras de contração lenta e metabolismo oxidativo; tibial anterior, com predominância de fibrasde contração rápida e metabolismo glicolítico e gastrocnêmio, músculo com características intermediárias;

2) Área seccional dos tipos de fibras delineados nos tres músculos (hipertrofia);

3) Expressão das isoformas NOS I e NOS III nos três músculos esqueléticos;

4) Ultraestrutura, densidade mitocondrial/fibra, densidade vascular/fibra, etc no sóleo

5) Remodelamento cardíaco;

6) Determinação do perfil lipídico;

7) Remodelamento do tendão de Achilles

3. CAPÍTULOS

Esta tese foi confeccionada de acordo com portaria CCPG/001/98-UNICAMP que regulamenta o formato alternativo para a tese de doutorado e permite a inserção de artigos científicos publicados, no prelo, submetidos ou em fase de submissão, de autoria ou co-autoria do candidato.

O exemplar de tese está composto por 6 artigos, sendo que 4 fazem parte do sistema locomotor, 1 do cardíaco, e 1 originado de trabalho satélite que foi desenvolvido paralelamente à tese. Destes, 2 artigos foram publicados (V; VI), 2 foram submetidos (II e IV), e 2 estão em preparação (I e III), conforme descrito abaixo:

3.1 Artigo I– Effects of anabolic steroids and high-intensity aerobic exercise on skeletal muscle of transgenic mice (Em fase final de redação).

3.2 Artigo II – Morphological changes in murine skeletal muscle in response to exercise and anabolic steroids (*Histochemistry and Cell Biology*. Submitted).

3.3 Artigo III – Until now concealed theme: Anabolic androgenic steroids affect the expression of nitric oxide synthase in skeletal muscles (Em preparação).

3.4 Artigo IV – Effect of high-intensity aerobic exercise and mesterolone on the Achilles tendon of transgenic mice (*The Journal of American Sports Medicine*. Submitted).

3.5 Artigo V - Adverse effect of anabolic androgenic steroid mesterolone on cardiac remodeling and lipoprotein profile is attenuated by aerobic exercise training. International Journal Experimental Pathology 2008; 89:358-366.

3.6 Artigo VI - Hepatocyte nuclear phenotype: the cross-talk between anabolic androgenic steroids and exercise in transgenic mice. Histology and Histopathology 2008; 23:1367-1377.

Capítulo I

Capítulo I:

Para alcançarmos os nossos objetivos, foram delineados os tipos de fibras musculares com base na reação de m-ATPase, bem como a ação miotrófica da mesterolona através da morfometria da área seccional transversa, do exercício ou de ambos combinados nos diferentes tipos de fibras musculares.

Capítulo I

Artigo em fase final de redação

Effects of Anabolic Steroids and High-Intensity Aerobic Exercise on Skeletal Muscle of Transgenic Mice

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Running title: Mesterolone and exercise in skeletal muscle in transgenic mice

ABSTRACT

Anabolic androgenic steroids have been used with success for therapeutic purpose however its indiscriminate use is a potential health risk. Herein, we analyzed the effects of mesterolone on *soleus* (SOL), *tibialis* anterior (TA) and *gastrocnemius* (GAS) muscles of sedentary and exercised mice. For this, mice heterozygous for the human CETP transgene and for the LDL-receptor null allele ($CETP^{+/-}LDLr^{-/+}$) sedentary and submitted to a 6-week-treadmill exercise program were compared. Mesterolone or gum arabic (vehicle) was administered orally in the last 3 weeks to both. Four groups were compared: Exercise plus Mesterolone (Ex-M), Exercise plus Gum arabic (Ex-C), Sedentary plus Mesterolone (Sed-M), Sedentary plus Gum arabic (Sed-C). The results showed that exercise retarded the body weight gain and mesterolone blunted this effect. Both mesterolone and exercise training increased muscles weight, but exercise surpassed mesterolone effect, except for TA ($P<0.05$). Mesterolone in sedentary mice increased the percentage of IC, IIAC, IIDA and IID/X, and decreased IIA in SOL, but did not affect TA and GAS, but mesterolone to exercised mice had no effect. Exercise *per se* increased the % of IID/X in SOL; IIA and IIDA in TA, and, IID/X in GAS, but decreased IIDA and IIDB in GAS. The exercise in mesterolone-treated mice caused no effect in the proportion of fibers in SOL, but increased I, IIA and IIDA in TA, whereas it decreased IIDA and IIDB and increased IIB fibers in GAS ($P<0.05$). Fiber hypertrophy was potencialized in Ex-M groups, however differently among the muscles by the steroid, exercise or both together. Concluding, mesterolone preferentially increased the mass of glycolytic muscle (TA) and least that of oxidative one (SOL); induced differentially adaptive changes in the *soleus*, *tibialis* anterior and *gastrocnemius* either sedentary or submitted to aerobic training which was reflected both in fiber composition and size.

Key words: Soleus, Tibialis Anterior, Gastrocnemius, Mesterolone, Physical exercise, Muscle Fiber type, Muscle fiber hypertrophy, mATPase

INTRODUCTION

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone which have been chemically modified to maximize anabolic and minimize undesirable androgenic effects (Haupt and Rovere, 1984). Given the powerful anabolic effect, AAS have been recommended for patients with hypogonadism, as well as those debilitated by chronic diseases and/or severe muscle catabolism (Basaria et al., 2001). Although banned from sports, the illicit use of AAS by professional and recreational athletes continues despite a long list of serious side effects (Maravelias et al., 2005). In general, these athletes are interested in decreasing body fat while increasing muscle mass and strength in an effort to enhance physical performance (Bahrke and Yesalis, 2004). It is well-known that the anabolic effects of androgens are mediated by androgen receptors (AR) (Inoue et al, 1993), which are found in both skeletal muscle fibers (Krieg, 1976, Max et al., 1981, Dorlochter et al., 1994, Kimura et al. 1993) and satellite cells (Doumit et al., 1996). Studies have shown a differential sensitivity of various muscles to androgens (Wilson, 1988), which may be related to the nonhomogeneous distribution of AR in different muscles (Janssen et al., 1994). In addition, AR content appears to be influenced by hormonal level and physical exercise (Rance and Max, 1984, Carson et al., 2002, Lee et al., 2003)

Skeletal muscle has the ability to respond to new endogenous or exogenous physiological demands by changing its phenotypic characteristics (Harridge et al., 1998; Gondim et al., 2005). The large diversity of myosin heavy chain (MHC) isoforms helps to form the basis for this remarkable plasticity. The expression or co-expression of the various myosin isoforms in a given fiber delineates an entire range of pure and hybrid fibers (Pette and Staron, 1990; Schiaffino and Reggiani, 1994, 1996; Pette and Staron, 2000). The prevalence of certain types of these fibers accounts for the functional and structural characteristics of a given muscle, and hence its phenotypic characteristics.

The present study was undertaken to examine the effects of mesterolone (an anabolic

androgenic steroid) on the fiber type composition and cross-sectional area of skeletal muscle fibers of sedentary and aerobically exercised transgenic mice. Three skeletal muscles with different metabolic and contractile properties were studied under these experimental conditions: *soleus* (SOL), *tibialis anterior* (TA) and *gastrocnemius* (GAS). We hypothesized that the administration of mesterolone would differentially affect the characteristics of the muscles, and that the high-intensity exercise program could have a modulating role on these effects. This work is part of a comprehensive study which has also investigated the effects of AAS on plasmatic lipid levels, tendon and cardiac remodeling, and on renal glomeruli alterations in this transgene heterozygous murine strain with a lipid profile similar to humans (Cazita *et al.* 2003; Casquero *et al.* 2006).

MATERIALS AND METHODS

Animals. The experimental protocol was approved by the University's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP, Protocol 700-1) and followed the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1996). The transgenic mice used in this study have been cross-bred and maintained in the Department of Physiology and Biophysics, Institute of Biology, UNICAMP. Mice heterozygous for the human CETP transgene and for the LDL-receptor null allele ($CETP^{+/-}LDLr^{-/+}$) were used. This transgenic mouse, as opposed to wild-type, exhibits a lipid profile closer to humans. Wild-type mice (WTM) have a deficiency in cholesteryl ester transfer protein (CETP) and thus, express large numbers of low density lipoprotein receptors (LDLr) which result in high circulating levels of HDL-c and low levels of LDL-c. Thus, by introducing the CETP transgene and reducing the expression of the LDLr gene, the lipemic phenotype of these transgenic mice more closely resembles that of humans (Cazita *et al.* 2003; Casquero *et al.* 2006). The mice were housed in a temperature and humidity controlled room (22 ± 1ºCand 55-65%, respectively), with a 12 h light/dark cycle and free access to water and food

(Nuvilab, Colombo, Paraná, Brazil).

Twenty-four 2-month-old male mice (19-20 g) were used. The animals were divided in four groups (n = 6/group): sedentary plus mesterolone (Sed-M); sedentary plus gum arabic (Sed-C); exercise plus mesterolone (Ex-M); exercise plus gum arabic (Ex-C). Ex-M and Ex-C animals had a week of training adaptation (Monday through Friday), in which a low to moderate exercise level was performed consisting of treadmill running (15 m/min during 20 min/day). After the adaptation period, trained mice were subjected to a gradual 6-week aerobic running program, 5 days a week (Table 1). The animals received mesterolone (M) (Proviron trademark of Schering, Schering do Brasil, São Paulo, SP, Brazil) (2 µg/g body weight) or vehicle (C) (=gum arabic, GA) (2 µg/g body weight) by orogastric tube during the last 3 weeks of the study (three days a week: Monday, Wednesday, and Friday) (Table 1). Gum arabic (compatible as a vehicle for the hydrophobic mesterolone) is inert, non-toxic and has been used to improve absorption in the small intestine (Codipilly et al, 2006).

Surgical procedures and body/muscle weights. At the end of the experimental period, fasted mice were deeply anesthetized with a 1:1 mixture of ketamine chloride (Dopalen, 100 mg/kg of animal) and xylazine chloride (Anasedan, 10 mg/kg,) (2 µl/mg body mass, i.p.). Both anesthetics were from Vetbrands (Jacareí, SP, Brazil). SOL, TA and GAS muscles were removed, weighed, and the animals were sacrificed with an overdose of the anesthetic. Body weight of the animals of each group was tracked weekly prior of any experimental procedure.

Muscle fiber typing. The middle portion of each muscle was separated, oriented in a mixture of gum tragacanth (Sigma-G1128, St. Louis, MO, USA) and Tissue-Tek embedding medium (Sakura Finetechnical Co., Tokyo, Japan), immediately frozen in isopentane cooled to -156ºC in liquid nitrogen, and stored at -70ºC. Transverse 12 µm thick cross sections were obtained in a cryostat,

collected on coverslips, and stored frozen at -40ºC until all samples were processed. Fiber types and subtypes were identified using myofibrillar adenosine triphosphatase (mATPase) histochemistry (Staron and Pette, 1993) following pre-incubation at pH 4.3, 4.5 (Brooke & Kaiser, 1970) and 10.5 (Guth & Samaha, 1970). The alkaline pH was slightly adjusted to optimize the reaction (10.57 and 10.6 for TA and GAS muscles, respectively). The muscles sections obtained at pH 4.5 were photographed and mounted as a plate. This plate (combined with the other slides) was used to identify the entire range of fiber types (I, IC, IIAC, IIA, IIAD, IID, IIDB, and IIB).

Histomorphometry. Image-Pro Express software (Media Cybernetics, Silver Spring, MD, USA) was used to calculate the cross-sectional areas (CSA) using a BX 51 Q Color 3 Olympus light microscope (Olympus, Japan). A minimum of 80 muscle fibers per type were analyzed in each group. The fields were randomly selected, and all muscle fibers encompassed in these fields were evaluated.

Statistical analysis. All numerical data are presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used to compare differences between groups. If an overall ANOVA revealed significant differences, a two-way ANOVA was used to determine how mesterolone treatment or exercise influenced body/muscle weight, fiber type composition or CSA changes, and if there was an interaction between exercise *vs*. mesterolone. Differences were considered significant at $P<0.05$. Origin software package (Microcal^{TM} Software Inc., Northampton, MA, USA) was used for all statistical analyses.

RESULTS

Body weight (BW): BW increased along the trial period however the smallest gain was achieved by exercised mice treated with gum arabic (Ex-C group) whereas the largest was achieved by Sed-M.

Mesterolone treatment counteracted the exercise effect, both in sedentary and exercised mice. Therefore, Ex-M achieved the values of body weight gain of the sedentary groups (Sed-C and Sed-M) as follows: Sed-M=3.88 g, Sed-C=3.84 g, Ex-M=3.55 and Ex-C=3.05 g. At the end of the sixth week, only Ex-C differed significantly from the other three groups in relation to the final body weight ($P<0.05$). Two-way ANOVA showed that there was no interaction between mesterolone and exercise for BW values.

Muscle weight (MW): The weight of *soleus*, *tibialis anterior* and *gastrocnemius* in sedentary and exercised animals treated with mesterolone or vehicle is shown in Table 2. It shows that the muscles weight was smallest in sedentary animals treated with vehicle (Sed-C) and largest in exercised animals treated with mesterolone (Ex-M) (Table 2A). The effect of mesterolone in sedentary mice (Sed-C *vs*. Sed-M) was stronger than in exercised mice (Ex-M *vs*. Ex-C), meaning that exercise was able to blunt the steroid effect. The highest impact of mesterolone, both in sedentary and exercised mice was in the *tibialis* anterior (40% and 20% increase, respectively) and the smallest in *gastrocnemius* (17% and 12%, respectively) (Table 2B). The effect of exercise *per se* in promoting MW increase was comparable among the muscles and was stronger than the promoted by the steroid, except for TA (compare Ex-C *vs*. Sed-C and Sed-M *vs*. Sed-C). The effect of exercise *per se* (Ex-C *vs*. Sed-C) in MW increase was stronger than when mesterolone was associated whether the mice were treated with GA or treated with mesterolene (Ex-M *vs*. Sed-M) indicating that mesterolone counteracted the effect of exercise, whose major expression was in the TA (Table 2B). Two-way ANOVA showed that there was no interaction between mesterolone and exercise for MW in the muscle.

Muscle fiber type: In this study, the classification of muscle fibers was based on the histochemical demonstration of mATPase. The pure (I, IIA, IID/X and IIB) and hybrid fiber (IC, IIAC, IIDA and

IIDB) types were determined in the muscles studied in all four experimental groups (Fig. 1) and the percentage of each was calculated.

SOL muscle: fiber I, IC, IIAC, IIA, IIDA and IID/X were delineated. Especially type I and IIA represented the majority of fibers in SOL muscle of Sed-C group, whereas type IID represented the minority. There was no significant effect of either the mesterolone or exercise or both combined in relation to type I fiber. However, mesterolone affected mainly the hybrid fibers increasing by 4-fold the IC, 3-fold IIAC, 1.5-fold IIDA and 3-fold IID, as well reduced to half the proportion of IIA in sedentary mice (comparison between Sed-C *vs*. Sed-M) ($P< 0.001$). As well, only type IID/X fiber proportion increased by 7-fold in exercised mice treated with GA (Sed-C *vs*. Ex-C). When combined exercise plus mesterolone the transformation of fibers was abolished (Table 3). Two-way ANOVA showed that there was interaction between mesterolone and exercise to IC fiber (P=0.0339); IIAC (P=0.0291); IIA (P=0.0258); IIDA (P=0.0424) and IID/X (P=0.0190).

In TA muscle, the fiber types I, IIA, IIDA, IID/X, IIDB and IIB were determined; fibers IC and IIAC were not found. A major percentage of types IID, IIDB and IIB fibers, and a minor of I was observed. The results showed that either mesterolone in sedentary (Sed-C *vs*. Sed-M), or in exercised mice (Ex-C *vs*. Ex-M) did not promote significant transition in any of the fiber types. The intensive physical exercise *per se* promoted significant increase in the proportion of the fibers IIA and IIDA (Sed-C *vs*. Ex-C). On the other hand, physical exercise in mesterolone-treated mice resulted in a significant 0.5-fold increase of type I, and potentiated the incremented proportion of IIA and IIDA (Sed-M *vs*. Ex-M) ($P< 0.001$) (Table 3). Two-way ANOVA showed that there was no interaction between mesterolone and exercise, whatever the fiber type.

In the GAS, among the fiber types I, IIA, IIDA, IID, IIDB and IIB delineated, IIB and IID had a larger percentage compared to the others; fibers IC and IIAC were not found. Mesterolone intake did not affect significantly the proportions of any of the fiber types in sedentary (Sed-C *vs*. Sed-M) and exercised mice (Ex-C *vs*. Ex-M). The physical exercise in animals treated with vehicle

(Ex-C group) compared to Sed-C caused a ~3-fold decrease in the proportion of types IIDA and IIDB, whilst increased by ~1/3 the IID/X fibers (P<0.001). The aerobic exercise in mice treated with mesterolone (Sed-M *vs*. Ex-M) maintained decreased the proportion of IIDA and IIDB, counteracted the increase of IID/X (seen in GA-treated mice) and turned into significant the increase of type IIB (Table 3) (P<0.001). Two-way ANOVA showed that there was interaction between mesterolone and exercise to IIDB fiber (P=0.0164) and IIB fiber (P=0.0391).

Cross-sectional area of muscle fibers (CSA): In the SOL muscle, type I and IIA fibers had the largest mean fiber area in all four experimental groups. This muscle showed the least variability in the size of their fiber types in sedentary mice treated with GA (Sed-C, control group). The administration of mesterolone promoted significant increase in CSA of type I (33.7%), IIA (18.5%) and IID/X (22.6%) fibers in sedentary mice (comparing Sed-C *vs*. Sed-M), however in trained mice (Ex-C *vs*. Ex-M) mesterolone increased by 7% the area of type I fibers. Contrary, the aerobic exercise promoted both in mice treated with gum arabic and mesterolone (comparison of Sed-C *vs*. Ex-C and Sed-M *vs*. Ex-M) a significant CSA increase in types I (101.3% and 61%), IIA (83.8% and 18.5%), IIDA (37.5% and 36%) and IID/X (55.8% and 31.7%), respectively (P< 0.001). Fiber type I was the most responsive to the trophic effects, whereas type IIDA was the least. Mesterolone and exercise effects were summed to cause the largest hypertrophy in the fibers (Ex-M group) (Table 4). Two-way ANOVA showed that there was interaction between mesterolone and exercise only for type I fiber (P=0.0028).

In TA, a descendant fiber size was seen from IIB → I, as follows IIB>IIDB>IID=IIA=IIDA>I in Sed-C mice. The results showed that the steroid in sedentary mice (comparison of Sed-C x Sed-M) caused significant hypertrophy in type I (14%), IIDA (65%) and IID/X (30%). In contrast,

mesterolone in trained mice (Ex-C *vs*. Ex-M) was able to increase only the size of type IIDA (22%) and IIDB (20%). A significant myotrophic effect caused by the aerobic exercise in GA-treated and M-treated mice (Sed-C *vs*. Ex-C and Sed-M x Ex-M) was seen in types I (76% and 85.6%), IIA (62.6% and 61.6%), IIDA (61.2% and 19.2%), IID/X (58% and 34.5%) and IIB (18.4% and 22%), respectively. As well, type IIDB fiber area increased by 37.2% in M-treated exercised mice (Table 4). Two-way ANOVA showed that there was interaction between mesterolone and exercise for IIDA fiber (P=0.0041) and IIDB (P=0.0144).

In terms of fiber size, the GAS of Sed-C mice showed IIB>IIDB>IID>IIDA>I=IIA. Mesterolone intake by sedentary mice promoted the hypertrophy of type I by 35.2%, IIA by 18.5%, IIDA by 18.2%, IID/X by 26.8% and IIB by 12.5% fibers (Sed-C *vs*. Sed-M); but when mesterolone was given to exercised mice (Ex-C *vs*. Ex-M) the degree of size increase varied, such as 20% for type I, 16% for IIA, 28.3% for IIDA, 43.4% for IID/X and 8.7% for IIB. On the other hand, the exercise in GA-treated mice (Sed-C *vs*. Ex-C) promoted significant hypertrophy of I (65.4%), IIA (35.3%), IIDA (26.4%), IID/X (19%) and IIB (20%) (P<0.001), whereas the exercise to mice to which was given the steroid (Sed-M *vs*. Ex-M) the hypertrophy committed types I (46.6%), IIA (31%), IIDA (37.2%), IID/X (34.6%) and IIB (16%) through a synergistic effect of both in relation to fiber IIDA and IID/X fibers. In general, the most responsive fibers to all the stimuli were I and IID/X in this muscle (Table 4). Two-way ANOVA showed that there was interaction between mesterolone and exercise only in relation to IIDA fiber (P=0.0158).

DISCUSSION

Fiber typing

The main goal of this work was to investigate whether anabolic androgenic steroids (AAS) affect differently the phenotypic characteristics of three hindlimb muscles which differ in their contractile

and metabolic attributes, and furthermore whether endurance aerobic exercise exerts a modulating role on these effects. In addition, the body and muscles weight were evaluated. The study was undertaken in transgenic mice which over-express CETP and are knockout for the LDL-receptor, and it follows the same experimental protocol as elsewhere (Fontana et al. 2008a, 2008b *in press*, 2008c, 2008d *submitted*). We used mATPase activity in alkali and acid pre-incubation to distinguish pure and hybrid types of fibers for characterization of the muscle phenotypic adaptation. This is an original study demonstrating that the mesterolone oral administration to sedentary mice induced significant fiber type transformation in the entire range of fiber types of the *soleus* with exception of type I which exhibited only a trend for decrease. When the mesterolone treatment was combined to exercise training the fibers transformation was abolished. In contrast, none of the types of fibers of the *tibialis* anterior and *gastrocnemius* was transformed by the steroid treatment alone. No data about the effects of AAS alone on the fiber type composition in mice are available in literature. A number of studies refers to humans and show that fiber types composition remains generally unaltered in different skeletal muscles, such as in *trapezius* (Kadi, 2000), deltoid (Hartgens et al., 2002), *vastus lateralis* (Sinha-Hikim et al., 2002), independent of the drug-regimen (single- or poly-drug regimen), or drug use duration (short or long). In rats, testosterone administration either to young or old animals also did not induce significant shift of fiber types in the *soleus* and EDL muscles (Isayama et al., 2006). Prezant et al. (1993) observed no significant alteration in the Type I and Type II fibers proportion in rat diaphragm both in males and females treated for short- or long-term with testosterone propionate. On the contrary, testosterone administration to female rats resulted in increased proportion of Type I and decreased Type II in the *soleus*, whereas fiber types in the *gastrocnemius* remained unchanged (Holmäng et al., 1990). The authors reported that Type II fibers were more affected than Type I, in agreement with our results where type I was unaffected and the entire range of Types II and besides type IC had their proportion altered. In our study, the fast oxidative type IIA fibers of sedentary *soleus* were recruited in favor of increased proportion of

fast glycolytic IIDA, IID/X and slow glycolytic-oxidative type IC and IIAC fibers after three weeks of mesterolone treatment. Increased performance in sports has been associated with the use of anabolic androgenic steroids (Bahrke and Yesalis, 2004), and this has been associated with increased muscle strength, a feature more related to Type II fibers (see also Hartgens and Kuipers, 2004). Although we did not evaluate muscle strength in these animals, it is conceivable that the animals improved muscle force since Type II fibers (except IIDA) were hypertrophied by the steroid as will be seen below, and since size increase has been associated with increased strength. The lack of fiber transition in fast- and intermediate-twitch muscles, such as the *tibialis* anterior and *gastrocnemius* muscles would likely be explained by the predominance of Type II fibers in these muscles. Moreover, our data showed that mesterolone given to exercised animals (Ex-C x Ex-M) did not produce any muscle fiber transition in *soleus* (and also in *tibialis* anterior and *gastrocnemius*) indicating that the aerobic exercise was able to blunt the steroid effect in these muscles. In male rats, castrated or administered nandrolone, the types of MHC of the *soleus* and *extensor digitorum longus* (EDL) muscles were shown also to be unchanged; however exercise plus nandrolone produced a shift from fast-MHC-2a to slow MHC-1 (Noirez and Ferry, 2000). In our study, the aerobic exercise program to which were submitted the mesterolone-treated mice (comparison of Sed-M x Ex-M) did not alter fiber composition in the *soleus*. However, it increased proportion of types I, IIA and IIDA in TA whereas it decreased IIDA and IIDB and increased IIB in the GAS, suggesting transition of TA to slower (oxidative) profile, whereas induced GAS towards to a faster (glycolytic) one. These results denote that the aerobic exercise prevented the effect of the steroid in the SOL (the exercise *per se* had only increased significantly the proportion of IID/X, and simply showed a trend to increase IC, IIDA, and decrease IIA in this muscle), whereas in TA the adaptive changes caused by the aerobic exercise were summed to the mesterolone effects, including in regard to type I fiber which had the proportion significantly increased. The results corroborate our former assumption that the *tibialis* anterior muscle acquired a slower profile. Concerning the

gastrocnemius, the intensive treadmill running undertaken by M-treated mice confirmed the tendency to assume characteristics more identified with a fast twitch phenotype given a significant decrease in the proportion of the hybrid types IIDA and IIDB and an increase of the pure type IIB, besides a trend to decrease type I proportion. It is well-known the effect of exercise in the adaptive response of skeletal muscles. Studies have shown that strength exercises in human increase proportion of MHC-IIA and reduce types IID/X, IIB and I in the *trapezius* muscle (Kadi and Thornell, 1999; Kadi, 2000), and that endurance exercise induces increase in type IIA in the *vastus lateralis* muscle (Staron et al, 1991; Kraemer et al 1995). Study on the effect of nandrolone cypionate and high intensity exercise (swimming) in rat medial *gastrocnemius* showed that either the exercise alone or the steroid alone were able to increase the fast oxidative-glycolytic fibers (FOG) and decrease the fast-glycolytic ones (FG), whereas fiber slow-twitch (ST) remained unaltered (Dimauro et al., 1992).

Concluding, considering the adaptive changes of each of the muscles studied, the *soleus* was the muscle to which mesterolone treatment was more effective in promoting recruitment of their fiber types (shifting). The extent of fiber transition varied depending on the hindlimb muscle and agrees with data reported in literature (see Baldwin and Haddad, 2001). The modulator role of exercise on the anabolic steroid treated mice differed in regard to the muscle contractile (and metabolic) uniqueness. Taking in consideration the fiber type quantitatively more representative in each of the muscles studied exercise to mesterolone-treated mice seems to drive *gastrocnemius* to faster-glycolytic profile, whereas *tibialis anterior* towards a slower-oxidative phenotype, whereas *soleus* muscle remained unchanged. Overall the adaptive transformation undertaken by the three hindlimbs here studied seems to be coordinated to promoted interactive adaptive transformation aimed to a balanced response of the organism.

Muscle weight and fiber hypertrophy

The differential response of the mice hindlimb muscles here studied was also observed through the muscle weight and cross-sectional area of the fibers. With exception of IIDB fiber in *gastrocnemius*, all other fiber types underwent hypertrophy by at least one of the treatment imposed to mice, i.e., mesterolone intake, physical training or both combined.

The hypertrophic effect caused by mesterolone was in general more moderate than the caused by the exercise training. This was reflected both by the intensity of fiber size change, and the amount of fiber types which were hypertrophied. Such a preponderance of exercise over the anabolic steroid was also reported by Soares and Duarte (1991) in the mouse *soleus*, with the steroid causing no significant myotrophic effect. One the other hand, nandrolone decanoate did not cause hypertrophy on *soleus* muscle when combined with resistance training (Cunha et al., 2006). Besides neither exercise (swimming) nor steroid treatment alone altered fiber diameter of the medial *gastrocnemius* in rats (Dimauro et al., 1992). No significant alteration in the Type I and Type II fibers area of rat diaphragm muscle was detected in males or females treated for short- or long-term with testosterone propionate (Prezant et al. 1993).

In contrast, our results showed that the anabolic steroid mesterolone induced significant size changes in the majority of fiber types of the muscles examined, except IIA, IIDB and IIB for TA, and IIDB for GAS. Such differences were reflected in the muscle weight gain at the final period of treatment. Mesterolone myotrophic action had as main target the fast fibers either with a glycolytic metabolism (IID/X), or oxidative metabolism (I and IIA) in case of a muscle predominantly slow-twitch and oxidative, as is the *soleus*. However, in a muscle predominantly fast-twitch and glycolytic in metabolism, such as the *tibialis* anterior, the main targeted fibers were I, IIDA and IID/X types, i.e., slow oxidative fibers and fast glycolytic fibers, respectively. In an intermediate muscle, as for the *gastrocnemius*, in addition to the fibers mentioned for TA, also IIA- and IIB-

typed fibers were affected by the steroid. The majority of these fibers were pure fibers; only the IIDA hybrid fiber types were stimulated to grow after 3 wks of mesterolone treatment. However, in exercised mice treated with mesterolone (Ex-C x Ex-M) a fewer number of fibers than the stimulated by the steroid alone underwent the myotrophic action of mesterolone, such as type I for SOL and type IIDA and IIDB for TA. Differently, in the GAS muscle the same fibers were affected by M alone or Ex+M, although diverse in degree. The results show the intricate interplay established between AAS and exercise resulting in fiber size alteration. This in turn suffers the influence of the internal milieu of the muscle, since a same fiber respond differently depending on its contractile and metabolic attributes. Taking in consideration the muscle weight and the fibers CSA gain, the 25% MW increase in the *soleus* can be ascribed to the increased proportion of type IC and all the Type II, as well to the increased area of fibers I, IIA and IID/X in M-treated sedentary mice (Sed-M). The MW gain was smaller in the *soleus* of M-treated exercised mice (18%) and correlates well with the increase of type I fiber area only, and none alteration of fiber proportion. In regard to TA, the muscle weight increased by 40% in Sed-M mice and by 20% in Ex-M mice giving a good correlation with the fewer number of fibers hypertrophied and none alteration of fibers proportion in the latter. However, such correlation was not observed for GAS (MW gain=17% *vs*. 12%, respectively caused by mesterolone alone, or mesterolone + exercise) where the most representative fiber types of the muscle showed prominent hypertrophy and no significant fiber transformation (compare Sed-C x Sed-M and Ex-C x Ex-M). Effects of training (swimming) and AAS treatment (nandrolone decanoate) in the murine *soleus* showed that there was increase of the fiber size (but the authors did not delineate the fiber types) in animals trained and decreased size both in animals treated with the steroid or trained and treated with the steroid (Soares and Duarte, 1991).

That AAS alone or associated to exercise increase muscle mass and fiber size has been well

documented in rodents and humans (Salmons, 1992; Kadi, 2000; Ustunel et al., 2002; Sinha-Hikim et al., 2002, 2003). However, why muscle with different metabolic and contractile activities show differential growth under the influence of equivalent anabolic androgenic steroid treatment or exercise program is less clear. Independent of differences which can be attributed to animal species, individual particularities, such as gender, age, physical conditions, AAS administration schema, type(s) of steroid administered, or exercise programmed, it is likely that molecular and cellular events specific to each muscle/fiber would respond for such distinctiveness. The differential rate of amino acid uptake and protein synthesis promoted by the muscle could be an explanation. Another explanation would be concerned with the density of androgen receptors in the muscle (Gustafsson et al., 1984; Janssen et al. 1994). It is well known the sexual hormone-susceptibility of different skeletal muscles, and that this action is mediated by the presence of androgen receptors (AR) in muscle fibers. The density of AR in a given muscle reflects its responsiveness to the action of androgen hormones or its derivatives, and would reflect ulterior differences in signaling pathways controlled genetically. Studies have shown that muscles from the upper region of the body show higher number of AR than the muscles of the lower regions (Hartgens and Kuipers, 2004). Muscles from different sites may have variable AR content, as for the *trapezius* muscle which contains higher proportion of AR than *vastus lateralis*. For instance, in humans, muscles of the neck and shoulder seem to exhibit higher density of AR than the muscles of the limbs; also, the type of involvement of different muscles in a specific exercise type would be reflected in the alteration of the AR muscles content (Kadi, 2000) and thereafter in type of MHC isoform of their fibers, and in its hypertrophic response.

Conclusion

This study provides evidences that the oral administration of mesterolone to transgenic mice with a lipemic profile close to the humans induced differentially adaptive changes in the *soleus*, *tibialis*

anterior and *gastrocnemius* either sedentary or submitted to intensive aerobic training. The effect in the hypertrophy of fibers was also distinct depending on the muscles. Exercise modulated differently the mesterolone action and vice-versa in the *soleus*, *tibialis* anterior and *gastrocnemius.*

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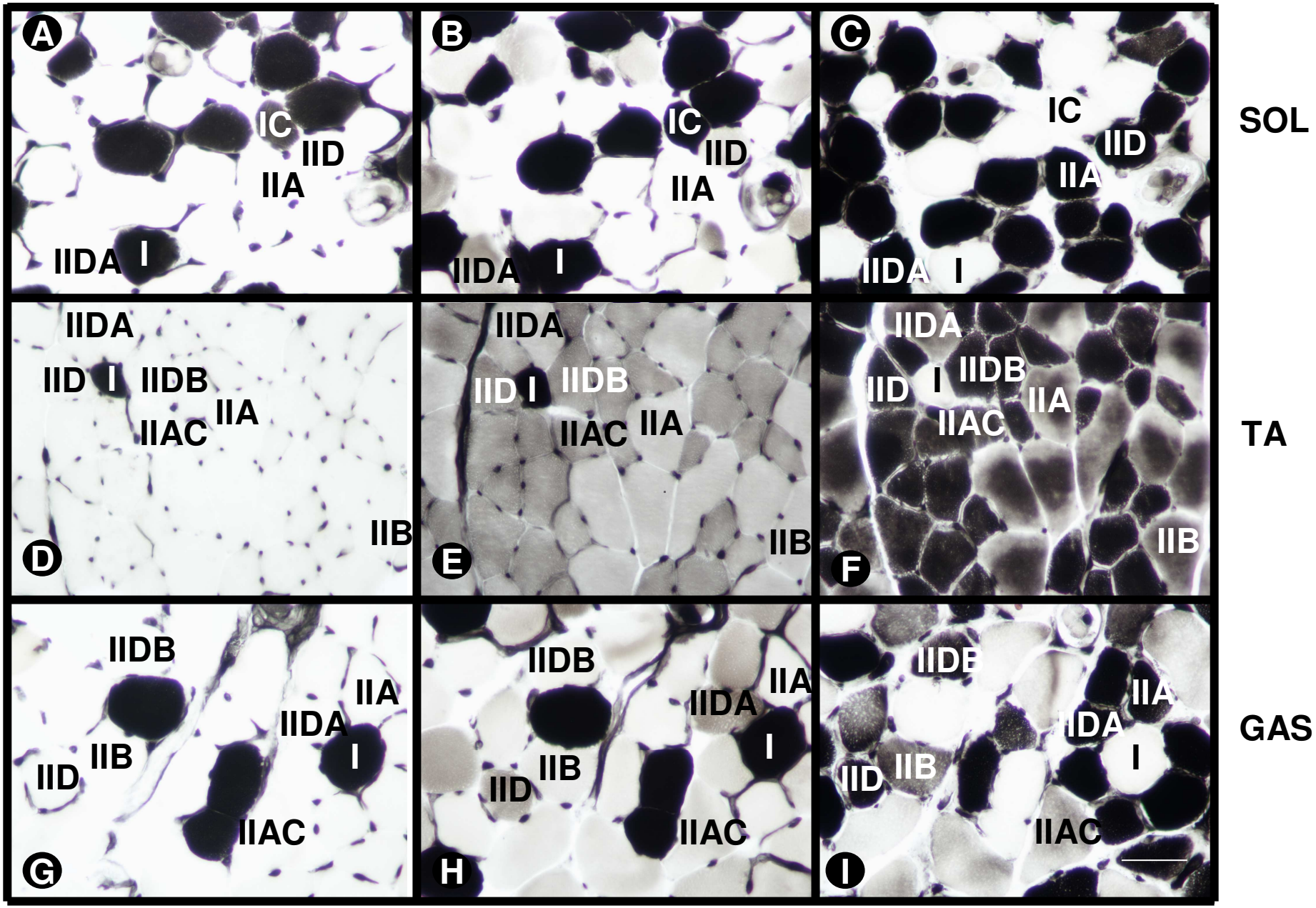

FIGURE 1. Histochemical mATPase reaction at pH 4.33 (a,d, g), 4.5 (b,e,h), 10.55 (c), 10.57 (f) and 10.6 (i) in serial sections of the SOL (a,b,c), TA (d,e,f) and GAS (g,h,i) muscles. 50µm.

Table 1 – Protocol of treadmill running program during six weeks (five days a week) in $CETP^{+/-}LDLr^{+/-}$ transgenic mice (adapted from Smolka et al., 2000). $^{(*)}$Mesterolone (M) or gum arabic (vehicle) (both 2 µg/g body weight) were administered at 10:00 a.m. (Monday, Wednesday and Friday) by orogastric gavage in the last three weeks (4^{th} to 6^{th}).

Weeks	Velocity (m/min)	Duration (min)
1	12.42	20
2	14.70	30
3	16.68	45
4-6 $^{(*)}$	17.04	60

Fontana et al., Table 1

Table 2 -Muscles weight (gram) in the $CETP^{+/-}LDLr^{-/+}$ transgenic mice sedentary (Sed) or submitted to exercise (Ex), half of which received either mesterolone (M) or vehicle (C). Values were expressed as means ± standard deviations (SD).

Groups \ Muscles	***Soleus*** (SOL)	***Tibialis anterior*** (TA)	***Gastrocnemius*** (GAS)
G1 - Ex-M	0.020 ± 0.002 †‡	0.067 ± 0.002 †‡	0.077 ± 0.001 †‡
G2- Ex-C	0.017 ± 0.001 #†	0.056 ± 0.002 #†	0.069 ± 0.001 #†
G3- Sed-M	0.015 ± 0.001 *‡	0.057 ± 0.006 *‡	0.057 ± 0.004 *‡
G4- Sed-C	0.012 ± 0.004 *#	0.041 ± 0.001 *#	0.049 ± 0.001 *#

The statistical significance among groups was determined by one-way ANOVA variance analysis followed for the Tukey test, with $P < 0.05$ indicating significance to all symbols.

Table 3- Distribuition of fiber type of *Soleus*, *Tibialis anterior* and *Gastrocnemius* muscles from transgenic mice. The same symbol in each column indicates significant difference of fiber proportion between groups. (P<0.05).One way ANOVA followed by Tukey test. The data were expressed by mean ± SD.

SOL / Groups	I	IC	IIAC	IIA	IIDA	IID/X
Ex-M	41.7± 5.2	12.6±5.3	10.1±3.5	27.6±2.7	5.9±2.4	2.1±0.7
Ex-C	38.0± 5.4	8.7± 1.5	8.6±3.1	35.8±8.4	5.3±3.1	3.7±2.4*
Sed-M	37.5± 2.4	13.8±3.0*	14.3±5.1*	23.3± 6.6*	9.6±2.3*	1.6±0.6#
Sed-C	39.9±2.2	3.1±3.7*	4.9±4.6*	47.6±12.2*	4.0±3.3*	0.5±0.2*#

TA / Groups	I	IC	IIAC	IIA	IIDA	IID/X	IIDB	IIB
Ex-M	1.1±0.2*	-----	-----	5.8±1.8*	8.7±1.8*	37.5±4.0	24.4±5.0	22.5±4.4
Ex-C	0.8 ±0.2	------	------	5.1±1.0 #	8.4±1.1 #	43.3±5.8	20.8±4.7	21.6±6.6
Sed-M	0.7±0.2*	-----	-----	3.6±0.3*	5.8±1.3*	43.0±5.7	27.1±4.4	19.8±6.9
Sed-C	0.6±0.2	-----	-----	3.1±0.2 #	4.7±0.4 #	46.4±3.6	25.4±4.6	19.9±3.8

GAS / Groups	I	IC	IIAC	IIA	IIDA	IID/X	IIDB	IIB
Ex-M	3.4±1.5	-----	-----	2.3±0.8	3.2±1.7*	25.5±5.3	6.6±2.2#	59.1±4.0 *
Ex-C	3.1±1.6	-----	-----	2.4±1.0	1.7±0.7 #	33.0±5.4*	8.7±2.6*	51.2±7.9
Sed-M	4.1±1.4	-----	-----	3.5±0.9	7.1±2.2*	19.7±3.9	23.1±4.1#	42.6±9.1*
Sed-C	4.2±1.9	-----	-----	2.2±1.4	5.2±1.7 #	23.0± 3.9 *	18.2±3.9*	47.1±5.6

Table 4- Relative cross sectional area (μm^2) of the I, IIA, IIDA and IID/X fibers from *Soleus* muscle and I, IIA, IIDA, IID/X and IIB fibers from *Tibialis anterior* and *Gastrocnemius* muscles. The same symbol in each column indicates significant difference of the mean cross-sectional area of a fiber type between groups. (P<0.05). One way ANOVA followed Tukey test. The data were expressed by mean ± SD.

SOL / Groups	I	IC	IIAC	IIA	IIDA	IID/X
Ex-M	1988± 14.0$^{\#\dagger}$	------	------	1806.6±17.2*	1284±17.7$^{\#}$	1357.2±37.3$^{\#}$
Ex-C	1858.6± 9.3*†	------	------	1744.7±36.6$^{\&}$	1168.7±42.1*	1308.5±25.8*
Sed-M	1234.3±27.3$^{\#\&}$	------	------	1124.6± 4.4*†	942.5±13.2$^{\#}$	1029.9±15.1$^{\#\dagger}$
Sed-C	923.3±13.4$^{*\&}$	------	------	948.9±11.7$^{\dagger\&}$	850.51±22.2*	839.5±5.8*†

TA / Groups	I	IC	IIAC	IIA	IIDA	IID/X	IIDB	IIB
Ex-M	848.2±13.7*	-----	-----	895±24.9*	933.8±18.3*†	924.8±16.5†	1114±33.1*$^{\&}$	2555.7±78.0*
Ex-C	706.1±65.0$^{\#}$	------	------	827.9±25.8$^{\#}$	765.3±19.1$^{\#\dagger}$	836.6±16.5*	928.1±22.5$^{\&}$	2301.2±19.8$^{\#}$
Sed-M	456.9±1.9*†	-----	-----	553.8±14.1*	783.4±7.8 *$^{\&}$	687.3±16.6$^{\dagger\&}$	811.7±15.1*	2093.5±29.3*
Sed-C	400.7±2.6$^{\#\dagger}$	-----	-----	508.9±19.2$^{\#}$	474.6±6.1$^{\#\&}$	529.3±26.6*$^{\&}$	818±21.0	1943±44.5$^{\#}$

GAS / Groups	I	IC	IIAC	IIA	IIDA	IID/X	IIDB	IIB
Ex-M	971.5±31.6$^{\#\&}$	-----	-----	752.5±5.5$^{\#\dagger}$	934.2±19.8 *†	1117.8±45.4$^{\dagger\#}$	949.3±39.8	2346.4±16.4*†
Ex-C	810.5±33.0*$^{\&}$	-----	-----	649.2±18.3$^{\dagger\&}$	728±5.0$^{\#\dagger}$	779.2±8.8*$^{\#}$	830.5±22.0	2157.2±25.3$^{\dagger\&}$
Sed-M	662.4±3.3$^{\#\dagger}$	-----	-----	574.2±10.7*$^{\#}$	680.8±4.7 *$^{\&}$	830.5±21.0$^{\dagger\&}$	821.9±18.4	2024.1±13.5*$^{\#}$
Sed-C	490±3.0*†	-----	-----	480±3.0*$^{\&}$	576±13.5$^{\#\&}$	654.8±6.5*$^{\&}$	767.9±6.1	1798.7±20.4$^{\#\&}$

Capítulo II

Capítulo II:

Para alcançarmos os nossos objetivos, ao microscópio de luz e eletrônico de transmissão foi avaliado no músculo *soleus*: número de miofibras, área seccional transversa das miofibras e das mitocôndrias, densidade mitocondrial/área de miofibra, número de capilares/miofibra, presença de células satélites ativadas, de "split fibers" (fibras fendidas) e a morfologia dos fusos musculares, bem como possíveis alterações da ultraestrutura em geral.

Capítulo II

Artigo II: Submetido ao Histochemistry and Cell Biology (em fase de resposta aos revisores)

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Morphological changes in murine skeletal muscle in response to exercise and mesterolone

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Abstract

Light and electron microscopy and quantitative morphometry were used to determine the effects of exercise and mesterolone on the soleus muscles of mice. Both exercise and mesterolone caused a significant hypertrophy of extrafusal muscle fibres but no hyperplasia. Mitochondria were more numerous and larger than in the muscles of sedentary animals. Capillarity increased and small, centrally nucleated muscle fibres appeared, usually in small clusters and most often in the muscles of animals exposed to mesterolone. A small proportion of satellite cells exhibited signs of activation but there were more in the muscles of mesterolone treated animals than after exercise. Muscles from animals that had been both exercised and treated with mesterolone exhibited the largest changes: muscle mass and muscle fibre hypertrophy was greater than in all other groups of animals, capillarity was higher and >30% of all recognized satellite cells exhibited signs of activation. Groups of small centrally nucleated muscle fibres were commonly seen in these muscles. They appeared to be the result of splits in the form of sprouts from existing muscle fibres. With both exercise and mesterolone, alone or in combination, there was an increase in the proportion of Type I muscle fibres and a decrease in the proportion of Type II.

Keywords: soleus muscle, mesterolone, physical exercise, hypertrophy, hyperplasia

Introduction

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone. Although AAS play a major role in the treatment of hypogonadism they are used extensively by a wide range of athletes attempting to increase muscle bulk and physical strength and to increase muscle:fat ratios (Bahrke and Yesalis, 2004). The increase in muscle mass that follows the non-clinical use of anabolic steroids, particularly when combined with exercise, appears to result primarily from an increase in muscle fibre cross-sectional area. This response is dose dependent and involves the activation of satellite cells and an increase in both myo- and satellite cell nuclei, the ratio between the two remaining constant (Kadi et al 2000, Sinha-Hikim et al 2003). There is no doubt that hypertrophy of the muscle fibre is a major component underlying the increase in muscle mass but the

possible role of muscle fibre hyperplasia is the subject of continuing debate. Antonio and Gonyea (1993) suggested that exercise-induced increases in muscle mass may involve both muscle fibre hypertrophy and hyperplasia but it is not always easy to differentiate between true hyperplasia (defined as an actual increase in the total number of individual muscle fibres in a skeletal muscle) and the splitting or branching of hypertrophied muscle fibres (Gonyea and Ericson, 1977, Eriksson et al., 2006). In this study we have examined the responses of murine soleus muscles to three different procedures-exercise alone, exposure to the anabolic steroid mesterolone alone and exercise plus mesterolone. We have been especially interested in the relative importance of muscle fibre hypertrophy and hyperplasia and we have also studied the effects of exercise and mesterolone on the ratio of slow (oxidative) to fast (oxidative-glycolytic, and glycolytic) muscle fibres in the soleus muscle, muscle fibre mitochondria, capillarisation and the intrafusal fibres of muscle spindles.

Materials and Methods

Animals

The mice used in this study were adult males aged two months and weighing between 19.5 and 22 g. They were heterozygous for the human CETP transgene and for LDL-receptor null allele ($CETP^{+/-}LDLr^{-/+}$) and have been described in detail elsewhere (Cazita et al., 2003; Casquero et al., 2006). They were cross-bred and have been maintained in the Department of Physiology and Biophysics, Institute of Biology, UNICAMP for ten years. The mice were housed in groups of three in a controlled environment room (temperature 22 ± 1ºC; humidity 55-65%;12 h light/dark cycle) with free access to water and standard chow (Nuvilab®, Colombo, PR, Brazil).

Mesterolone and Gum arabic

Mesterolone (1alpha-methyl-5alpha-androstan-17beta-ol-3-one) is a non-17 alpha-alkylated derivative of testosterone, commercially distributed as Pro-Viron® by Schering do Brazil, São Paulo, SP, Brazil. It is used routinely to treat hypogonadism. Gum arabic, also called gum acacia, is a complex polysaccharide produced by a large number of different species of shrubs of the genus *Acacia.* The gum, purchased from Sigma (St.

Louis, MO, USA), forms a thick mucilage when dissolved in water and is commonly used in the formulation of suspensions of hydropathic pharmaceuticals.

In these experiments gum arabic was made up as a 2% w/v solution in distilled water and was administered at a dose of 10 ml Kg^{-1} by orogastric intubation. Mesterolone was suspended in gum arabic solution at a concentration of 200 µg ml^{-1} and was administered at a dose of 2 mg Kg^{-1} body weight during weeks 4-6 of the exercise programme as described below.

The anaesthetic agents ketamine chloride (Dopalen®) and xylazine chloride (Anasedan®) were obtained from Vertbrands, Jacarei, SP, Brazil.

Exercise programme

The mice were divided into five groups: Group 1 (Sed) was entirely sedentary; Group two (Sed-C) was sedentary and had been given gum arabic; Group three (Sed-M) was sedentary but had been given gum arabic containing mesterolone; Group four (Ex-C) was exercised using the programme summarized in Table 1 and had been given gum arabic; Group five (Ex-M) was exercised and had been given mesterolone in gum arabic. Exercised mice were allowed to adapt to the treadmill for one week prior to the start of the experimental protocol. The adaptation programme consisted of low to moderate level exercise on the treadmill daily for 5 days (15 $m.min^{-1}$ for 20 $min.day^{-1}$) followed by two days rest. After adaptation, exercised mice were submitted to 6 weeks of involuntary intensive exercise training (treadmill running) on a weekly cycle of 5 consecutive exercise days followed by two days rest, as outlined in Table 1. This programme is a form of endurance training and does not compare with power training. During weeks 4-6 of the programme mice received either gum arabic or mesterolone suspended in gum arabic on three alternate days (see Table 1).

At the end of the experimental programme mice were fasted overnight and terminally anesthetized with a 1:1 mixture of ketamine chloride, 100 mg Kg^{-1} body weight, and xylazine chloride, 2 mg Kg^{-1} body weight, i.p. The soleus muscles were removed with tendons of origin and insertion intact and processed for transmission electron microscopy (TEM) or light microscopy.

The study protocols were approved by UNICAMP's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP, Protocol 700-1) and complied fully with the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1996).

Transmission Electron Microscopy

The isolated muscles (n=3 mice per group) were pinned out at 1.2 x resting length and fixed in Karnovsky's fluid (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) overnight, rinsed in 0.05 M PBS, post fixed for 2 h in 2% OsO_4, and rinsed again in 0.05 M phosphate buffered saline (PBS). The samples were dehydrated in graded acetone (30–100%) and embedded in Epon-Araldite resin. Resin-blocked samples were trimmed perpendicular to the long axis of the muscle fibres and 60–70 nm thick ultra thin sections were collected on Formvar-coated copper slot grids and stained with 2% uranyl acetate in 0.05 M maleate buffer and lead citrate. All samples were examined using a Philips CM100 Compustage TEM (EM Research Services, Newcastle University) and images were collected with an AMT40 digital camera.

Morphometry

Systematically sampled random images were taken at 800x and 7,900x magnification. The lower magnification electron micrographs were used to estimate capillary number per fibre and for the examination of muscle spindles. Approximately 50 fibres per animal were photographed. The higher magnification electron micrographs were analyzed at 21,000x to estimate mitochondrial number per unit area and cross-sectional area of mitochondria. An average of 20 micrographs per animal (n = 3 mice per group) were analyzed. Images of all satellite cells were collected at 13,500x if the entire profile of the cell could be observed. The satellite cells were classified as resting if they had a low cytoplasm/nucleus ratio, a clear cytoplasm and a condensed chromatin, and activated if the cytoplasm/nucleus ratio was high, the cytoplasm contained membranous organelles and polyribosomes. Mitotic figures were seen in some but not all activated cells. Split fibres were also noted on these micrographs. All images were analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

For the estimation of mitochondrial number per unit area and mitochondrial cross-sectional area, a calibrated non-biased counting frame and grid of points were superimposed on each image. The number of mitochondrial profiles (N) was counted

along with the number of points hitting mitochondria (P_M). Mean mitochondrial area (A_M) was then calculated as $A_M = P_M \times A_P / N$ where A_P = area per point. Damaged mitochondria (identified by the presence of distorted and disrupted cristae) were counted and expressed as a percentage of all mitochondria in the same field.

Light Microscopy

The central belly portion of the isolated muscles was isolated and oriented in a mixture of tragacanth gum (Sigma-G1128, St. Louis, MO, USA) and Tissue-Tek (Sakura Finetechnical Co., Tokyo, Japan), and immediately frozen in isopentane cooled to -156ºC in liquid nitrogen, after which it was stored at -70ºC until use. To study the gross morphology of the muscles transverse sections 6 µm thick were cut from frozen muscle and collected on silane-coated glass slides, air dried at room temperature and stained with Harris' haematoxylin. To determine the respective proportions of fast- and slow-type muscle fibres transverse sections 12 µm thick were cut from frozen muscle and collected on silane-coated glass slides, air-dried at room temperature and stained for myofibrillar myosin ATPase activity after incubation at pH 4.3, 4.5 and 10.5. (Brooke and Kaiser, 1970; Guth and Samaha, 1970; Staron and Pette, 1993). Fibre types I, IC, IIAC, IIA, IIDA, IID/X were identified and counted. To determine the proportion of Type I and Type II fibres, the values of types I and IC for Type I, and types IIAC, IIA, IIDA and IID/X for Type II were summed and a mean value was obtained which was used for comparison among groups. The total number of fibre/muscle was estimated in frozen sections of n = 6 mice per group.

Semithin sections 0.5 µm thick stained with Toluidine blues were used for the estimation of muscle fibre cross-sectional area using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Between 500 and 800 fibres (without fibre type identification) from each group of muscles were measured using a 10x eye piece and a 40x objective.

Statistical Analysis

All numerical data are expressed as mean ± standard deviation (SD) and were analyzed using Graph Pad Prism version 4.0 (San Diego, CA, USA). The correlation coefficient (*r*) was used to determine the degree of relationship between two variables. The statistical

significance of differences among groups was determined by one-way ANOVA followed by the *post-hoc* test of Bonferroni for multiple comparisons. A p value <0.05 indicated statistical significance.

Results

Control data

As mesterolone was administered as a suspension in gum arabic, the appropriate control group of animals was that comprising sedentary animals that had been given oral gum arabic (i.e.Group Sed-C). To determine the effects of gum arabic itself on muscle mass we compared the muscles of the Sed-C group with those of a purely sedentary group of age and sex-matched mice (Group Sed). We noted no differences in behaviour between the two groups of animals. There was a significant, 14%, increase in the mean muscle fibre cross-sectional area in the Sed-C group of animals from 739 ± 9 μm^2 in Sed animals to 843 ± 61 μm^2 in Sed-C and a corresponding significant increase in capillarisation from 1.68 ± 0.33 to 1.81 ± 0.13 capillaries per muscle fibre (not shown). In neither group did we observe any indication of degeneration or regeneration of muscle fibres, there was no change in muscle fibre numbers in the Sed-C group and mitochondria were similar in size and number to those in muscle from the Sed group. In this communication we have used data from the Sed-C group as our controls.

Animals

There was no mortality or morbidity in the animals used in this study. Animals given mesterolone became aggressive, more likely to bite the handler and fight with fellows. The exercise programmes were tiring for the animals involved and animals retired to rest for up to two hours after the completion of a session of exercise. Thereafter their behaviour was indistinguishable from that of the sedentary animals.

Muscle mass and muscle fibre numbers

Soleus muscles in the Sed-C group of animals weighed 12 ± 0.4 mg. The average muscle contained 599 ± 111 extrafusal muscle fibres with an average cross-sectional area of 843 ± 61 μm^2 (Fig 1A-C). There was no inflammation or edema in the muscles, no signs of

degenerating muscle fibres and no muscle fibres were centrally nucleated. Forty three (43 ± 2.9) % of muscle fibres were classified as Type I (mean value of type I + type IC numbers) and 57 ± 5.1 % as Type II (mean value of type IIAC, IIA, IIDA and IID/X numbers) (Fig 1D).

Treatment with mesterolone alone (Sed-M) caused no significant change in the total number of extrafusal fibres per muscle (590±76 *cf* 599 ± 111) but there was a significant increase in muscle wet weight to 15 ± 0.1 mg, an increase in the range of fibre size (Fig 1) and, overall, a small but significant increase in mean muscle fibre cross-sectional area to 883 ± 48 μm^2. There was an increase in the proportion of Type I fibres from 43 ± 2.9 % to 51 ± 2.7 % and a decrease in the proportion of Type II from 57 ± 5.1 % to 49 ± 3.7 % ($p < 0.05$).

Exercise alone (Group Ex-C) caused a significant increase in mean muscle wet weight, from 12 ± 0.4 mg in group Sed-C animals to 17 ± 0.1 mg in Ex-C animals and this increase in wet weight was significantly larger than the increase in wet weight in response to the administration of mesterolone alone (see above) . Mean muscle fibre cross-sectional area (1414 ± 178 μm^2) was also significantly larger than in both Sed-C and Sed-M groups. There was no significant change in muscle fibre number (541 ± 66) ($p < 0.05$). Compared with the control muscles (Sed-C) there was a trend to increase the proportion of Type I fibres in the muscle (from 43 ± 2.9 % in Sed-C to 47 ± 2.3 % in Ex-C) and a corresponding reduction in the proportion of Type II (from 57 ± 5.1 % in Sed-C to 53 ± 4.3 % in Ex-C) but the changes in this group of muscles were not as marked as in the Sed–M group (51 % and 49 %, respectively-see above).

The combination of exercise and the administration of mesterolone (group Ex-M) led to the greatest increase in muscle wet weight (to 20 ± 0.2 mg) and mean muscle fibre cross-sectional area (to 1936 ± 60 μm^2) ($p < 0.05$), but not in muscle fibre number (625 ± 135 *cf* 599 ± 111 in Sed-C animals). The shift in muscle fibre composition towards a slower type seen in both Sed-M and Ex-C groups was more marked in the Ex-M group of animals where 54 ± 5.2% of the muscle fibres were Type I and 46 ± 2.3 % Type II.

The increase in mean muscle fibre cross-sectional area reported above was seen in all muscle fibre types but was particularly pronounced in the Type I fibres. For example, the

cross-sectional area of Type I and Type II fibres increased respectively by 34% and 22% between Sed-C and Sed-M groups, by 100% and 60% between Sed-C and Ex-C groups and by 120% and 70% between Sed-C and Ex-M groups.

The data on muscle wet weight, muscle fibre number and cross-sectional area and muscle fibre type are summarized in Figs 1A-D. Increased cross-sectional area in Sed-M, and Ex-M groups is also illustrated in Fig 2 (compare the size of extrafusal muscle fibres from Sed-M in panel B, and from Ex-M in panels C-D with those from Sed-C muscles illustrated in panel A).

Centrally nucleated muscle fibres

Centrally nucleated extrafusal muscle fibres were not seen in any control muscle studied (Fig 2A). Occasional, isolated centrally nucleated fibres were seen in the muscles of the sedentary animals treated with mesterolone (i.e the Sed-M group). These fibres were typically of a diameter similar to those of the rest of the muscle fibres present (Fig 2B). Equally rarely were signs of splits in some of the muscle fibres (not shown). Centrally nucleated fibres, both small and large were common in the Ex-M group of muscles (Fig 2C-D). Centrally nucleated fibres were also found in thin sections of muscles of both exercised groups and it was clear that in many cases the centrally nucleated fibres arose as splits from larger muscle fibres (Fig 3).

Capillarisation and mitochondria

Capillarisation, defined as the number of capillaries per muscle fibre, increased from 1.81 ± 0.13 in muscles from animals in group Sed-C, to 1.94 ± 0.07 in Sed-M, 2.25 ± 0.36 in Ex-C and 3.56 ± 0.19 in Ex-M. Differences were significant only between Sed-M vs. Ex-M ($p < 0.05$) (Fig 4). The increase in capillarisation was linearly related to cross-sectional area (Fig 5). Similarly mitochondrial density, defined as the number of mitochondria per μm^2 of muscle fibre matrix, and mitochondrial cross-sectional area increased progressively with the increase in muscle fibre cross-sectional area. For example, mitochondrial density significantly increased from 0.24 ± 0.0007 μm^{-2} in Sed-C muscles to 0.35 ± 0.0003 μm^{-2} in Sed-M, 0.55 ± 0.04 μm^{-2} in Ex-C and 0.89 ± 0.16 μm^{-2} in Ex-M, and mitochondrial area increased from 0.09 ± 0.01 μm^2 in Sed-C to 0.12 ± 0.01 μm^2 in Sed-M, 0.23 ± 0.04 μm^2 in Ex-C and 0.26 ± 0.06 μm^2 in Ex-M ($p < 0.05$). The increase in

mitochondrial density was also linearly related to the increase in mean muscle fibre cross-sectional area (Fig 5). Damaged mitochondria were seen occasionally in all groups of muscles. They comprised between 4% and 9% of all mitochondria encountered and were most commonly seen in the animals of groups Ex-C and Ex-M ($p < 0.05$).

Satellite cells

Relatively few satellite cells were recognised unequivocally in these studies. Thirteen satellite cells were identified in muscle fibres of sedentary animals, only one of which appeared to be activated. Ten were recognized in muscle fibres of Sed-M animals, 3 of which appeared to be activated. Only 1 of 9 cells in Ex-C muscles was activated but 4 of 13 in Ex-M muscles were activated. A representative image of an activated satellite cell is shown in Fig 6.

Intrafusal muscle fibres

Muscle spindles were regularly encountered. We did not routinely prepare serial sections through the spindles or compared images of spindles cut through the equatorial region but our impression was that intrafusal fibres were routinely hypertrophied in both groups of muscles from exercised animals and in sedentary animals treated with mesterolone (i.e. Ex-C, Ex-M and Sed-M) and typical images are shown in Fig 7.

Discussion

The relationship between exercise, the use of anabolic steroids and the structure and function of skeletal muscle is a significant area of contemporary research but is a field complicated by much variability in terms of experimental protocols, animals used and steroids involved. The experiments described here were made using a standardized set of protocols and were designed to allow us to determine directly the effects of exercise alone, anabolic steroids alone and the combined use of both. Mesterolone, the anabolic steroid used in this study, is in regular clinical use, but the dose used did not equate to that used in clinical practice. In our study, we elected a supra-physiologic dose (2 μg/g/day) to be administered to this inbred mice, since 1 μg/g/day is considered an adequate replacement dose to mimic physiological levels in castrated mice (Tang et al.,

1991), and also to match with doses which athletes use to achieve good performance. CEPT$^{+/-}$/LDLr$^{-/+}$ mice were used as they have a plasma lipid profile similar to that of humans and thus we did not need to concern ourselves with the potential problem that data generated in the mice would be irrelevant to those studying human subjects because variations in the plasma lipids could influence the response to anabolic steroids.

In the first part of this study we concerned ourselves with changes in muscle mass and muscle fibre hypertrophy. We found that both exercise alone (Ex-C) and the administration of mesterolone alone (Sed-M) caused an increase in muscle mass and an increase in muscle fibre cross-sectional area and that exercise alone was significantly more effective than treatment with mesterolone alone. The increase in muscle fibre cross-sectional area was seen in both Type I and Type II muscle fibres but was greater in the Type I. There was a small increase in the proportion of Type I fibres from 43% to 47% in the muscles of group Ex-C. In muscles from mice of group Sed-M 51% of muscle fibres were Type I. The combined use of exercise and mesterolone (Ex-M) was particularly effective in increasing muscle mass and muscle fibre cross-sectional area. Both mean muscle mass and mean muscle fibre cross-sectional area were significantly larger than in Sed-C, Ex-C and Sed-M groups of animals. Both Type I and Type II fibres in the muscles were hypertrophied and in each case the degree of hypertrophy was greater than in any other group of muscles. There was a similar shift in fibre type to a slower profile as seen in muscles from the Sed-M group of animals.

There was no statistically significant difference in the number of muscle fibres per muscle between the four groups of animals (Sed-C, Sed-M, Ex-C, Ex-M) and we can confidently assert that the increase in muscle mass seen in animals treated with mesterolone and exercise is entirely the consequence of muscle fibre hypertrophy.

Centrally nucleated muscle fibres were recognized in three groups of muscles; Sed-M, Ex-C and Ex-M. Although it has long been known that regenerating and immature muscle fibres of rodents are characterized by the retention of a centrally located nucleus (Clark, 1946) it is important to ask whether the centrally nucleated fibres seen in the muscles of the Sed-M, Ex-C and Ex-M mice can be explained by proposing a cycle of degeneration and regeneration. Could the centrally located nuclei represent a residual invading inflammatory or phagocytic cell? If the centrally located nuclei are myonuclei,

are the relevant muscle fibres new muscle fibres at an early stage of development or outgrowths of an existing healthy muscle fibre?

Inflammation was not a feature of any of the muscles we isolated from any of the experimental animals used in this study. No invasive cells were seen in any of the very many muscle fibres we examined during the ultrastructural studies. We doubt that there was an inflammatory response in the muscles exposed to exercise or following the administration of mesterolone and doubt that the centrally located nuclei we saw in some muscle fibres of groups Sed-M, Ex-C and Ex-M were the nuclei of invading cells.

The occasional centrally nucleated muscle fibre with no other clinical or pathological signs is seen routinely in both medical and veterinary practice (Cordy, 1963; Schwartz et al., 1976; Cardinet and Halliday, 1979). In both human and non-human subjects up to 5% of muscle fibres with centrally located nuclei would be considered within normal limits. In exercised human subjects the proportion of muscle fibres with centrally located nuclei is higher than 5% and the same is probably the case in animals. The occasional isolated centrally nucleated muscle fibre probably represents a muscle fibre that has regenerated after minor vascular insufficiency or a physical tear.

It is more difficult to offer an unequivocal explanation for the split muscle fibres and clusters of small centrally nucleated fibres seen in muscles from both Ex-C and Ex-M groups of animals. It is possible that the clusters of small muscle fibres represent regenerating muscle fibres following a highly localized vascular infarct affecting a group of previously healthy muscle fibres but we saw no evidence, such as grouped fibre necrosis or atrophy that might favour such a hypothesis. Ultrastructural studies showing the apparent emergence of small centrally nucleated fibres from the periphery of existing muscle fibres suggested strongly that the small centrally nucleated fibres represent multiple regenerative outgrowths from a small number of single mature muscle fibres (Pearson, 1963). Hypertrophy and split muscle fibres are often seen in the skeletal muscles of patients and animals with degenerative diseases of the lower motor neuron and skeletal muscle and are generally considered to reflect a response of a relatively undamaged muscle fibres to the additional work required when many muscle fibres or motor units are structurally, metabolically or physiologically compromised (Schwartz et al., 1976). Eriksson et al. (2006) in their study of muscle fibre hypertrophy in power

lifters using anabolic steroids also suggested that muscle fibre splits and the presence of small centrally nucleated muscle fibres represented a form of regeneration distinct from hyperplasia. Perhaps the split fibres and the small centrally nucleated fibres seen in these and related studies are associated with the activation of satellite cells following exposure to exercise and anabolic steroids. This is not an entirely novel suggestion. Harris and his colleagues have described the intense activation and signs of budding of satellite cells in undamaged muscle fibres in muscles partially destroyed by myotoxic peptides (Klein-Ogus and Harris, 1983; Maltin et al., 1983). In those experiments it was suggested that some of the satellite cells might enter a pool of circulating pre-myoblasts that could initiate the development of a new muscle fibre and that others might provide a site for the development of a split fibre.

Exercise-induced increases in both muscle fibre capillarity and in the density and area of mitochondria were fully consistent with the increased demand for the provision of nutrition to and the removal of metabolic waste from the hypertrophied extrafusal muscle fibres of working animals (Klausen et al., 1981; Poole and Mathieu-Costello, 1996; Gute et al., 1996; Howald et al., 2002; Jensen et al., 2004; Tarnopolsky et al., 2007).

It has been suggested by many authors (e.g. Kadi, 2000; Sinha-Hikim et al., 2003, Eriksson et al., 2006; O'Connor and Pavlath, 2007) that muscle fibre hypertrophy involves the activation of muscle satellite cells and the incorporation of the activated cells into the parent fibre (see review, Bhasin et al., 2003). Our data on the activation of satellite cells must be considered preliminary in view of the small numbers of cells we encountered. Despite that caveat, our data do indicate that muscles from the animals treated with mesterolone exhibited a larger proportion of activated satellite cells than those from sedentary animals and those who were simply exercised. The biological receptors for anabolic steroids in mammalian skeletal muscle are present in the cytosol of both the muscle fibres and satellite cells (Saartok et al., 1984; Sinha-Hikim et al., 2004) and the direct activation of satellite cells by the anabolic steroid may be the explanation for the increased proportion of activated satellite cells found in these muscles.

The combination of both exercise and mestelorone (Ex-M) was a particularly potent stimulus for an increase in muscle mass. Muscle fibre hypertrophy was also greater in this group of animals than in any other group, as was muscle fibre mitochondrial cross-

sectional area, mitochondrial number and capillarity. Foci of small centrally nucleated muscle fibres were seen in all muscles examined from this group of animals.

We had not planned to study the response of intrafusal fibres to exercise and exposure to mesterolone and so our observations on these structures were incidental. It was our impression that muscle fibre hypertrophy was not confined to extrafusal muscle fibres but also involved the intrafusal muscle fibres of the muscle spindle. We do not have enough information on spindle physiology during either exercise or AAS treatment to be able to offer any credible explanation for this observation but we suggest that a formal study of the pathology and pathophysiology of these major sensory structures would be timely.

Acknowledgments

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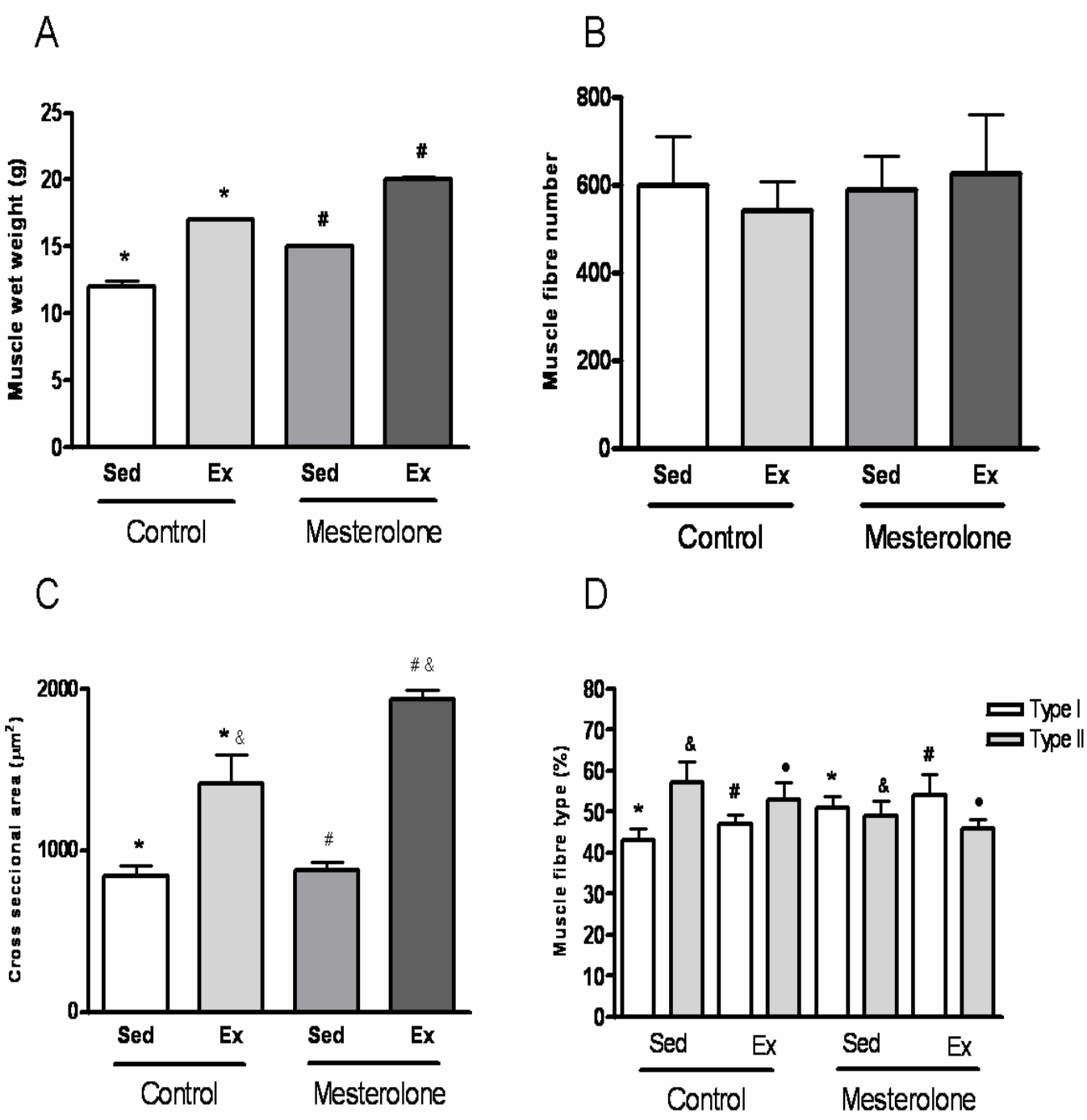

Figure 1 The effects of exercise and mesterolone treatment on muscle mass and muscle fibres in soleus muscles of mice. Both exercise and mesterolone treatment caused an increase in muscle wet weight (A). There was no significant hyperplasia (B) but a significant increase in muscle fibre cross sectional area (C). Both exercise and mesterolone treatment caused an increase in the proportion of Type-I muscle fibres and a decrease in the proportion of Type-IIA (D). Note that the changes were larger when exercise was combined with mesterolone treatment than with either exercise or mesterolone treatment alone. The same symbol over the bars indicates significant differences among groups ($p < 0.05$). One-way ANOVA followed by Bonferroni test. The data were expressed in mean ± SD.

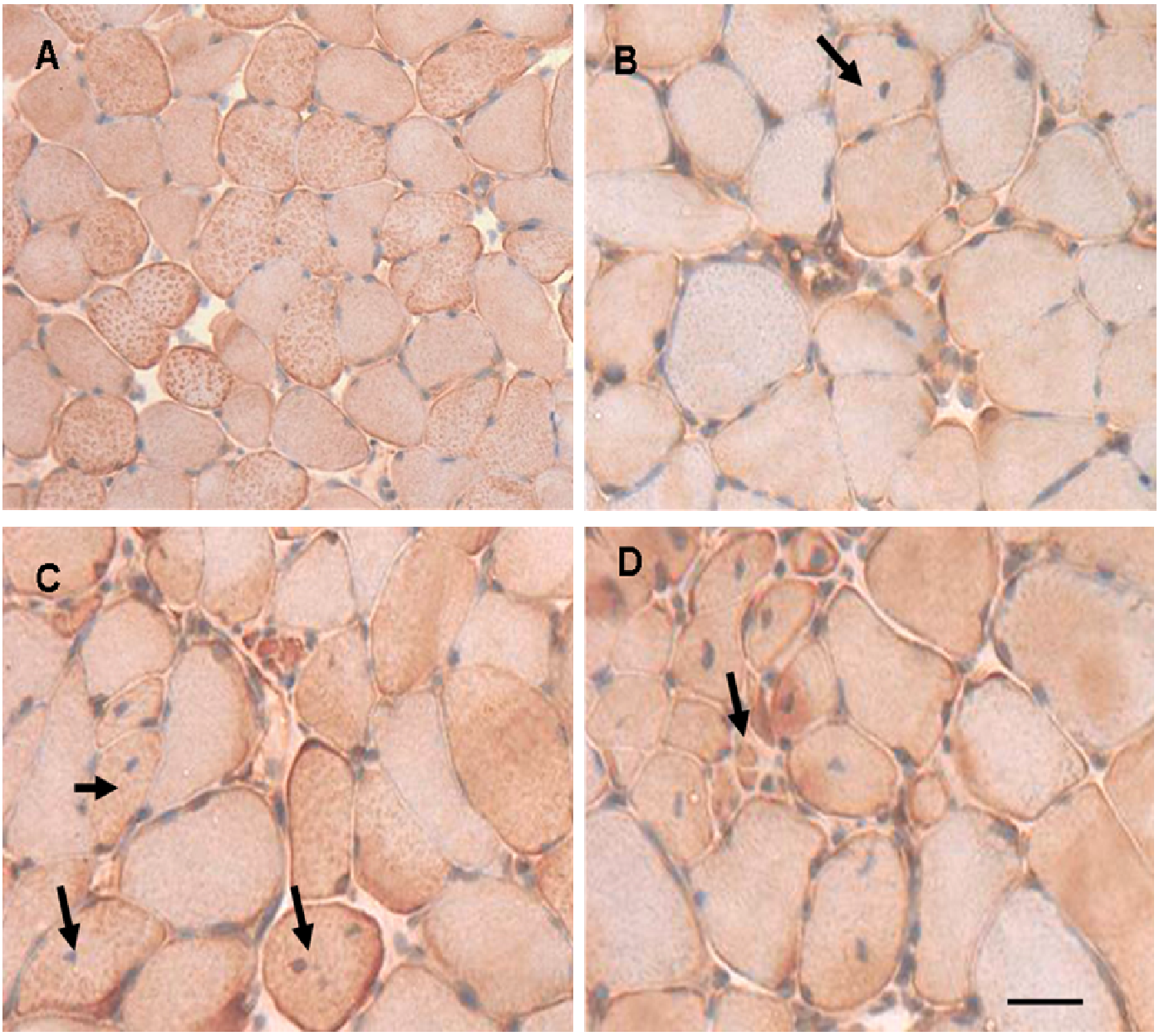

Figure 2 Transverse sections of frozen soleus muscles from a sedentary mouse (A), a sedentary mouse treated with the anabolic steroid mesterolone (B), and mice treated with mesterolone and exercised on a treadmill (C, D). Note the regular shape and size of muscle fibres in (A) and the increased size of muscle fibres in (B, C and D). Apart from the occasional, centrally located myo-nucleus of normal-sized muscle fibres seen in sedentary mice treated with mesterolone (arrow, B) clusters of small centrally nucleated muscle fibres shown in (D) were seen only in the muscles of mice that had been both exercised and treated with the anabolic steroid mesterolone.
Sections 6 μm thick were stained with Harris' haematoxylin. Scale bar = 30 μm.

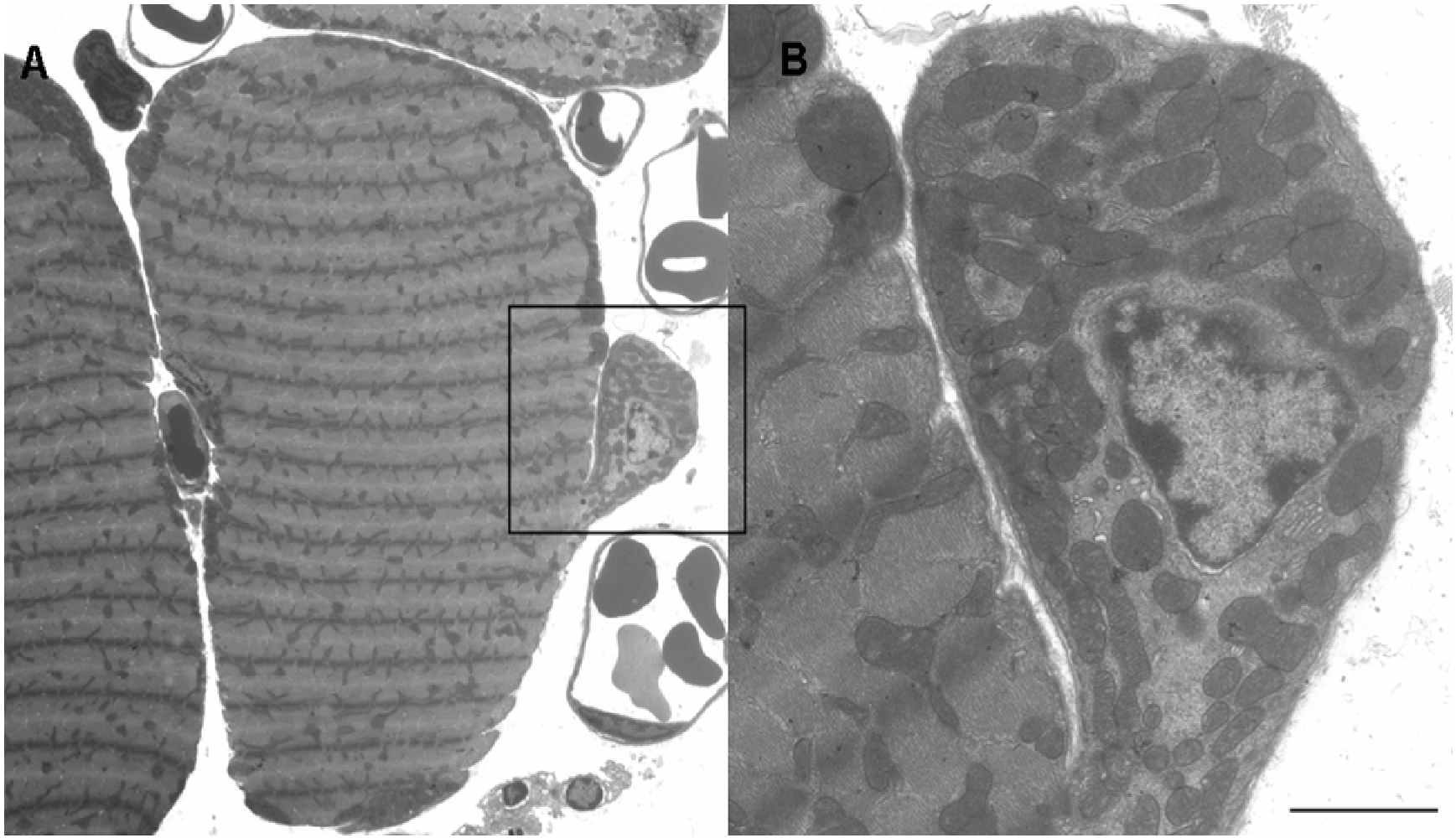

Figure 3 Ultra-thin section from resin-impregnated mouse soleus muscle cut 60-70nm thick. (A) This image shows the splitting of a small, centrally nucleated daughter muscle fibre from a hypertrophied muscle fibre in the soleus muscle of a mouse exercised on a treadmill and also treated with the anabolic steroid mesterolone (B) An enlarged image of the daughter fibre.
Scale bar =10 μm (A), 2 μm (B).

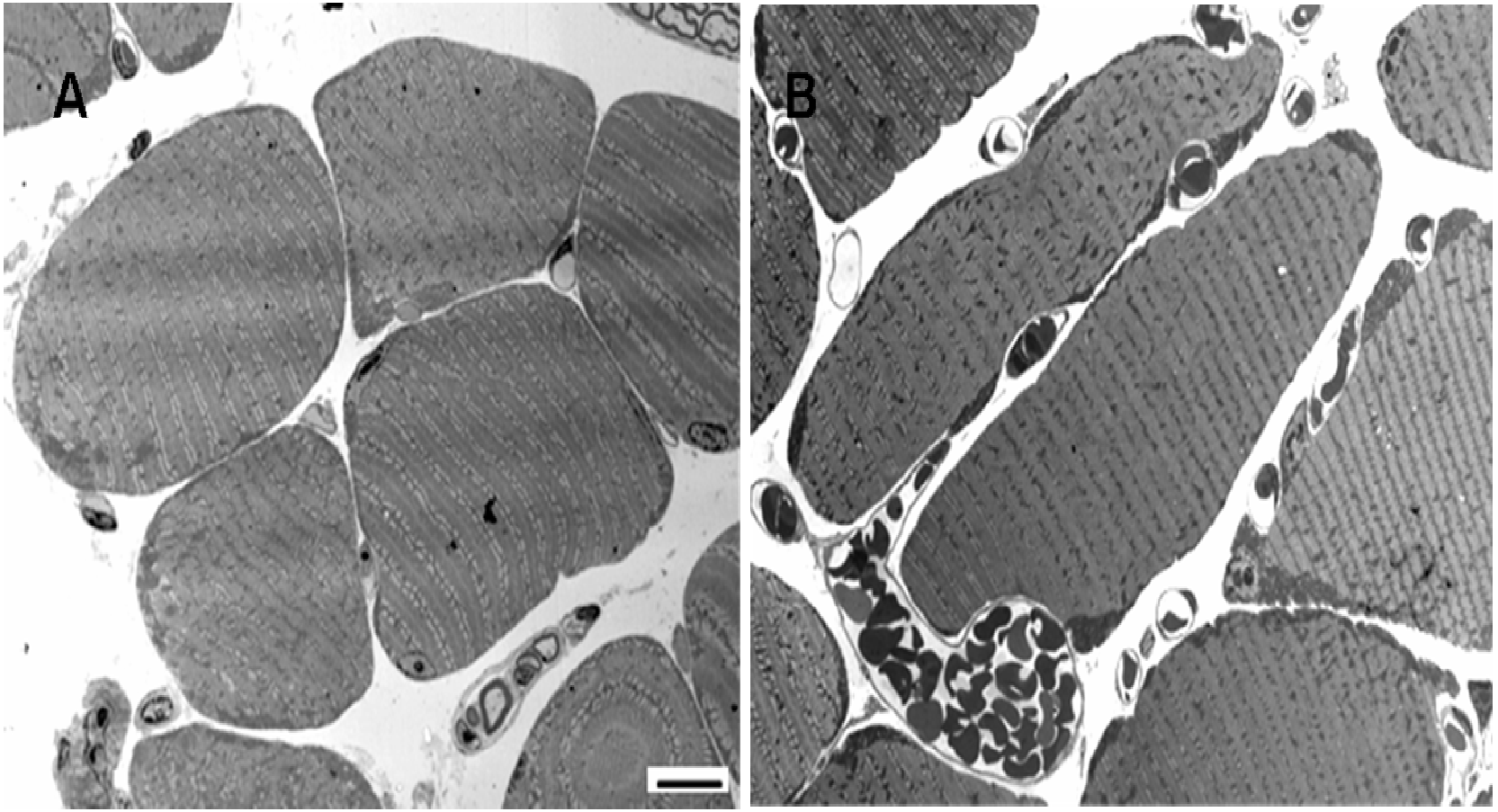

Figure 4 Muscle fibre capillarity in the soleus muscle of a sedentary mouse treated with gum arabic alone (A) and in the soleus muscle of a mouse exercised on a treadmill and also treated with mesterolone (B). Note the very marked increase in capillarity.
Ultrathin sections from resin-impregnated muscles cut 60-70 nm thick. Scale bar =10µm.

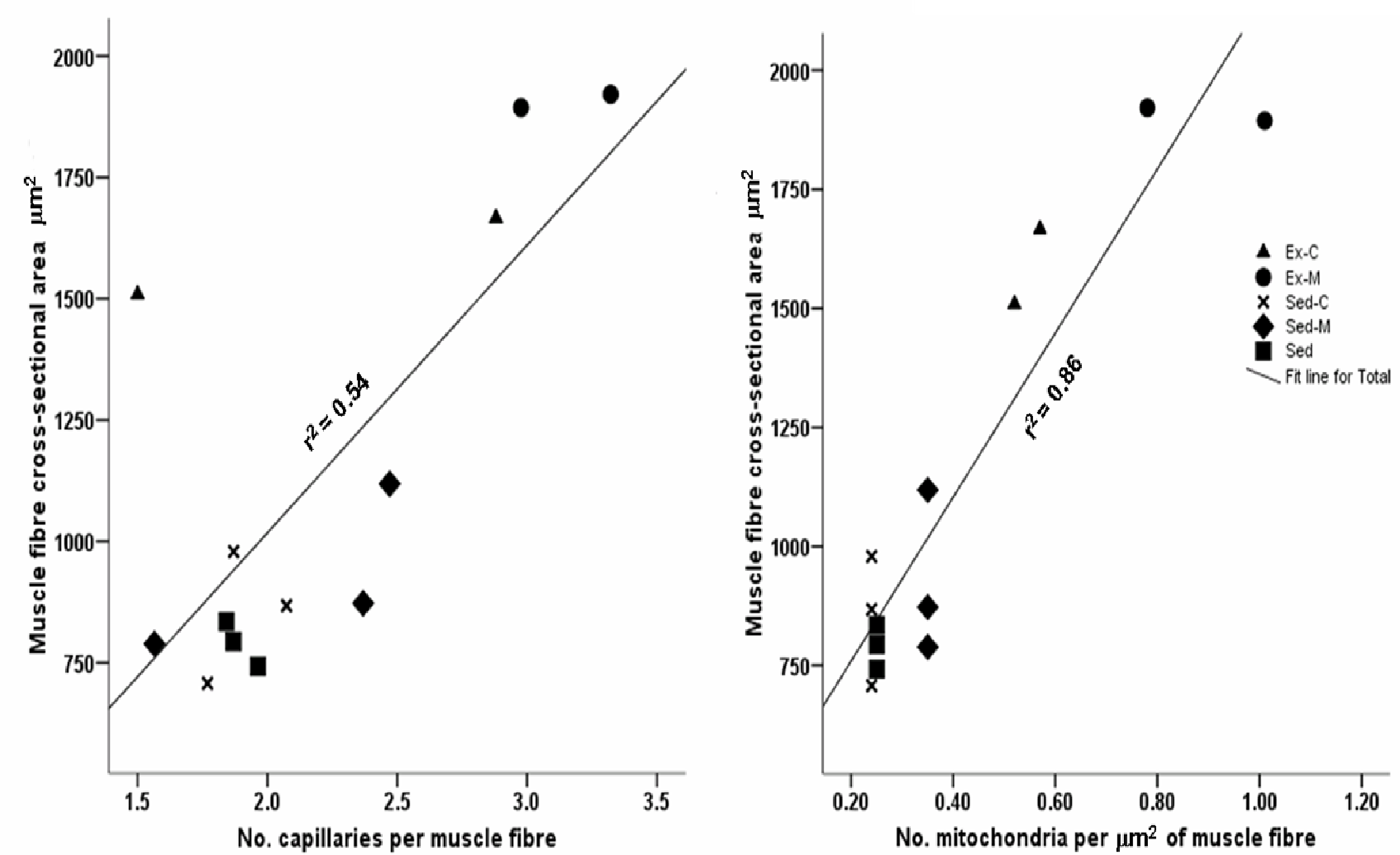

Figure 5 The relationship between muscle fibre cross-sectional area and (A) capillarity and (B) the number of mitochondria per μm2 muscle fibre matrix in the soleus muscles of the four groups of mice.

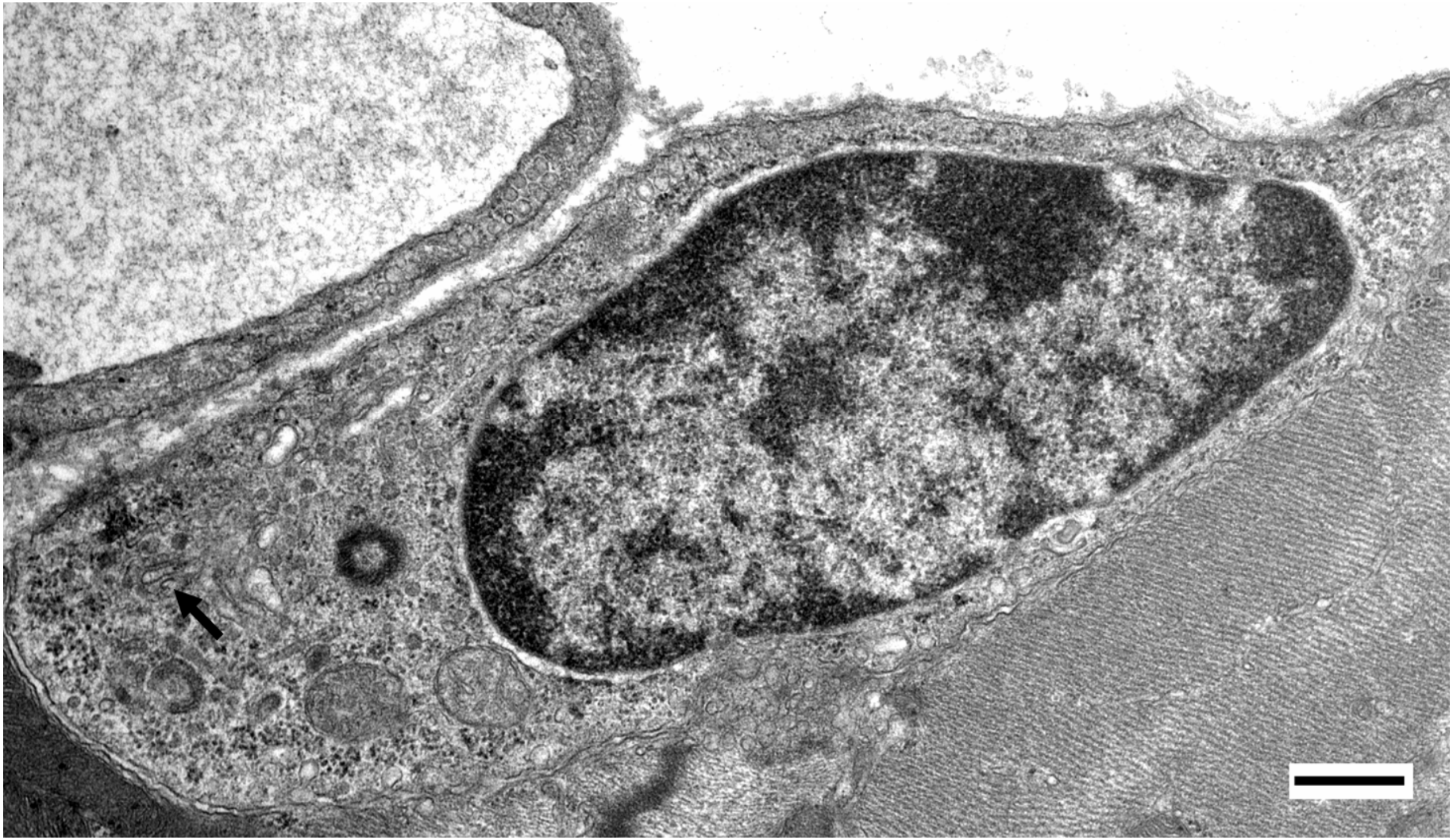

Figure 6 Typical image of an activated satellite cell. Note the relatively large cytoplasm/nuclear volume and the membranous structures of Golgi, endoplasmic reticulum, mitochondria, centriole and cross- and longitudinally-sectioned microtubules, in the cytoplasm. Scale bar = 500 nm.

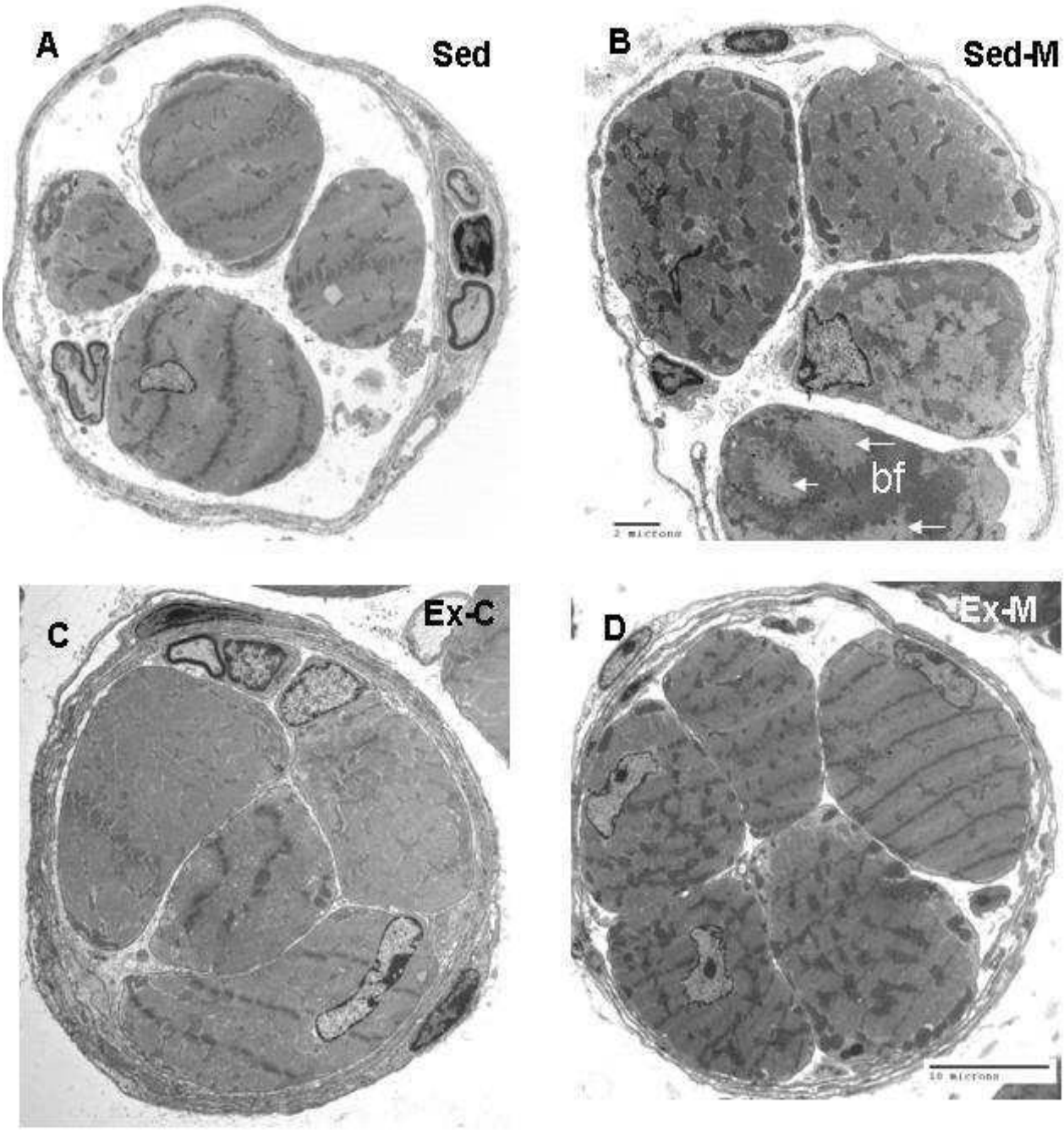

Figure 7 Muscle spindles in the soleus muscles of a sedentary mouse (A), a mouse treated with mesterolone (B), a mouse exercised on a treadmill (C) and a mouse exercised on a treadmill and also treated with mesterolone (D). Note the increase in intrafusal muscle fibre cross-sectional area, the unusual polygonal shape of closely apposed fibres and the complete filling of the spindle capsule in C and D. Note in B the presence of nuclear bag fibres (bf) with three nuclei (arrows). In sedentary mice (A) intrafusal fibres show the typical round profile.
Ultrathin sections from resin-impregnated muscles cut 60-70 nm thick. Scale bar =10 µm.

Capítulo III

Capítulo III:

Para alcançarmos os nossos objetivos, utilizamos as técnicas de imunohistoquímica e western blotting para a detecção da expressão das isoformas óxido nítrico síntase neuronal (NOS I) e óxido nítrico síntase endotelial (NOSIII).

Artigo III: Processo de redação ainda em curso

Until now concealed theme: Anabolic androgenic steroids affect the expression of nitric oxide synthase in skeletal muscles

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Running title: Nitric oxide synthase expression and skeletal muscle

Abstract

Abstract

In skeletal muscle NO-mediated signaling has been demonstrated in myogenic differentiation, force development, fiber growth and in muscle adaptation to exercise. Although many studies have shown that anabolic androgenic steroid (AAS) use or abuse have the same targets, none have demonstrated that the AAS effects could be mediated by nitric oxide signaling. To address this issue we examined the localization and expression of nitric oxide synthase (NOS) isoforms I and III, in mice *soleus, tibialis anterior* and *gastrocnemius* muscles of sedentary and exercised mice treated with mesterolone employing immunohistochemistry and Western blotting. The results were compared to adaptive changes in fiber composition and fiber hypertrophy underwent by the muscles using the same experimental protocol and murine model. Mice were submitted to 6-week aerobic treadmill running (one hour/day, 5 days a week) and mesterolone (or gum arabic, vehicle of the steroid) was administered during alternate days in the last three weeks by gavage. Sedentary mice also received mesterolone or gum arabic. TA showed the strongest labeling and SOL the slowest for both isoforms, but with predominance of NOS I. Mesterolone administration to sedentary or exercised mice significantly upregulated the expression of both isoforms but preferentially NOS I in tibial anterior muscle and soleus whereas gastrocnemius was unaffected. The exercise training in mice treated with gum arabic upregulated significantly the expression of NOS I and NOS III in soleus, and NOS III in gastrocnemius, but lesser than the caused by the steroid. Tibial anterior did not change the NOS expression in response to exercise. The exercise to mice treated with mesterolone increased significantly the expression of NOS I in all three muscle, however, it only increased NOS III expression in tibial anterior. The confrontation of these data with the adaptive transformation in the soleus, tibial anterior and gastrocnemius phenotype seen in our previous study using this protocol demonstrate that NO has a role in these fiber type transition and fiber hypertrophy.

Key words: mesterolone, nitric oxide synthase neuronal and endothelial, treadmill exercise, transgenic mice

Introduction

The advantages attained from a controlled exercise program have been exhaustively divulged in scientific and non-scientific publications. Despite the knowledge that regular exercise practice is important to prevent risk of chronic diseases development, such as hypertension, osteoporosis, diabetes, obesity, muscle wasting, cancer, emotional stress, depression, memory, among others, the adherence of people to a such habitual scheme is highly poor (Kruk, 2007). The practice of physical exercise have been associated with regulation of both coronary (Duncker and Bache, 2008) and skeletal muscle blood-flow, the latter with a good repercussion on the adaptive changes in cardiovascular system (McAllister et al., 2008). In part such gains have been ascribed to exercise modulated endothelium-dependent vasorelaxation, in which the nitric oxide plays a key-role (Green et al., 2004). Nitric oxide (NO) originally identified as the endothelial-derived relaxing factor (Furchgott and Zawadzki, 1980), is a labile, lipid soluble gas synthesized from the amino-acid L-arginine through the action of some isoforms of the nitric oxide synthase (NOS) (Palmer et al. 1988). The constitutive isoforms I (neuronal or nNOS) and III (endothelial or eNOS) have been expressed in skeletal muscle, among which NOS I seems to be ubiquitous in muscle fibers (Planitzer et al., 2001), whereas NOS III has its major expression in endothelial cells of blood vessels (Momken et al., 2004). Nevertheless, such an enzyme-linked location has been shown to be not strict and both isoforms are expressed in muscle fibers. In skeletal muscle, NO mediates strength development (Förstermann et al., 1998), fiber growth (Sheehy et al., 1997) and myogenic differentiation (Kaliman et al., 1999), roles also shared by exercise (Longhurst and Stebbins, 1997; Wackerhage and Ratkevicius, 2008; Wahl et al., 2008) and by anabolic androgenic steroid through androgenic receptor (AR)-mediated signaling (Jasuja et al., 2005a, 2005b). Other effects caused by AAS use, such as muscle mass boost, fat body decrease, improvement of strength and physical performance (Hartgens and Kuipers, 2004),

and adaptive responses of muscle through fiber-type transition and fiber hypertrophy are also shared by controlled exercise program (Kadi 2000; Fontana et al., unpublished). Much of these events were also shown to have the involvement of nitric oxide synthase (Smith et al., 2002). Overall, it becomes obvious that anabolic androgenic steroids, exercise training and nitric oxide have roles in muscle plasticity likely by action on targets in common. A number of studies have shown the interaction between exercise and AAS use on the adaptive changes of skeletal muscle (see Kadi, 2000). Likewise a number of studies showed that the muscle adaptive changes caused by exercise are mediated by nitric oxide (Maiorana et al., 2003). However, as far as we know, until date no data were available on the effect of AAS on production of nitric oxide, or whether the effects of AAS on skeletal fibers could be mediated by nitric oxide. Our hypothesis is that NO can be a candidate for the role of mediator on the reported effects of AAS on skeletal muscle. In previous studies in which we used the same experimental protocol of AAS administration associated or not with aerobic exercise in a murine transgenic model (see Fontana et al., 2008) we found differential response of the *soleus*, *tibialis* anterior and *gastrocnemius* muscles in regard to fiber hypertrophy and fiber-type transition (Fontana et al. in preparation). In the present study, the expression of NOS I and NOS III was evaluated in the same experimental model to test indirectly the hypothesis that NO production could be affected by AAS use in sedentary or exercised mice submitted to intense aerobic exercise, and hence to suggest that NO may intermediate AAS-induced skeletal muscle remodeling. The expression of NOS I and NOS III was assayed through western blotting and immunohistochemistry.

Material and Methods

Animals

The experimental protocol was approved by the University's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP) and followed the "Principles of Laboratory Animal Care" (NIH

publication no. 85-23, revised 1996). The transgenic mice ($CETP^{+/-}LDLr^{-/+}$) used in this study have been cross-bred and maintained in the Department of Physiology and Biophysics, Institute of Biology, UNICAMP. The mice (C57/BL6 strain) were heterozygous for the human CETP transgene and for the LDL-receptor null allele (Cazita et al. 2003, Casquero et al. 2006). The mice were housed in a temperature-controlled room (22 ± 1ºC), humidity of 55-65%, 12 h light/dark cycle and had free access to water and standard food (Nuvilab, Colombo, Paraná, Brazil).

Twenty four young adult male mice ($CETP^{+/-}LDLr^{-/+}$) with 2-month-old (~20-22 grams) at the beginning of the experiment and 3.5 month-old at the sacrifice were used. The animals were divided in two groups (n = 12/group), two sedentary (Sed) and two exercised (Ex). Half of each group received gum arabic (Sed-C and Ex-C) and half received mestolone (Sed-M and Ex-M). Exercise consisted in treadmill running in a motorized device with 12 separate lanes and controlled speed, each mouse submitted to involuntary running in separate lanes. After a 5-day of low to moderate level running adaptation (15 m/min during 20 min/day) on a 0 degree grade, the velocity and the duration of daily training was progressive from the 1^{st} to 3^{rd} week (12.42 m/min during 20 min/day to 16.68 m/min for 45 min/day, respectively), after which the speed were set stationary at 17.04 m/min speed for 60 min/day during the last three weeks of training (from 4^{th} to 6^{th} wk), always 5 days/week as outlined in detail in Fontana et al.[13]. The animals received either mesterolone (AAS) (Proviron trademark of Schering, Schering do Brasil, São Paulo, SP, Brazil) or gum arabic (vehicle) (Sigma Chemical, St. Louis, MO, USA) (2 µg/g body weight) by orogastric via during the last 3 weeks (three days a week: Monday, Wednesday and Friday) of training or sedentary period. Gum arabic (extracted from Acacia genus), commonly used in the formulation of suspensions of hydropathic pharmaceuticals, was used as vehicle given its non-toxic and pro-absorptive effect in small intestine and (Codipilly et al., 2006).

Tissue Collection

At the end of the experimental period, overnight fasted mice were deeply anesthetized with a 1:1 mixture of ketamine chloride (Dopalen, 100 mg/kg of animal) and xylazine chloride (Anasedan, 10 mg/kg,) (2 µl/mg body mass, i.p.). Both anesthetics were from Vetbrands (Jacareí, SP, Brazil). SOL, TA and GAS muscles were removed, embedded in gum tragacanth and immediately frozen in isopentane cooled to -159 ºC in liquid nitrogen. The tissues were then stored at -70 ºC until processed.

Western blotting

SOL, TA and GAS muscles were homogenized in an extraction cocktail (10 mM EDTA, 2 mM PMSF, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 µL of aprotinin/mL and 100 mM Tris, pH 7.4). The homogenates were centrifuged at 3.000 g for 10 minutes and the supernatants collected and stored at -70ºC until used for electrophoresis gel. Protein concentrations were determined with a Bio-Rad protein assay kit. Aliquots of SOL (70 µg), TA and GAS (50 µg) were applied to 6% polyacrylamide gels (Hames, 1990). After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane by electroblotting (Towbin et al., 1979), and the membrane then blocked overnight at 4ºC in Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween 20 with 5% non-fat milk. The membranes were incubated for 2 hours at RT with polyclonal anti NOS I and monoclonal anti NOS III antibodies (both diluted 1:50 in TBS; Transduction Laboratories, Lexington, KY, USA). Subsequent to incubation, the membranes were rinsed six-times (10 minutes each) in TBS and then incubated with respective HRP-conjugated secondary antibody (Sigma, St. Louis, MO, USA) (diluted 1:1000 in TBS) for 1.5 hour at RT. After rising in buffer, the immunoreactive bands were detected by chemiluminescence (Super Signal, Pierce West Pico Chemiluminescent Substrate, Rockford, IL,

USA) using X-ray film (BioMax XAR Film Kodak, Rochester, NY, USA). Densitometric analysis were done using UN- SCANN- IT version 6.1 (Silk Scientific Corporation, Utah, USA).

Immunohistochemistry

The samples from SOL, TA and GAS muscles were obtained through cryostat cross-sections (6 µm thick) and were collected on silane-coated glass slides, air dried and fixed in cold acetone (4ºC) for 4 minutes at room temperature (RT). The sections were washed in 0.05 M Tris-buffered saline, pH 7.4 (TBS) for 5 minutes at RT. To quench endogenous peroxidase acitivity, sections were treated with methanol containing 0.3% H_20_2 for 30 minutes at RT, after which they were rinsed four times with TBS (5 minutes each). Non-specific sites were blocked with TBS containing 5% non-fat milk at RT for 1 hour. The sections were then incubated at 4°C overnight with primary antibody to NOS I and NOS III (diluted 1:25 in TBS containing 1% non fat-milk; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in TBS, the bound primary antibodies were detected by incubating the sections with biotinylated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (diluted 1: 4 serum blocking solution) for 30 minutes at RT, followed by washing in TBS and incubation with HRP-streptavidin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 minutes. The sections were subsequently washed four times in TBS (5 minutes each). The reaction was developed using 3,3-diaminobenzedine (DAB, Sigma Chemical, St. Louis, MO, USA) and stopped with distilled water. The slides were counterstained with Harris's hematoxylin, mounted with Canadian Balsam and examined by light microscopy. For negative controls, the primary antibodies were omitted.

Statistical analysis

All numerical results were analyzed using the Origin software package (Microcal^{TM} Software Inc., Northampton, MA, USA) and expressed as the mean ± standard deviation (DP). The statistical

significance among the control and treated groups was determined by One-way ANOVA followed for the Tukey test. A value of $P<0.05$ indicated statistical significance. In addition, two-way ANOVA was used to determine how a response was affected by two factors, i.e. exercise training and mesterolone use.

Results

Western blotting

The results showed that NOS I and NOS III had their highest expression in TA muscles with prevalence of the latter in relation to the former. The SOL muscle showed the least expression of both enzymes. NOS I expression in TA was higher by 2.5-fold than in SOL and by 2-fold than in GAS. NOS III expression in TA was higher by 4-fold than in SOL and by 2-fold than in GAS. Figure 1 and Table 1 summarizes the effects of mesterolone alone (Sed-C x Sed-M), exercise alone (Ex-C x Ex-M), the effect of mesterolone in exercised mice (Ex-C x Ex-M), and the effect of exercise in the mesterolone-treated mice (Sed-M x Ex-M).

The treatment with mesterolone in sedentary mice (Sed-C x Sed-M) increased significantly the expression of NOS I and NOS III in all the three muscle, with exception of GAS where mesterolone did not affect the expression of NOS I ($p < 0.05$).

The exercise *per se* (Sed-C x Ex-C) increased significantly the expression of NOS I (by 13.7%) and NOS III (by 7.4%) in SOL, and NOS III (5.1%) in GAS. The aerobic exercise did not significantly affect the expression of both enzymes in TA and NOS I in GAS ($p < 0.05$).

The exercise to mice to which was administered mesterolone (Sed-M x Ex-M) increased significantly the expression of NOS I in all three muscle, however, it only increased NOS III expression in TA ($p < 0.05$). Seemingly, mesterolone intake by trained mice (Ex-C x Ex-M) caused increases in NOS I expression in all muscles assayed, whereas it promoted increase of NOS III in SOL and TA and not

in GAS ($p < 0.05$). Two-way ANOVA showed that there was interaction between exercise and AAS for NOS III expression from GAS muscle.

Immunohistochemistry analysis

Imunohistochemistry was undertaken to detect the possible alterations in the location of NOS I and NOS III isoforms in the muscle tissue of SOL, TA and GAS. Figure 2 shows cross-sections of the muscles of sedentary mice treated with vehicle (gum arabic/placebo group, Sed-C). Qualitative variation in the immunolabeling of NOS I and NOS III was detected in all experimental groups (not shown). Both isoforms were immunolabeled in the sub-sarcolemmal region and sarcoplasm in fibers of all muscles, except for SOL where the labeling of NOS I isoform was faint in some fibers and absent in others. The strongest labeling was exhibited by the TA, followed by GAS. These finding paralleled with the immunoblotting findings. Labeling of NOS I or NOS III was seen in muscle spindle (Fig. 2D,F), nerves (Fig. 2A,E,F) and blood vessels (Fig. 2A,F).

Discussion

Our study used three hind limb muscles which differ in metabolic and contractile responses to assess the effects of mesterolone treatment in mice sedentary or submitted to intense involuntary aerobic training in the expression of neuronal and endothelial NOS isoforms. The TA muscle presented the highest expression of both isoforms of NOS in all groups of animals, sedentary mice treated with vehicle (placebo, control group taken as reference) or with mesterolone and the matched controls. The mesterolone major effect was on the expression of NOS I which was by 30.4% upregulated in TA in comparison to SOL (11.7%) and GAS (where no significant increase was observed). In a previous study using the same study design and murine model (Fontana et al., in preparation) mesterolone did not cause significant fiber type transformation in TA and GAS of sedentary mice

(Sed-M group animals) but promoted significant hypertrophy of type I, IIDA and IID/X fibers in TA, and type I, IIA, IIDA IID/X and IIB in GAS. In contrast, mesterolone promoted significant *Soleus* adaptive remodeling by increasing the proportion of type IC, IIAC, IIDA and IID/X fibers and decreasing IIA (a fast oxidative fiber type); the steroid also caused significant hypertrophy of type I, IIA and IID/X fibers. The results suggest that in a muscle fast-twitch, preponderantly glycolytic, such as TA, has adaptive changes induced by the steroid mediated by NO than the slow-twitch predominantly oxidative muscle SOL (GAS had no involvement). In contrast, NOS III expression increased by 9% in SOL and GAS, whereas the lowest increase was for TA (by 3.7%). The involvement of NOS I in animals submitted to aerobic exercise and treated with gum arabic (comparison of Sed-C x Ex-C) was only seen in SOL (13.7% increase), whereas the involvement of NOS III was seen in SOL and GAS (7.4% and 5.1% increase, respectively). When mesterolone-treated animals were submitted to exercise (comparison between Sed-M and Ex-M) the expression of NOS I increased by 95.3% in GAS, 22.3% in TA and 11.6% in SOL. NOS III expression was increased only for TA (4.5%) ($p<0.05$), suggesting that steroid affects positively on production of NO mainly in muscle oxidative. The findings showed that NO mediated signaling was more prominent for the anabolic steroid mesterolone than for the aerobic exercise. On the other hand, when exercised mice were treated with mesterolone (comparison between Ex-C vs. Ex-M) involvement of NOS I increased by 95.2% for GAS, 56.6% for TA and 9% for SOL, meaning that NO mediation was favorable to intermediate muscle with oxidative-glycolytic metabolism. NOS III expression increased only by 5.5% in SOL, 7% in TA and GAS was no affect. Some authors report that NOS I is connected with type IIB fibers - fast glycolytic fibers in rodents and the NOS expression does not correlate to hybrid types (Kobzick et al., 1994; Grozdanovic et al., 1995).

In this study, NOS III expression, for the three muscles studied with four different experimental groups, showed slightly altering in NO production. A reduction in NO synthesis can be interpreted as

a compensatory mechanism to improve cellular respiration in deficient mitochondria, as shown previously with other types of stress (Brookes et al., 2001; Venkatraman et al., 2004).

In current study, Punkt et al., 2006, showed the connection of NOS immunoreactivity to fast oxidative glycolytic (FOG) fibers in human muscles and observed that the NOS is susceptible in the oxidative and glycolytic fiber. Moreover, fibers with these metabolisms showed faint NOS expression, suggesting that NO might be involved in the oxidative metabolism in correlation with fast force development which occurs in FOG fibers.

Momken et al., 2002 observed that deficiency in NOS III reduces oxidative capacity in slow twitch skeletal muscle. Mice knockout to NOS III show reduced angiogenic ability (Murohara et al., 1998). On the other hand, in mammals training induces an increase of vascularization in muscles that strongly correlates with mitochondrial volume density and enzymes involved in energy metabolism (Holloszy and Coyle, 1984) and increased functional capacity and cardiac protective effects associated with higher physical activity (for review, see Kingwell 2000).

In conclusion, three different muscles with differences metabolism showed evidence of NO expression affects mainly the fast glycolytic fibers (TA) than fast-oxidative fibers (GAS) and slow oxidative (SOL). TA muscle express higher concentrations of NOS I and NOS III than GAS and SOL ones and may be enclose the highest quantity of potential target molecules of the NO produced. So, fast oxidative and oxidative metabolism can signal by small differences in the NO meditation.

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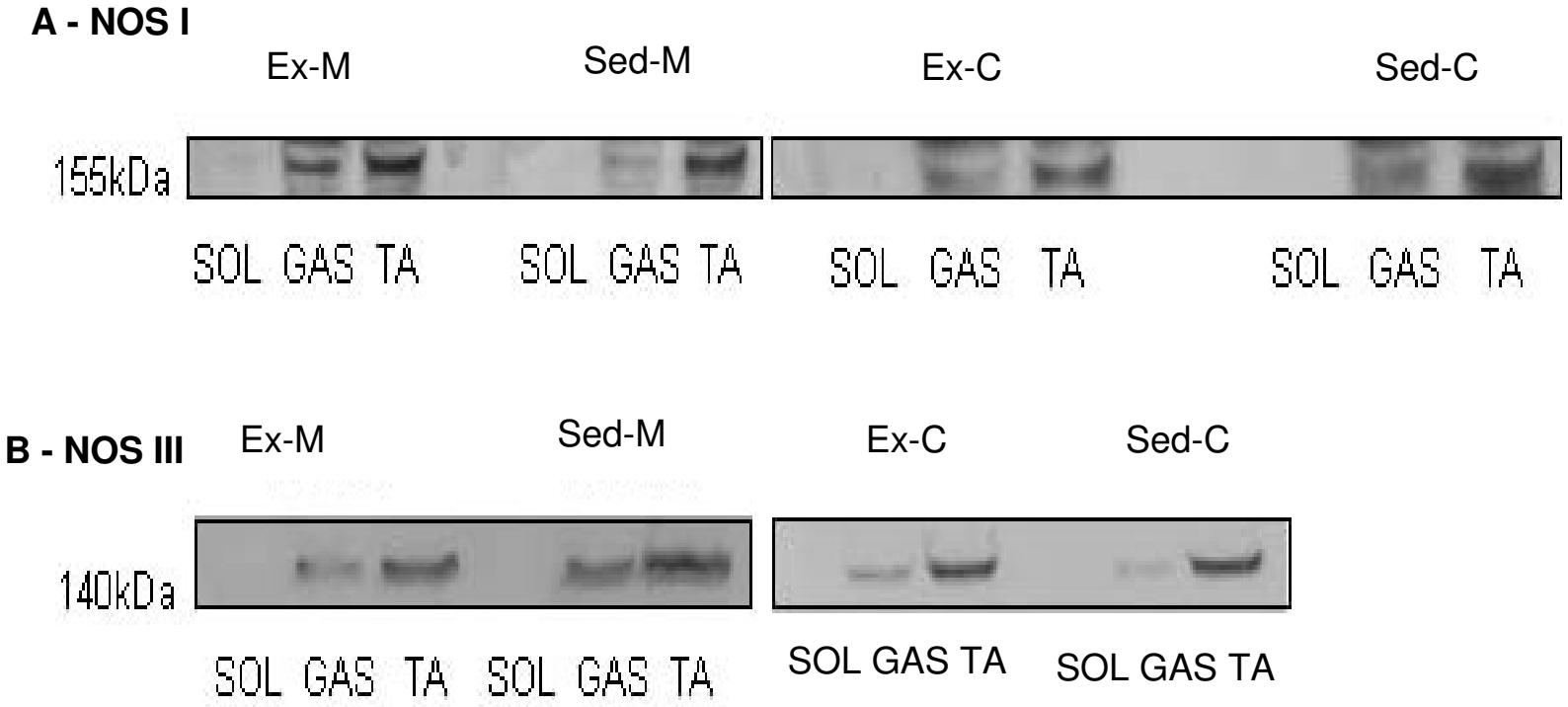

Figure 1 - Western blotting for analysis of the NOS I (A) and NOS III (B) isoform expression in the four experimental groups. Note the faint expression of NOS I and NOS III to SOL muscle and the strong expression to TA and GAS muscles.

Table 1. Quantitative analysis (by pixels) of NOS I and NOS III expression in the $CETP^{+/-}LDLr^{-/+}$ transgenic mice.

	NOS I			NOS III		
muscles / groups	**SOL**	**TA**	**GAS**	**SOL**	**TA**	**GAS**
Sed-C	$18.60\pm0.56^{\#\&\dagger}$	$42.03\pm0.78^{\#\&}$	$23.55\pm0.42^{\#}$	$19.14\pm0.60^{\#\&\dagger}$	$78.43\pm0.41^{\#\&}$	$37.16\pm0.77^{\#\&\dagger}$
Sed-M	$20.66\pm1.08^{\#\S}$	$54.82\pm0.89^{\#\dagger\Delta}$	$23.94\pm0.45^{\&}$	$20.91\pm0.36^{\#}$	$81.37\pm0.55^{\#\dagger\Delta}$	$40.51\pm0.56^{\#\S}$
Ex-C	$21.15\pm0.67^{\&\Delta}$	$42.82\pm1.01^{\dagger\blacktriangledown}$	$23.96\pm0.19^{\dagger}$	$20.55\pm0.37^{\dagger\S}$	$79.60\pm1.02^{\S\Delta}$	$39.07\pm0.81^{\&\S}$
Ex-M	$23.05\pm0.63^{\dagger\S\Delta}$	$67.04\pm0.91^{\&\Delta\blacktriangledown}$	$46.76\pm0.93^{\#\&\dagger}$	$21.69\pm0.52^{\&\S}$	$85.01\pm1.30^{\&\dagger\S}$	$39.70\pm0.40^{\dagger}$

Statistical analyses showed that there were significant differences as follows:
NOS I: **SOL:** $^{\#\&\dagger\S}p<0.001$ and $^{\Delta}p<0.01$**; TA:** $^{\#\&\dagger\S\Delta\blacktriangledown}p<0.001$**; GAS:** $^{\#\&\dagger}p<0.001$**.**
NOS III: SOL: $^{\#\&}p<0.001$ and $^{\dagger\S}p<0.01$; **TA:** $^{\#\&\dagger\S}p<0.001$ and $^{\Delta}p<0.05$; **GAS:** $^{\#\&\dagger}p<0.001$ and $^{\S}p<0.01$. (One way ANOVA followed by Tukey test).

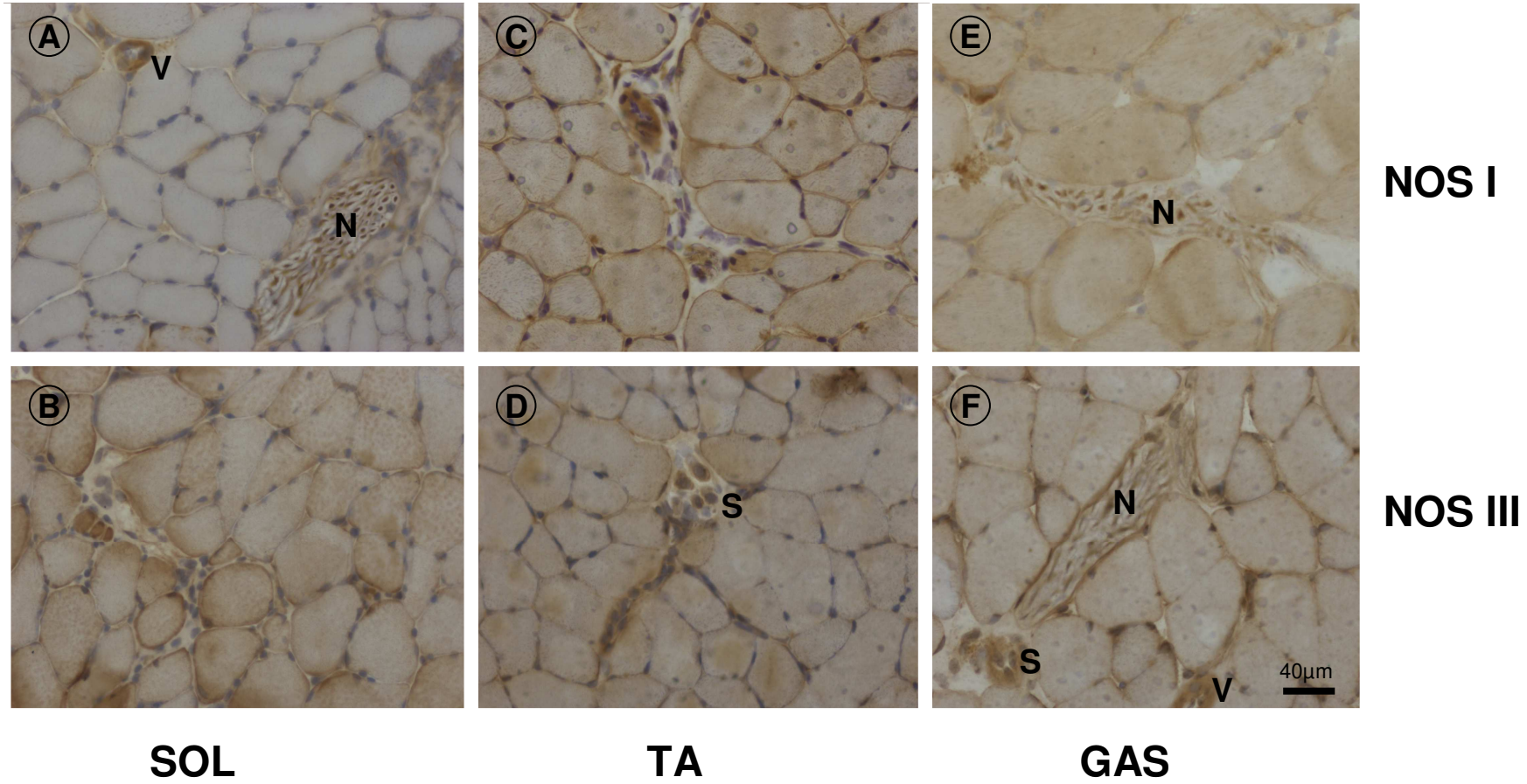

Figure 2 - Imunohistochemistry of the expression of NOS I and NOS III in transversal sections in SOL, TA and GAS muscles from sedentary animals treated with arabic gum. Note: TA showed the stronger labeling (C-D), followed by GAS (E-F) and SOL the fainter labeling (A-B). The isoforms were expressed in subsarcolemal regions and mitochondria regions (intermyofibril and subsarcolemal), spindle fiber (S), vessel (V), nerve (N). Barra: 40 µm.

Capítulo IV

Capítulo IV:

Para alcançarmos os nossos objetivos, foram realizados: Gel SDS-PAGE para detecção das proteínas colagênicas e não colagênicas, dosagem de hidroxiprolina, ultra-estrutura dos fibroblastos, morfometria do diâmetro e da área seccional transversa das fibrilas de colágeno.

Artigo IV: ***The Journal American of Sports Medicine*** **– Submetido.**

EFFECT OF HIGH-INTENSITY AEROBIC EXERCISE AND MESTEROLONE ON THE ACHILLES TENDON OF TRANSGENIC MICE

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Running title: AAS and exercise effects in Achilles tendon

Abstract

Background: Anabolic-androgenic steroids use is widespread among professional and recreational athletes; therapeutically it can be used in sarcopenia-linked clinical conditions.

Purpose: To study anabolic-androgenic effects combined with a high intensive exercise program in the Achilles tendon.

Study Design: Controlled laboratory study.

Methods: Sedentary mice treated orally with mesterolone (Sed-M) or vehicle (Sed-C, placebo/control) and matched exercised (Ex-M and Ex-C) were used. Tendons were excised and processed for analysis by SDS-polyacrylamide gel electrophoresis for collagen bands detection and for determination of hydroxyproline concentration. Determination of collagen fibrils diameter and the area of fibrils contained in an area probe and ultrastructure of fibroblasts was made with transmission electron microscopy.

Results: Remarkable presence of collagen in all groups. The collagen content in Sed-M, Ex-C and Ex-M was higher than in Sed-C, what was validated by hydroxyproline quantification. Noticeable bands of non-collagenous proteins were found in Sed-M and Ex-M, whereas just traces were found in Ex-C, but β-chain appeared in Ex-C. The number of fibrils into the area probe increased markedly in Sed-M and Ex-C (12-fold), but their diameter and area were unchanged compared to Sed-C. In Ex-M, the number of fibrils decreased by 3-3.5-fold compared to Sed-M and Ex-C, whereas diameter and area increased significantly. Sed-C tenocytes looked quiescent, whereas in the other groups looked activated with hypertrophied processes, well developed nucleolus and organelles

Conclusion: Aerobic exercise and mesterolone, separately or associated, have ergogenic effects which are reflected on tendon remodeling and likely on tendon strength.

Clinical Relevance: Anabolic-androgenic plus exercise improves synthesis of collagenous and non-collagenous proteins, increases diameter and area of collagen fibrils, the knowledge of which could have impact in clinical sports medicine.

Keywords: Anabolic androgenic steroid, fibril collagen diameter, fibril collagen area, hydroxyproline

INTRODUCTION

Anabolic androgenic steroids (AAS) although benefic when administered therapeutically to elderly, hypogonadal or sarcopenic patients[2] could lead to serious long-term health complications when abused. Despite banned from sports competition, AAS self-administration continues to be practiced both by elite athletes and people training at gyms that aspire body sculpturing, strength, aggressiveness and enhanced athletic performance.[26] At higher doses and combined with a training program AAS intake leads to increased muscle power and mass.[1] Androgens elicit their anabolic effects by acting through androgen receptors (ARs) present in muscle fibers[11,17,21,23,29] the expression of which can be upregulated by hormones and physical exercise [7,25,43.] ARs have been also detected in other cell types, including in different populations of fibroblasts, such as dermal fibroblasts,[42] sciatic nerve endoneurial fibroblasts[19] and fibroblasts from skeletal muscle.[35] Monks and collaborators[35] also reported that castration decreased AR(+) fibroblasts in muscle and testosterone treatment prevented such castration effect. No available information about the presence of AR(+) fibroblasts in tendons (tenocytes) was

found in literature. However, tendon adaptive changes in response to inactivity, exercise or steroid hormones level has been associated with changes in the morphology, mechanical properties and molecular biology of the tendons[4,5,18,22,27,28,44,46] among which some showed evidences of tendon-induced pathologies.[12,24,32,34,48] Since, tendons connect muscles to bones and have a key role in musculoskeletal biomechanics it is likely that the augment of muscle power and mass would reflect in tendon response to the new loading condition.

The main aim of this study was to analyze the hydroxyproline content of the Achilles tendon (AT) to infer on the collagen synthesis in mice administered orally mesterolone combined or not with high-intensity exercise training. Also, we measured the total area of the fibrils contained into an area probe, fibril diameter and analyzed the ultrastructure of tendon fibroblasts by transmission electron microscopy. The study is part of a comprehensive study dealing with the effects of AAS use and intensive exercise in several biological systems.

MATERIALS AND METHODS

Animals

The mice (C57/BL6 strain) used in this study were adult males aged two months and weighing 20 - 22 g at the beginning of the study. They were heterozygous for the human CETP transgene and for LDL-receptor null allele ($CETP^{+/-}LDLr^{-/+}$) what gives to this mice a lipoprotein profile very close to the human profile, and have been described in detail elsewhere.[8,9] The mice were housed in groups of three in a controlled environment

room (temperature 22 ± 1ºC; humidity 55-65%;12h light/dark cycle) with free access to water and standard chow (Nuvilab®, Colombo, PR, Brazil). See Fontana et al.[13] for additional information.

Mesterolone and Gum arabic

Mesterolone (AAS, 1alpha-methyl-5alpha-androstan-17beta-ol-3-one) is a non-17 alpha-alkylated derivative of testosterone, commercially distributed as Proviron® by Schering do Brazil, São Paulo, SP, Brazil. Gum arabic (GA) is a complex polysaccharide produced by a large number of different species of shrubs of the genus *Acacia.* The gum was purchased from Sigma (St. Louis, MO, USA) and used as vehicle for mesterolone.

Study groups and Experimental Protocol

Twenty four young male mice were divided into four groups (n = 6/group) of which two were sedentary and the other two were submitted to involuntary running in motorized treadmill always during morning. Both in the sedentary and exercised groups, the animals were given either gum arabic (Sed-C and Ex-C, control groups) or mesterolone (Sed-M and Ex-M). Exercised groups were allowed to adapt to the treadmill for one week prior to the start of the experimental protocol. The adaptation period comprised low to moderate exercise level on the treadmill daily for 5 days (15 $m.min^{-1}$ for 20 $min.day^{-1}$) followed by two days rest. After adaptation, exercised mice were submitted to 6 weeks of intensive aerobic exercise training (treadmill running) on a weekly cycle of 5 consecutive exercise days followed by two days rest. Both the velocity and duration of running was progressive from the 1st to 3rd week (12.42 $m.min^{-1}$ for 20 min to 16.68 $m.min^{-1}$ for 45

min, respectively), after which they were set stationary in 17.04 $m.min^{-1}$ for 60 $min.day^{-1}$ during the last three weeks of training (4 to 6), as outlined in detail in Fontana et al.[13] This program is a form of endurance training and does not compare with power training. During weeks 4-6 mice (sedentary and exercised) received either gum arabic or mesterolone suspended in gum arabic on three alternate days (2 µg/g body weight) by gavage, always at 10:00 a.m.

At the end of the experimental program mice were fasted overnight and terminally anesthetized with a 1:1 mixture of ketamine chloride (Dopalen®, 100 mg kg^{-1} body weight) and xylazine chloride (Anasedan®, 10 mg kg^{-1} body weight) i.p.(2 µg/mg body mass) both anesthetics from Vertbrands, Jacarei, SP, Brazil. The Achilles tendons were dissected and processed for transmission electron microscopy (TEM), or were frozen in isopentane cooled to -156ºC in liquid nitrogen, after which it was stored at -70ºC for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for hydroxyproline (HO-Pro) dosage.

The "Principles of Laboratory Animal Care" of the Brazilian College in Animal Experimentation – COBEA were followed and the study design, protocol and euthanasia of the animals were approved by Institute of Biology's Committee for Ethics in Animal Experimentation (CEEA/IB) of the UNICAMP (Protocol nr. 700-1)

Extraction procedures and Sodium dodecyl sulfate-electrophoresis (SDS-PAGE)

The tendons were treated with 25 volumes of 4 M guanidine chloride (GuHCl), containing 1 mM PMSF and 20 mM EDTA in 50 mM sodium acetate buffer, pH 5.8[16] at 4°C for 24 h. The mixture was then centrifuged (13.000 x g, 4°C, 30 min) and the supernatant was precipitated in acetate-ethanol and analyzed by SDS-PAGE according to

Zingales[51] using gradient gels (4-16%). Staining was done with Coomassie brilliant blue R-250.

Hydroxyproline quantification

Hydroxyproline (HO-Pro) was used as an indicator of the collagen content. The tendons were hydrolyzed in 6 N HCl (10 mg of tissue/ml) for 4 h at 130°C, after which the hydrolysate was treated with chloramine T and perchloric acid/aldehyde. After incubation for 15 min at 60°C, the material was cooled and the absorbance at 550 nm was read. The amount of HO-Pro in the sample was calculated by comparison with a standard curve of HO-Pro and expressed as mg/g of wet tissue.[47]

Transmission Electron Microscopy (TEM)

Collagen fibril diameter: For diameter measurements of collagen fibrils by TEM Achilles tendon fragments taken at the tension region (proximal) were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, at pH 7.2 for 24 h, then post fixed with 1% osmium tetroxide in the same buffer at 4ºC for 1 h, dehydrated in acetone and embedded in epon-araldite resin mixture. Ultrathin sections (20-60 nm) were stained with 2% uranyl acetate (25 min) and 2% lead citrate (7 min) prior to observation with TEM (Zeiss, Leo 906) operated at 60 kV. Transversal sections of three tendons were analyzed by TEM. Images with magnitude of 464 640 x obtained from six non-overlapping randomized areas analyzed per tendon were examined by software ITEM 5.0 (Soft Imaging

System, Olympus, Japan). Collagen fibrils were measured into an area probe of 5 mm^2 dimension and the diameter of about 150±35 fibrils per group were determined.

Determination of the area of collagen fibrils: An average of six digitized electron microscopy images was obtained from a known area of 5 mm^2 from each cross-section of the tendons of the four experimental groups (n=3 mice/group). The collagen fibrils contained within the area probe were counted and their diameter measured. To calculate the total area (A = sum of the collagen fibrils area located inside the probe area) the formula: $A = \pi.r^2$ x number of fibrils into the probe area.

Fibroblast ultrastructure: For the ultrastructure analysis of fibroblasts, the dissected soleus muscle-Achilles tendon complex at the calcanneal insertion region (n=3/group) was excised, cut transversally and fragments were processed as described above.

Statistical analysis

All numerical data were presented as mean ± standard deviation (SD). We used one-way ANOVA variance analysis followed for the Tukey test to compare differences among groups. A value of $P<0.05$ indicated statistical significance. Two-way ANOVA was used to determine whether mesterolone treatment or the exercise training influenced hydroxyproline quantification and collagen fibril diameter, and whether there was interaction between exercise and mesterolone. Graph Pad Prism version 5.0 (San Diego, CA, USA) was used for all statistical analyses.

RESULTS

Biochemical analysis

SDS-PAGE electrophoresis analysis showed a remarkable presence of α1 and α2 collagen chain which was gradually more prominent in Sed-M, Ex-C and Ex-M groups. Moreover, β-chain bands were prominent only in exercised animals treated with gum arabic (Ex-C group). On the other hand, while exercise *per se* reduced markedly the presence of non-collagenous proteins (NCP) in Achilles tendon (comparison of Sed-C *vs*. Ex-C), mesterolone *per se* maintained almost all the bands displayed in Sed-C and besides exhibited other bands of such proteins (compare Sed-C *vs*. Sed-M). Interestingly, the exercise in animals treated with mesterolone accentuated (additive effect) the appearance of NCP (compare Sed-M and Ex-M). In contrast, mesterolone intake by exercised animals blunted the effect of exercise especially in regard to NCP (compare Ex-C and Ex-M) (Figure 1A).

Quantification of HO-Pro content corroborated the SDS-PAGE profile. Mesterolone exhibited a trend to increase HO-Pro content in sedentary mice (see Sed-C *vs*. Sed-M), whereas an effective increase was seen when M was administered to exercised mice (see Ex-C *vs*. Ex-M). Significant differences were observed between Sed-C *vs.* Ex-C (7.3 ± 1.9 *vs.* 20.6 ± 5.0), Sed-M *vs*. Ex-M (13.1 ± 4.9 *vs*. 37.3 ± 6.2) and Ex-C *vs*. Ex-M (20.6 ± 5.0 *vs*. 37.3 ± 6.2) ($p<0.001$), indicating (indirectly) that exercise effect surpassed the mesterolone effect in promoting synthesis of collagen. In addition, exercise alone (Ex-C mice) exhibited a striking band of collagen (β-chain), not observed in Sed-C, Sed-M and Ex-M. Given that the Ex-M group exhibited the highest value of

HO-Pro content, it can be inferred that a synergism existed between the aerobic exercise and the steroid oral intake (Figure 1B). Two-way ANOVA showed that there was interaction between mesterolone and exercise in HO-Pro concentration ($p=0.0115$).

Transmission Electron Microscopy

Collagen fibril diameter: No change in the ultrastructural characteristics of collagen fibrils sectioned transversally was noticed (not shown). Collagen fibrils diameter measurements of all groups showed sizes ranging from 37.6 to 215.6 nm. Group-specific size ranges were 37.6 – 41.2 nm for Sed-C; 37.6 to 42.4 nm for Sed-M; 47.2 to 50.1 nm for Ex-C and 168.2 to 215.6 nm for Ex-M. Mesterolone alone to sedentary mice did not alter the fibrils size (Sed-C *vs.* Sed-M), neither exercise alone (Sed-C *vs*. Ex-C). However, both mesterolone intake by animals submitted to intensive aerobic exercise as well as the exercise training to mesterolone-treated animals produced a remarkable increase in fibrils diameter, as shown by the significant differences observed between Sed-M *vs.* Ex-M (40.3 ± 1.9 nm *vs.* 185.9 ± 15.5 nm) and between Ex-C *vs.* Ex-M (48.6 ± 1.2 nm *vs.* 185.9 ± 15.5 nm) ($p<0.001$) (Figure 2A). Two-way ANOVA confirmed interaction between mesterolone and exercise in collagen fibrils diameter increase ($p<0.0001$).

Determination of the area of collagen fibrils: Fibers were counted in a probe area in each group and the area calculated. The number of fibrils within the area probe was different between groups. In sedentary mice treated with mesterolone (Sed-M) the number of

collagen fibrils increased by 12-fold the number seen in sedentary treated with vehicle (Sed-C mice); exercise alone (Ex-C) also elevated by 12-fold the number of fibrils in comparison to Sed-C; exercised animals treated with mesterolone (Ex-M) showed 3 to 3.5-fold decrease of fibrils than Ex-C and Sed-M, respectively. Collagen fibrils area calculation of all groups showed sizes ranging from 0.01 mm^2 to 3.11 mm^2. Group-specific area ranges were 0.01 – 0.026 mm^2 for Sed-C; 0.10 to 0.42 mm^2 for Sed-M; 0.10 to 0.56 mm^2 for Ex-C and 0.8 to 3.11 mm^2 for Ex-M. Inter-groups comparison showed significant differences in the area occupied by fibrils (sum of fibrils areas) into the area probe (5 mm^2 dimension). The exercise effect on mesterolone-treated mice (Ex-M) increased by 7.2-fold the area in comparison to Sed-M); mesterolone effect on exercised mice (Ex-M) increased by 5.8-fold the area in regard to Ex-C ($P < 0.001$), indicating a synergic effect of M and Ex. Likewise, exercise and mesterolone alone did not cause significant alteration of the sum of fibril area (Figure 2B). The results indicate that when combined (Ex + M) exercise neutralizes the effect of the steroid in regard to the number of fibrils contained into the area probe (since there was decrease of fibril in Ex-M compared to Ex-C and Sed-M); however, but they are synergic in regard to the fibril size (since the size of fibrils increased in Ex-M in relation to Ex-C and Sed-M). Two-way ANOVA confirmed interaction between the steroid and aerobic exercise ($P=0.0037$).

Fibroblast ultrastructure: Ultrastructural analysis showed that fibroblasts from Sed-C were rarer and smaller cells than in animals from Sed-M, Ex-C or Ex-M groups. The cytoplasm around the nucleus was scanty and devoid of well-developed organelles, the more visible being free ribosomes dispersed in the cytoplasm ring. Because the cell

processes were poorly-developed when cross-sectioned only a few round in shape bodies having about 0.3 μm in diameter were seen scattered among the collagen fibrils bundles (Figure 3A). In sedentary animals treated with the steroid (Sed-M) fibroblasts were more numerous, as were so the number of sectioned cell processes dispersed among the collagen fibrils; well-developed rough endoplasmic reticulum (RER) and numerous mitochondria were also seen (Figure 3B). Exercised mice either treated with vehicle or mesterolone (Ex-C or Ex-M) had also more fibroblasts at the AT-soleus-calcanneal insertion region with developed long cell processes containing widened RER cisternae and mitochondria. Nuclei with prominent nucleolus were more frequent (Figures 4A,B). Such characteristics indicate activated fibroblast for synthetic activity, which was in accordance with the biochemical results (Figures 1- 4).

DISCUSSION

The present study examined parameters such as SDS-PAGE profile, hydroxyproline quantification, measurements of collagen fibrils diameter and calculation of the sum of the area of fibrils contained in an area probe, and ultrastructure of fibroblasts to test the hypothesis that collagen synthesis can be altered in Achilles tendons of mice treated with the AAS mesterolone combined or not with an intensive aerobic exercise program. The results indicated that mesterolone alone (Sed-M) induced a trend to increase the collagen content, estimated by the HO-Pro dosage, whereas exercise alone (Ex-C) caused effective increase of collagen content ($p<0.001$).

The analysis in SDS-PAGE showed that collagen bands of Achilles tendon of Ex-C were constituted of striking presence of collagen β-chains in comparison with the other groups. Given that β-chain result from cross-linked dimers composed of two α-chains, the marked prominent band of it in Ex-C suggests that the intensive aerobic training induced increased amounts of cross-links between the collagen chains (compare Ex-C with the other groups). Curiously, the β-chains were not observed in Ex-M, suggesting that mesterolone blunted the exercise effect, probably by impairing the formation of cross-links among the collagen chains, a fact that needs further confirmation. Studies correlate positively the mechanical strength of tendons with increase in fibril diameter and paralleled intermolecular covalent cross-links.[10,20,38,49] Our study failed in showing increase of fibrils size after six-week of resistance training (Sed-C compared to Ex-C), despite significant 2.8-fold increase of HO-Pro in Ex-C. Data on the effect of increased physical exercise on fibril size alteration are not unanimous. There are reports on the increase of fibril size by exercise,[30,41] its decrease[30] and none effects,[16,40] the latter in agreement with our results. We suggest that a possible increase in the quantity of intermolecular cross-links promoted close-packing of aggregated molecules what could explain the non-significant increase of fibrils diameter by exercise alone. Another possibility was the occurrence of fibrillogenesis, where new fibrils were formed in animals submitted to intensive training. In both hypothesis, collagen synthesis was needed, a possibility that has support in the increased HO-Pro concentration indicative of the presence of larger collagen amounts. In support of this, morphological evidences suggested that fibroblasts (tenovytes) from Ex-C, Ex-M and Sed-M were engaged in

active synthesis of proteins. In soft connective tissues, AASs enhance collagen synthesis in a dose-dependent manner.[39]

Our findings showed Ex-C tendons with α-1 and α-2 chain bands more widened than the observed in Sed-C and Sed-M, but less widened than in Ex-M. In agreement, the comparative analysis of the four groups showed that the highest level of HO-Pro was achieved in Ex-M mice. Moreover, the variations in the area of the fibrils (contained into a 5 mm^2 area probe) showed a direct correlation between the variation in the HO-Pro content and the increment of the collagen fibrils area. In accord, our results showed that the major diameter of fibrils was found in Ex-M tendons. It was also observed that mesterolone treatment or the exercise training, separately, resulted in higher number (12-fold) of fibrils than in sedentary animals treated with gum arabic (Sed-C *vs*. Sed-M, and Sed-C vs. Ex-C). Conversely, comparison of Ex-M x Sed-M, or Ex-M x Ex-C showed that Ex-M mice had by 3 to 3.5-fold less number of fibrils than Sed-M or Ex-C into the area probe. However, the collagen fibrils of Sed-M and Ex-C were significantly smaller in diameter and area than that of Ex-M. Hence the sum of areas of Sed-M and Ex-C fibrils contained within a known tendon area probe (frame) was significantly 7.2-fold and 5.8-fold, respectively smaller than the sum of areas of the fibrils found in Ex-M, corroborating the fibrils diameter of Ex-M being by 4.6-fold and 3.8-fold larger than Sed-M and Ex-C, respectively, what allows establish relationship with the remarkable increase of HO-Pro seen in Ex-M. We suggest that a larger number of collagen cross-links could have been promoted by exercise associated to mesterolone leading to the largest diameter and area of collagen fibrils in Ex-M. Such findings indicate that there was a synergic effect of exercise plus steroid in regard to fibril diameter and area, but

antagonistic in regard to fibril number. An interesting finding was that in tendons of exercised mice treated with mesterolone (Ex-M) the average of fibrils diameter was 185.9 nm, whereas in Sed-C, Sed-M and Ex-C the average ranged from 37.6 to 50.1 nm. Further studies to determine whether these changes were associated with increased tendon strength in this experimental model and design would help to advance in the understanding of the subject. Studies have shown that mechanical strength of tendon is related upon the extracellular aggregation of collagen molecules into fibrils with stabilizing molecular cross-links[20] and with fibril diameter distribution, such that large fibrils could resist higher tensile forces.[38] Experimentally, it has been reported that fibril diameter increases during development[38] and decreases with disuse[37] and aging,[36] what indirectly correlates fibril diameter with strength.

In the current study, Ex-M tendons showed the highest content of collagen apparently with a major contribution given by increased collagen α-1 and α-2 chains. Studies in rats have shown that AAS treatment by six weeks did not produce any visible change in the appearance of collagen fibrils, nor in fibril diameter or shape, but biomechanical evaluation showed tendon rigidity and less capacity of absorbing energy despite no changed in tendon strength was detected.[16] Whether the rigidity caused by AAS was associated with decreased amount of cross-links was not investigated by the authors. Our study also did not show morphological alterations of collagen fibrils by TEM, but modification in the crimp pattern and biomechanical properties[50] and tendon rupture[12] by the steroids have been reported. Other studies have also associated the use of AAS to inefficacious tendon extensibility,[34] tendinopathies,[48] tendon rupture[3,45] some

associated with impairment of collagen homeostasis[24,31,32,39] AAS administration was shown to modify the biochemical properties of tendon by altering collagen structure.[12,24]

Another interesting finding observed on the SDS-PAGE analysis was the remarkable presence of non-collagenous proteins (NCP) mainly in Sed-M and Ex-M mice, but also in Sed-C, and as such remarkable, the absence of a great number of them in Ex-C mice. These results and the related to collagen proteins indicated that mesterolone alone or associated with intensive aerobic training caused influence not only in the collagen content but also in non-fibrous proteins. NCP are not structural proteins and hence they were not directly involved in formation of structural components in the tendon. Nevertheless, they are essential in cell matrix modulation, in interaction amongst the extracellular matrix components and in the cell function.[6] *In vitro*, such proteins may regulate the adhesion, migration and cellular survival, activity of NF-$_k$B, and NO synthesis.[14] NCP remain at basal level in adult tissue, but are more intensely expressed during development and have its expression increased in inflammation process and tissue remodeling,[33] indicating that a possible regulatory role is reserved to these proteins. In the current study, expressive bands of non-collagenous proteins were observed in Achilles tendons of mice treated with mesterolone, regardless sedentary or trained (although some differences in the detected bands exist among Sed-M and Ex-M). Analyzing the results of SDS-PAGE it was observed NCP presence in all groups. Special attention should be done to the Ex-M, where the presence of NCP was more pronounced, and in Ex-C, where faint bands of NCP were observed. The sum of the effects of exercise and anabolic steroids treatment may be the reason of a noticeable presence of NCP in Ex-M. On the other side, in Ex-C the faint bands may be result of remaining proteins after the action of proteases,

which probably pay a role in the remodeling process that occurs during intense exercise. Also, it should be considered that during intense exercise there is a possibility of reduction in the expression of several NCP. In spite of this hypothesis can be considered highly speculative they serve to address future investigation. Also, the hypothesis that these proteins could have a role in stimulating the synthesis of some proteins like the collagenous for instance cannot be discarded.

Ultrastructural analysis showed fibroblasts from Sed-M, Ex-C and Ex-M with activating-looking characteristics in comparison to Sed-C, and this can be related to increased amount in collagenous and non-collagenous proteins. Whether tenocytes within the tendon are direct targets of anabolic androgens action we do not know. Nevertheless, expression of AR were shown in fibroblasts from endoneurium of sciatic nerve[19] and from skeletal muscle, where their expression was particularly enhanced in myonuclei of neuromuscular synapse sites.[35] Whether this differential AR expression in skeletal muscle could be reflected in animals subjected to intense treadmill running program and interfere with changes observed in Achilles tendon is unknown.

In conclusion, our results showed that mesterolone combined with highly aerobic exercise stimulates the synthesis of collagenous and non-collagenous proteins. This paralleled with increased concentration of hydroxyproline, highest diameter achieved by fibrils as well as the highest sum of fibrils area inside an area probe. The results indicate that when combined (Ex + M) the exercise counteracts the effect of steroid and vice-versa in relation to collagen fibrils number, but are synergic in regard to the increased fibril size (diameter/area), HO-Pro concentration and collagen content. Increase in fibril size could reflect aggregation of collagen molecules, whereas increase in fibrils number

could be evidence of fibrillogenesis. The findings also paralleled with improvements in tenocytes synthetic machinery, as seen morphologically (TEM observations). Whether the tendon remodeling, seen in this work, was mediated by putative androgen receptors (AR) present in tenocytes, or either it was an indirect result from increased loading to which Achilles tendons were submitted, or both, is an interesting matter to be investigated.

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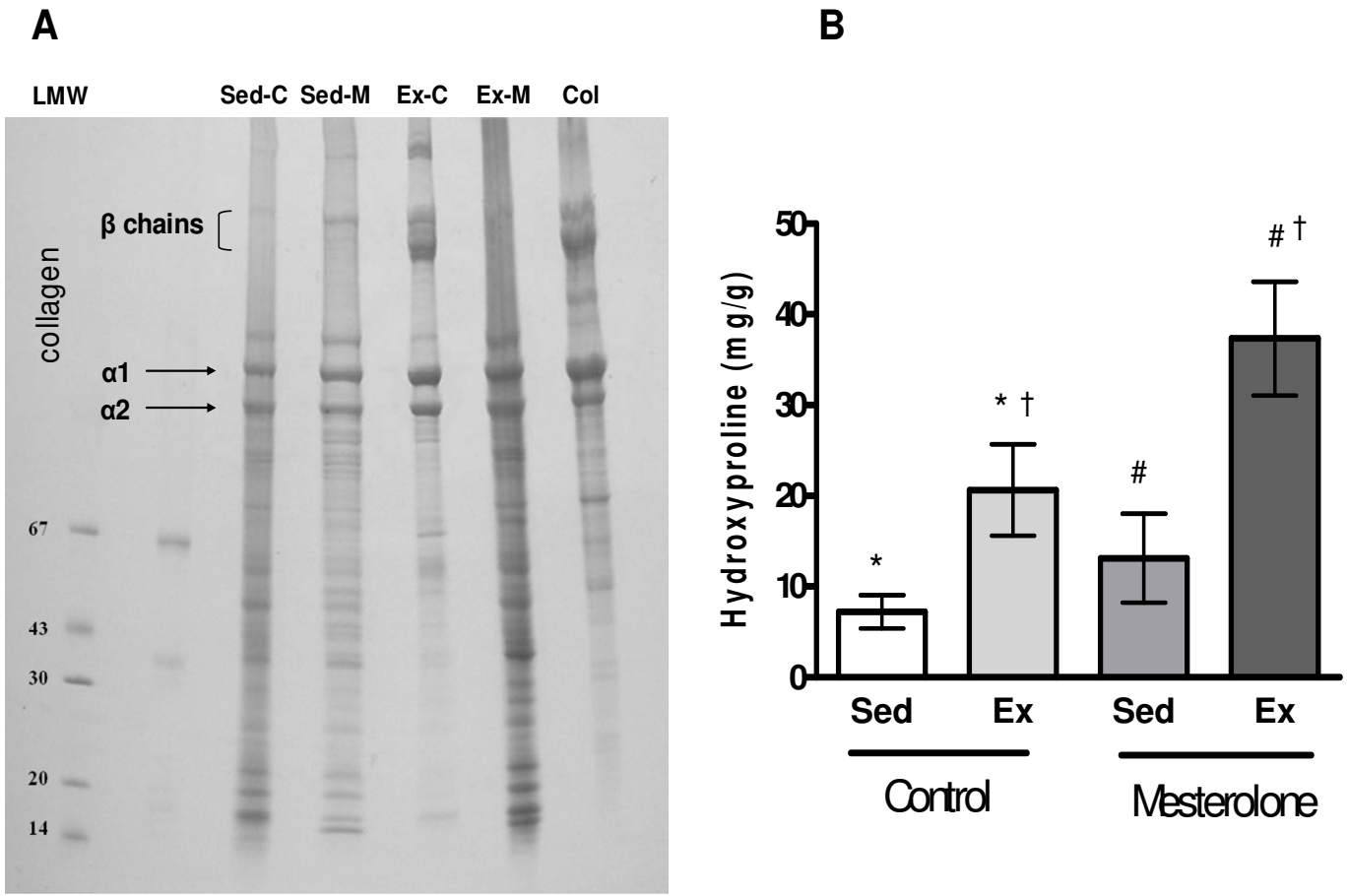


Figure 1: A - Analysis in SDS-PAGE showed that collagen increased gradually in Achilles tendons of sedentary mice treated with mesterolone (Sed-M), in treadmill-exercised animals (Ex-C group) and in exercised animals treated with mesterolone (Ex-M group), whereas non-collagenous proteins are abundant in Ex-M and scarce in Ex-C; **B - :** Quantification of hydroxyproline (HO-Pro) in the Achilles tendon (indirect evidence of collagen synthesis). Observe that there was a gradual increase in the HO-Pro by mesterolone *per se* (Sed-M), exercise *per se* (Ex-C) and exercise training plus mesterolone treatment (Ex-M). Exercise alone and exercise plus AAS produced a synergistic significant increase in the HO-Pro concentration (comparison of Sed-M *vs*. Ex-M). The same symbol over the bar indicate significant differences among groups ($p<0.05$). Data were expressed in mean ± SD. One way ANOVA followed by Tukey test.

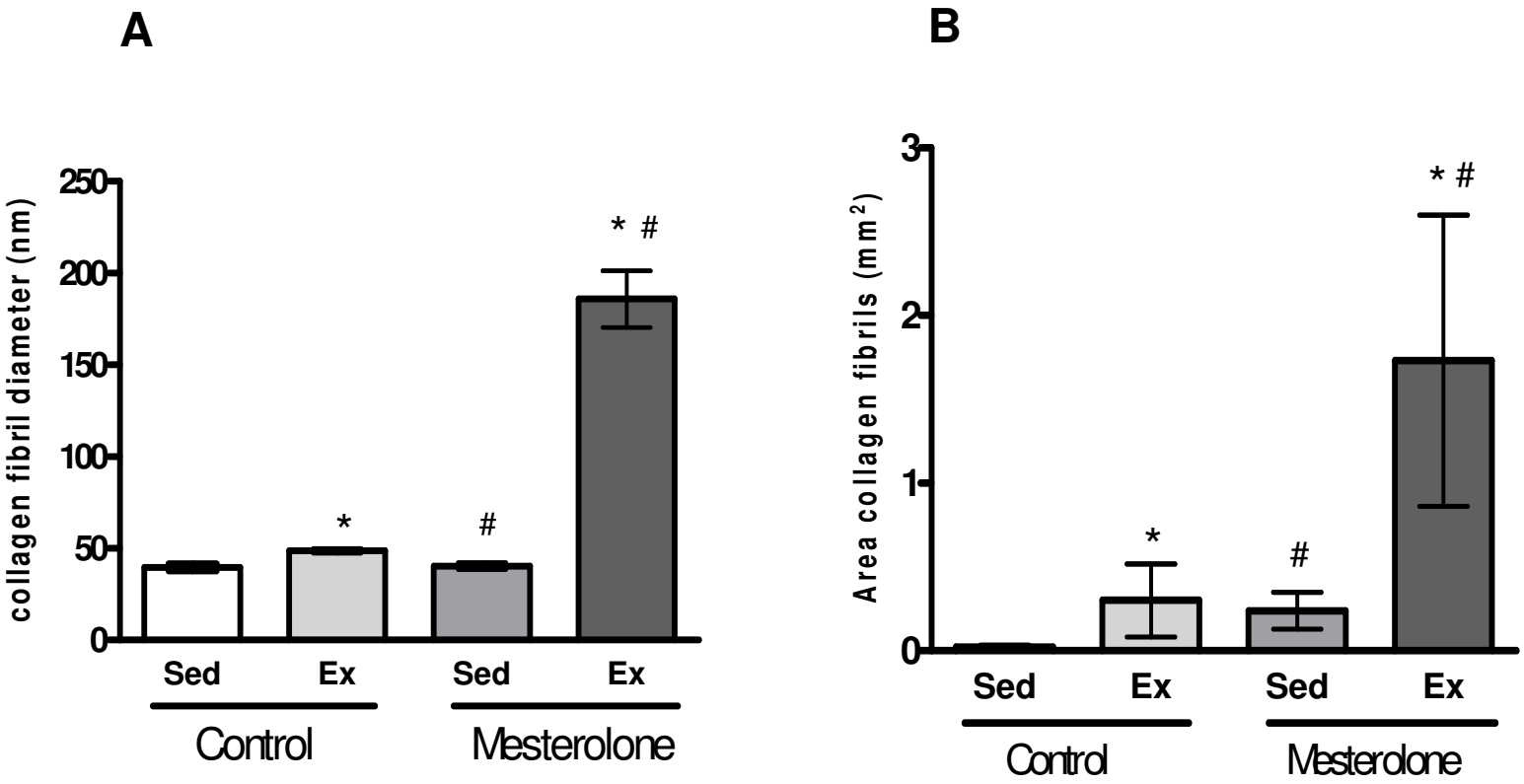
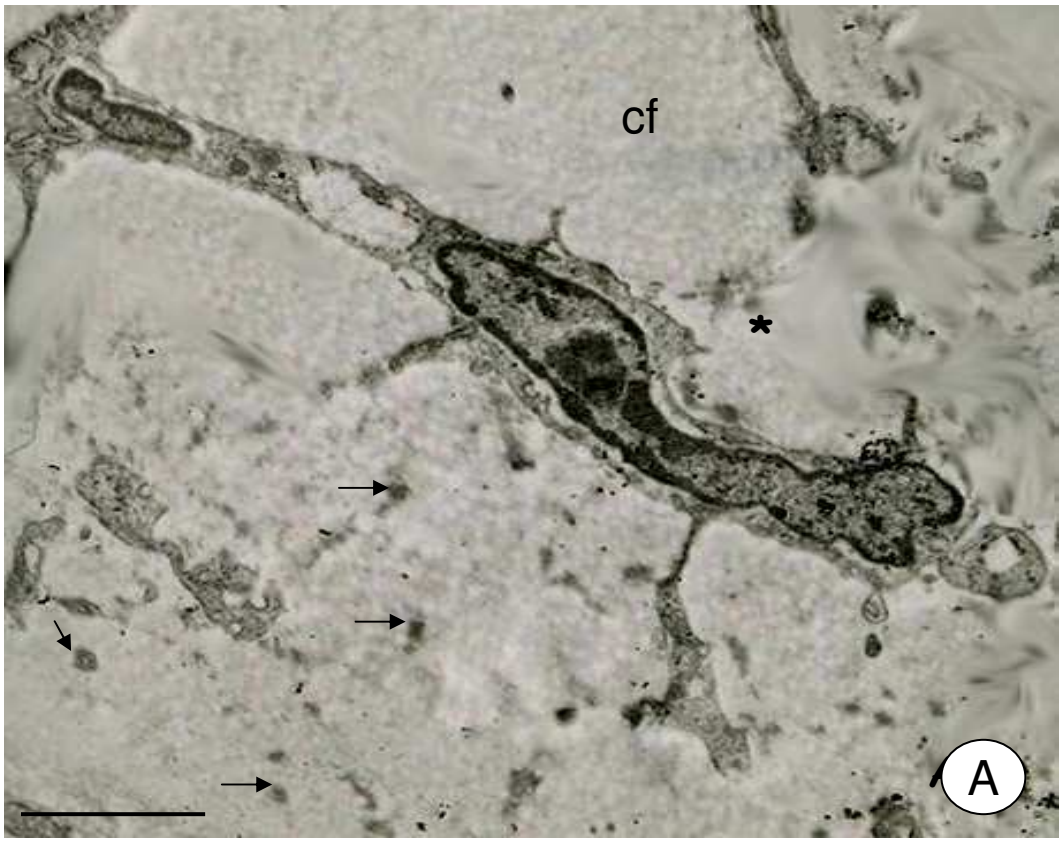


Figure 2: A-: Histogram showing collagen fiber diameter (nm) and **B**-: showing area collagen fiber (mm2) from Achilles tendon in the sedentary plus gum arabic (Sed-C), sedentary plus mesterolone (Sed-M), exercised plus gum arabic (Ex-C) and exercised plus mesterolone (Ex-M). The same symbols over the bar indicate significant differences among groups ($p<0.05$). Data were expressed in mean ± SD. One way ANOVA followed by Tukey test.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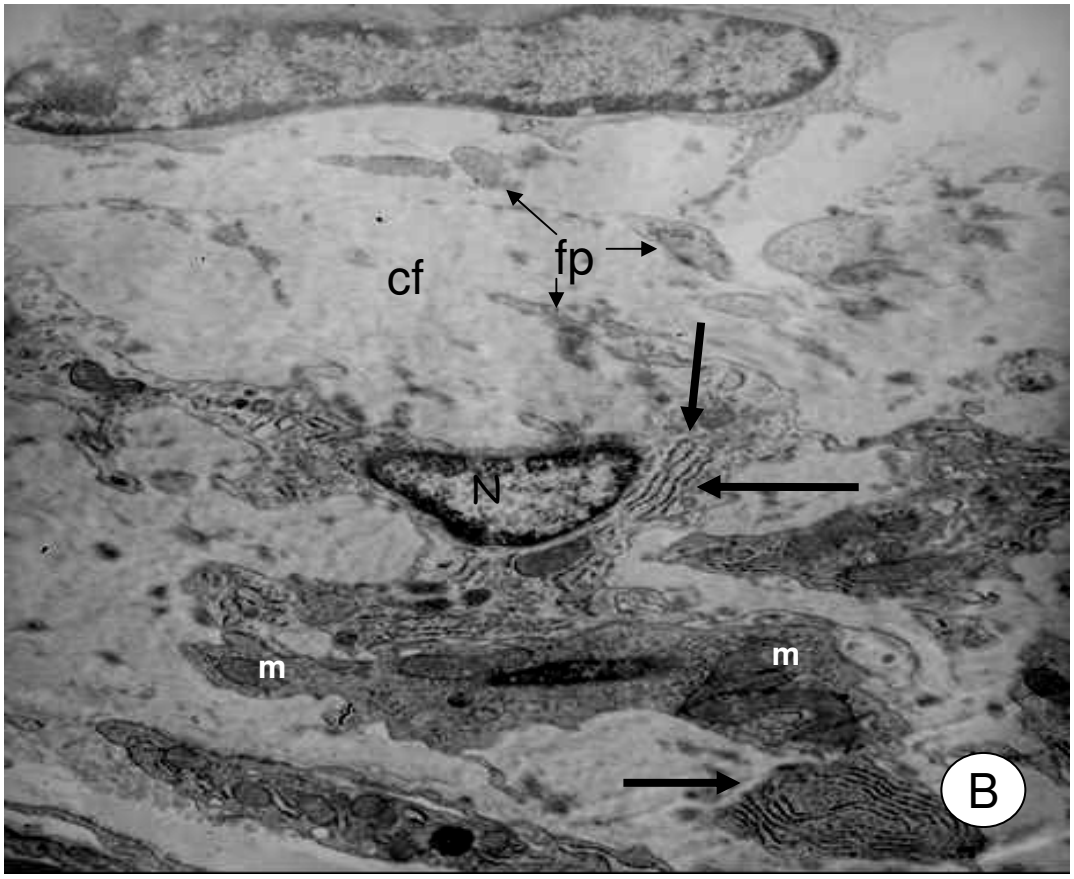
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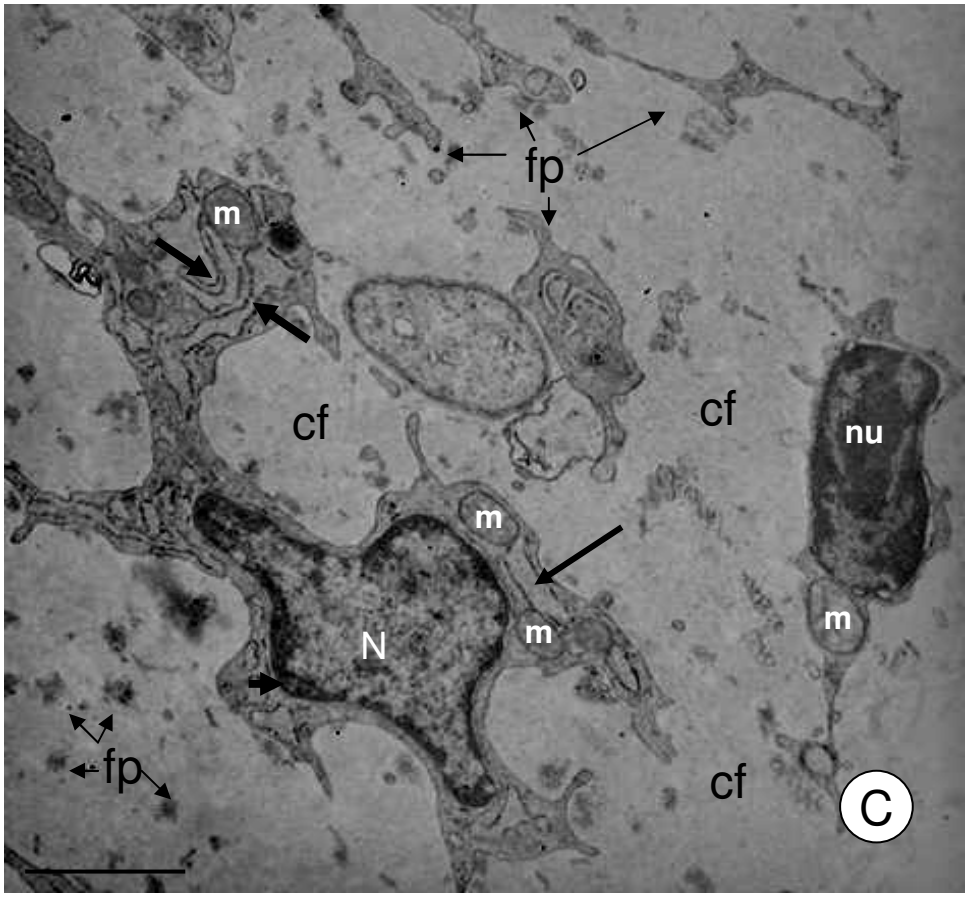
Figure 3: Transmission electron micrograph of the Achilles tendon of sedentary mice close to the insertion region in the soleus muscle (AT-calcanneal-soleus complex). **A – Sed-C**: sedentary mice treated with vehicle (gum arabic). Observe a fibroblast with a narrow ring of cytoplasm around the nucleus, slender cytoplasmic processes containing very few free ribosomes were sectioned longitudinally and transversally (arrows). A dense extracellular matrix with bundles of cross-sectioned collagen fibrils (cf) was seen surrounding the fibroblast; **B – Sed-M**: sedentary mice treated with mesterolone. The number of fibroblasts and sectioned cell processes (fp) was higher than in Sed-C; mitochondria (m) were numerous and stacked lamellae of rough endoplasmic reticulum (arrows) indicate activated fibroblasts. Bar = 3 µm.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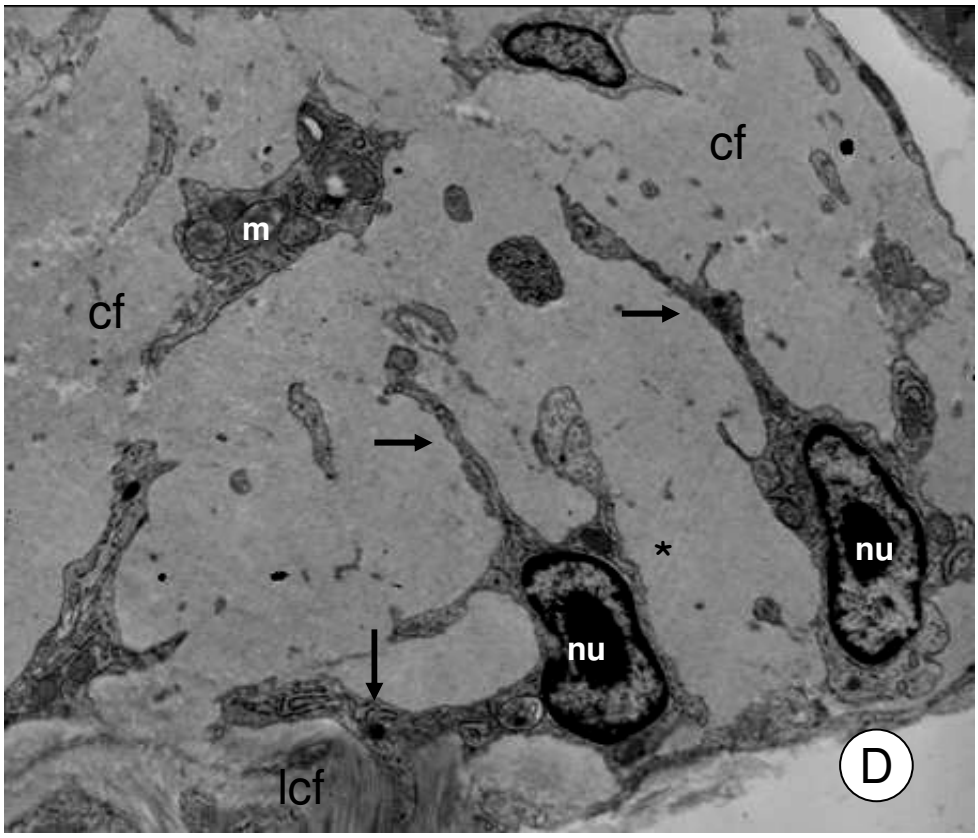
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Figure 4: Transmission electron micrograph of the Achilles tendon of treadmill-exercised mice at the insertion region (AT-calcanneal-soleus complex). **C – Ex-C**: exercised mice treated with vehicle (gum arabic). Fibroblasts exhibited a well-developed endoplasmic reticulum apparently containing secretory substances into the widened cisternae (arrows); numerous fibroblast processes (fp) are dispersed within a dense extracellular matrix (*) composed of bundles of cross-sectioned collagen fibrils (cf); nu = nucleolus. **D – Ex-M**: exercised treated with mesterolone. Highly-branched fibroblasts with larger cytoplasmic processes (large arrows) show several profiles of rough endoplasmic reticulum (arrows) and mitochondria (m); the nuclei showed developed nucleolus (nu); cf = cross-sectioned fibrils; lcf = longitudinally-sectioned fibrils Bar = 3 μm.

Capítulo V

Capítulo V:

Para alcançarmos os nossos objetivos, foram avaliados: índice de hipertrofia cardíaca, dano cardíaco através dos níveis plasmáticos de troponina T, estereologia (densidade de volume dos cardiomiócitos, densidade de volume do interstício e microvascularização) e análise dos níveis plasmáticos de lipoproteínas e lipídes.

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ORIGINAL ARTICLE

Adverse effect of the anabolic–androgenic steroid mesterolone on cardiac remodelling and lipoprotein profile is attenuated by aerobicz exercise training

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Summary

Abuse of anabolic–androgenic steroids (AAS) for improving physical performance is associated with serious, sometimes fatal, adverse effects. The aim of the present work was to investigate the effects of AAS on the cardiac structure and the plasma lipoprotein profile isolated and in combination with exercise. Transgenic mice with a human lipaemic phenotype (expressing cholesteryl ester transfer protein on the LDL receptor knockout background) were used in this study. Sedentary and exercised mice (treadmill running, five times per week for 6 weeks) were treated with mesterolone (2 μg/g body weight) or vehicle (control-C) in the last 3 weeks. Four groups were compared: (i) exercise + mesterolone (Ex-M), (ii) exercise + vehicle (Ex-C), (iii) sedentary + mesterolone (Sed-M) and (iv) sedentary + vehicle (Sed-C). Arterial blood pressure and body mass increased in all groups along time, but Sed-M reached the highest values and Ex-C the lowest. Treatment with mesterolone increased total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-c) and very LDL-c (VLDL-c) plasma levels. However, exercise blunted some of these deleterious effects by increasing high-density lipoprotein cholesterol and decreasing LDL-c, VLDL-c and triglycerides. Exercise training induced beneficial effects, such as physiological cardiomyocyte hypertrophy, increase in myocardial circulation and decrease in cardiac interstitium. However, mesterolone impaired such physiological gains and in addition increased troponin T plasma levels both in sedentary and exercised mice. Thus, while mesterolone induced pro-atherogenic lipoprotein profile and pathogenic cardiac hypertrophy, exercise counteracted these effects and modified favourably both the lipoprotein profile and the cardiac remodelling induced by mesterolone.

Keywords
cardiac interstitium, cardiomyocytes, lipid profile, mesterolone, transgenic murine model, ventricular hypertrophy

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Androgenic–anabolic steroids (AASs) are synthetic derivatives of the male hormone testosterone. The therapeutic use of AASs is indicated in endocrine dysfunction of the testes, age-related and HIV-related muscle wasting (Vermeulen 2001). From clinical trials, it seems likely that appropriate prescription of AASs may have positive anabolic effects on cachexia associated with HIV, cancer, burns, renal and hepatic failure and anaemia associated with leukaemia or kidney failure, which overcome the threat of side effects (for review, see Basaria *et al.* 2001).

The non-therapeutic use of AASs is a common practice among athletes for improving physical performance given its putative capacity of enhancing muscle mass and strength (Hartgens & Kuipers 2004). Generally administered in supraphysiological doses, the abuse of AASs by healthy athletes has been connected with the incidence of cardiovascular disease (CVD), such as development of hypertension, cardiomyopathy, atrial fibrillation, cerebrovascular accident, myocardial infarction, disturbances in haemostatic system, ventricular thrombosis and systemic embolism and acute heart failure (Dickerman *et al.* 1996; Sullivan *et al.* 1998). In addition, it has been shown that testosterone may affect negatively the recovery of postischaemic myocardial infarction by activating pro-inflammatory cytokine production (Wang *et al.* 2005).

Studies have also shown that the use of AASs induces atherogenic lipoprotein profile with a decrease in high-density lipoprotein cholesterol (HDL-c) and apolipoprotein Al and an increase in low-density lipoprotein cholesterol (LDL-c) plasma levels (Glazer 1991). On the other hand, exercise training has been associated with a reduced risk of CVD, possibly because it leads to an improvement of the lipoprotein profile (LaRosa 1992).

On the other hand, experimental and clinical evidence suggests androgen deficiency as a potential risk factor for CVD (for review, see Winkler 1996). Therefore, testosterone deficiency in mice resulted in increased plasma levels of apolipoprotein B-containing lipoproteins [very LDL (VLDL) and LDL] and decreased HDL-c, but transgenic mice that express the cholesteryl ester transfer protein (CETP) had such effects significantly attenuated (Casquero *et al.* 2006). This transgenic mice model is interesting because its lipoprotein profile is closer to that of the humans than the wild-type mouse. Contrary to humans, wild-type mice show very low levels of LDL and high levels of HDL. These differences are attributed to the lack of expression of the CETP, which increases HDL-c, and to the high number of LDL receptors, which reduce plasma LDL-c in the wild-type mice. Thus, by introducing the expression of the CETP gene and reducing the expression of the LDL receptor (LDLr) gene, the lipaemic phenotype of these transgenic mice resembles those of humans (Cazita *et al.* 2003; Casquero *et al.* 2006).

In the present study, the effects of mesterolone (17-beta-hydroxy-1-alpha-methyl-5-alpha-androstan-3-one, $C_{20}H_{32}O_2$), a synthetic steroid with anabolic and androgenic activities, were evaluated for its potency in inducing cardiac structure remodelling and altering lipoprotein profile in this transgenic murine model. In addition, we verified whether exercise training modulates such effects.

Materials and methods

Animal protocols

The experimental protocol was approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP) in accordance with the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The transgenic mice used in this study were cross-bred and maintained in the Department of Physiology and Biophysics (Institute of Biology, UNICAMP) for 10 years. Mice heterozygous for the human CETP transgene and for the LDL-receptor null allele ($CETP^{+/-}LDLr^{-/+}$) have been described elsewhere (Cazita *et al.* 2003; Casquero *et al.* 2006). The plasma CETP activity, expressed as % cholesteryl ester transferred from LDL to HDL, in this model is 36.6 ± 1.6 (n = 24) while wild-type mice are completely deficient of circulating CETP. The mice were housed in a temperature-controlled room (22 ± 1 °C), humidity of 55–65%, 12 h light/dark cycle and had free access to water and food standard chow (Nuvilab, Colombo, PR, Brazil).

Experimental protocol

Twenty-four adult male mice aged 2 months were divided into four groups of six animals: the control groups, sedentary (Sed-C) and exercised (Ex-C), received gum arabic [2 μg/g body mass (BM) via orogastric] during the last 3 weeks of training or sedentary period and the experimental groups, sedentary (Sed-M) and exercised (Ex-M), received the AAS mesterolone (Proviron™; Schering, Sao Paulo, SP, Brazil; 2 μg/g BM via orogastric) during the last 3 weeks of training or sedentary period. Gum arabic was used as vehicle given its non-toxic and pro-absorptive effects in small intestine (Codipilly *et al.* 2006). Exercised mice were allowed to adapt to treadmill running for 1 week prior to the use of the experimental protocol. This consisted of low to moderate level exercise of the treadmill running carried out daily for 5 days (15 m/min for 20 min/day). After

adaptation, exercised mice were subjected to 6 weeks of intensive exercise training (treadmill running), 5 days a week, as scheduled in Table 1 (adapted from Smolka *et al.* 2000). Blood pressure (BP) and BM were monitored weekly prior to any experimental procedure. All animals were habituated to the BP measurement device for 7 days before the onset of the experimental period. Blood pressure was measured weekly through the non-invasive computerized tail-cuff plethysmography (RTBP 1000; Kent Scientific Co., Litchfield, CT, USA) in conscious mice.

At the end of the experimental period, overnight fasted mice were deeply anaesthetized with a 1:1 mixture of ketamine chloride (Dopalen, 100 mg/kg of animal) and xylazine chloride (Anasedan, 10 mg/kg; 2 μl/mg BM, i.p.; both anaesthetics from Vetbrands, Jacarei, SP, Brazil). Blood samples were taken from the right atrium of the heart for plasma lipid analyses. Hearts were removed and the atria were separated from the ventricles and the left ventricle with the interventricular septum (LV) was weighed and then cut into two halves, put with the sectioned face down and then sectioned perpendicular to the ventral face at random. The LV fragments fixed with freshly prepared 4% (w/v) formaldehyde in 0.1 M phosphate buffer, pH 7.2 for 48 h, were then routinely processed until embedding in Histosec (Merck, Rio de Janeiro, RJ, Brazil). Sections (3 μm thick) stained with haematoxylin–eosin and Masson's trichrome were used for stereology. The LV mass/BM ratio was used as an index of cardiac hypertrophy.

Plasma lipid and troponin T analysis

Levels of total cholesterol (TC), triglycerides (TG), HDL in plasma were determined by enzymatic assays using commercially available kits (Roche, São Paulo, SP, Brazil) according to the instructions given by the manufacturer's protocol. Very low-density lipoprotein cholesterol was estimated as TG/5 and LDL-c by the Friedewald's formula (Friedewald *et al.* 1972). Troponin T (TnT) was determined by one-step enzyme immunoassay based on third-generation ECLIA assay on an Elecsys 2010 analyzer (Roche Diagnostics, Basel, Switzerland).

Stereology

A video microscopic system composed of a Leica DMRBE microscope, Kappa videocamera (Gleichen, Germany) and Sony Trinitron monitor (Pencoed, UK) was used. For stereology, the myocardium of the LV consisting of cardiomyocytes (cmy) plus cardiac interstitium (int, the latter consisting of connective tissue and intramyocardial vessels, ve) was analysed. Ten random histological sections were observed from the various LV fragments of each animal (n = 5 mice/group) and 10 random microscopic fields per animal were analysed blindly with a planachromatic objective 40× (Leica, Wetzlar, Germany) by moving the stage of the microscope. The stereological analysis used a test system with 36 test points and a known area. The volume densities of the structures were estimated as: V_v [structure] = P_p [structure]/P_T, where P_p is the number of points that hit the structure and P_T is the total number of test points (36 in the present study) contained in the frame. The mean cross-sectional area of the cardiomyocyte $A_{(cmy)}$ was estimated as: $A_{(cmy)} = V_{v(cmy)}/2{\cdot}Q_{A(cmy)}$, where V_v is the volume density of the cardiomyocytes and Q_A is the number of cardiomyocyte nuclei within the frame whose area is known respecting the 'forbidden line' (Gundersen 1977). The LV intramyocardial vascularization was analysed by the $V_{v(ve)}/V_{v(cmy)}$ ratio (Mandarim-de-Lacerda 2003).

Statistical analysis

The data were tested for deviations from Gaussian distribution using the Kolmogorov–Smirnov test (GRAPH PAD PRISM version 5.0; GraphPad Software, San Diego, CA, USA) and expressed as mean ± SEM. The statistical comparison among the control and treated groups was determined using one-way ANOVA (pair-wise) followed by the *post hoc* test of Tukey. In addition, two-way ANOVA was used where appropriate to determine how mesterolone treatment or exercise training affected the results, and whether there was an interaction between these two conditions. A P-value of 0.05 indicated statistical significance.

Results

Blood pressure and body mass

The weekly variation in BP and BM during the 6-week period of experimentation is shown in Figures 1 and 2 respectively. Blood pressure tended to increase along the

Table 1 Exercise (6 weeks) and anabolic–androgenic steroid (AAS) or vehicle administration (3 weeks) protocol

Weeks	Velocity (m/min)	Duration (min)
1	12.42	20
2	14.70	30
3	16.68	45
4–6*	17.04	60

*AAS or gum arabic (vehicle) administered orally by gavage at 10.00 AM (Monday, Wednesday and Friday) in the last 3 weeks.

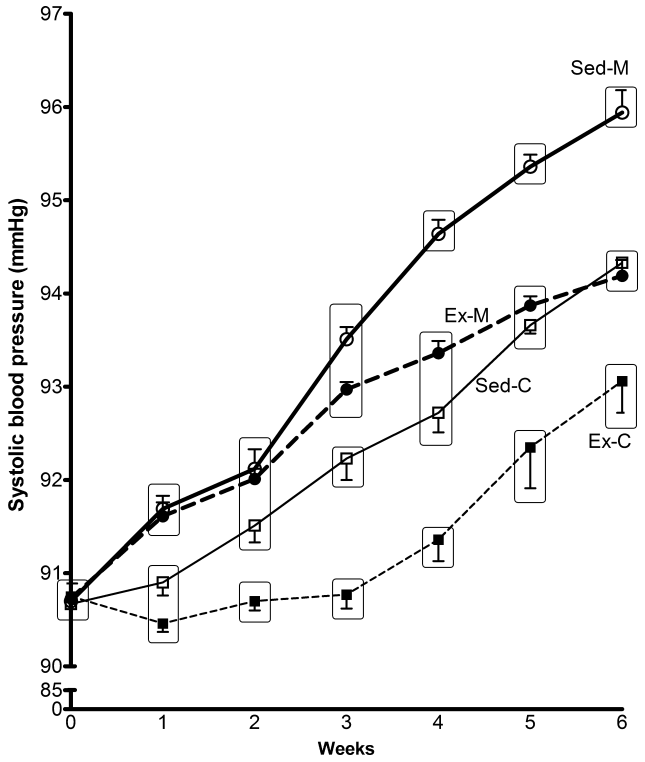

Figure 1 Caudal blood pressure (systolic-like) evolution in control groups sedentary (Sed-C) and exercised (Ex-C) treated with gum arabic (vehicle), and the experimental groups sedentary (Sed-M) and exercised (Ex-M) treated with mesterolone of $CETP^{+/-}LDLr^{-/+}$ transgenic mice. When the groups were put together (into a rectangle), they did not show statistically significant difference, but among the rectangles the difference was significant ($P < 0.05$). One-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

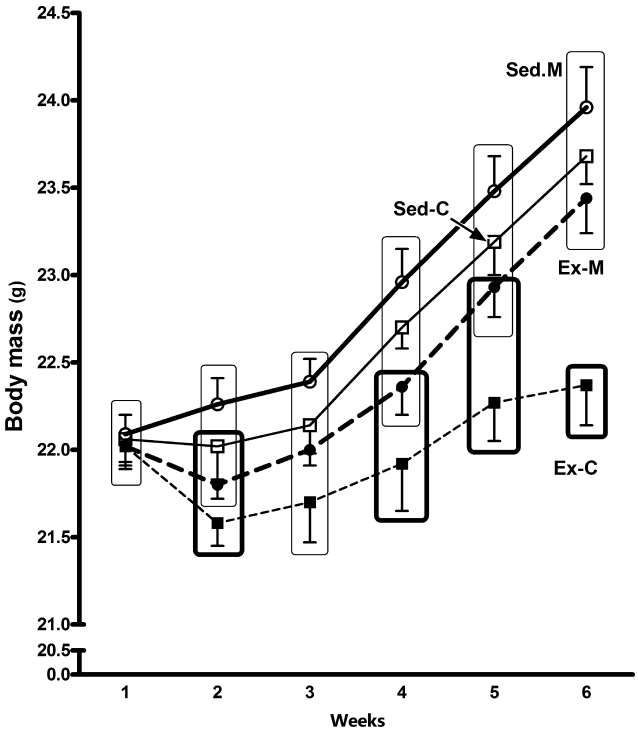

Figure 2 Body mass (BM in grams) evolution during the 6 week period in the gum arabic- (vehicle) and mesterolone-treated sedentary (Sed-C and Sed-M) or exercised (Ex-C and Ex-M) $CETP^{+/-}LDL^{-/+}$ transgenic mice. Sed-M exhibited the highest weight gain whereas Ex-C exhibited the lowest (Sed-M = Sed-C = Ex-M > Ex-C). Groups inside rectangles not inter-sectioned to each other present a week-by-week significant difference as follows: Ex-C ≠ Sed-M (in all weeks, except in the third); Ex-C ≠ Sed-C (fourth, fifth and sixth); Ex-C ≠ Ex-M (sixth) ($P < 0.05$). One-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

trial period, particularly from the third week onwards in the mesterolone groups (highest BP values in both the Sed-M and Ex-M groups). The BP values at the sixth week were significantly different from the initial values in the same group ($P < 0.05$). It is noteworthy that exercise training delayed the rhythm of increase of BP in both the mesterolone-treated and vehicle-treated groups. Despite the significant difference in BP between Ex-C and Sed-M ($P < 0.05$), the little variation was not relevant from the physiological point of view.

Figure 2 shows that BM gain was lower in exercised than in sedentary mice. Sed-M showed better week-by-week BM increase and the highest BM gain at the end of the sixth week. Because in Ex-C, BM was maintained practically at a constant level since the beginning of the experimental period, a slight, but statistically significant difference was found comparing Sed-M *vs*. Ex-C ($P < 0.05$). However, the effect of exercise in maintaining low BM gain was not enough to abolish the gain promoted by mesterolone and hence there is no significant difference between Sed-M and Ex-M.

Plasma lipids

The plasma levels of TC, TG and cholesterol distribution in lipoprotein fractions (HDL and LDL) are shown in Figure 3. Considering TC plasma levels in the Sed-C group as reference (86.5 ± 1.8), there was a significant increase in Sed-M ($P = 0.00004$) and Ex-C ($P = 0.02$). Compared with the Sed-C group (92.5 ± 0.7 mg/dl), the TG plasma levels in the Sed-M group were increased by 38% ($P < 0.0001$), in the Ex-M group decreased by 15% ($P < 0.0001$) and in the Ex-C group decreased by 30% ($P = 0.003$). These findings

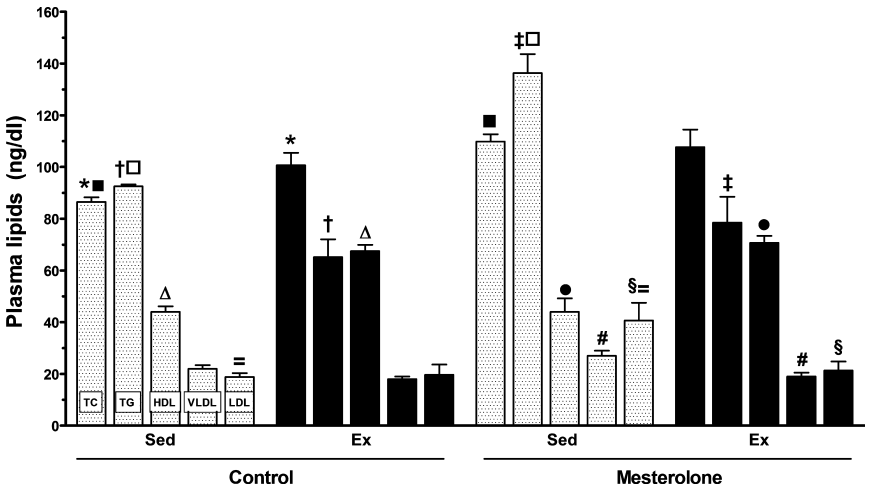

Figure 3 Plasmatic lipid and lipoprotein levels in the CETP$^{+/-}$LDLr$^{-/+}$ transgenic mice in control groups treated with gum arabic (vehicle), sedentary (Sed-C) or exercised (Ex-C) and matched groups treated with mesterolone (Sed-M and Ex-M). The same symbol over the bar indicates significant differences among groups ($P < 0.05$). One-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

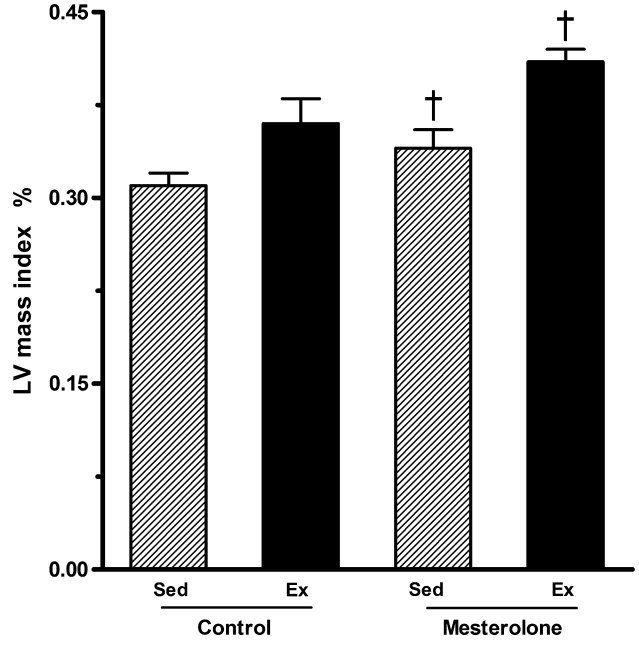

Figure 4 Left ventricular mass index in exercised and sedentary CETP$^{+/-}$LDLr$^{-/+}$ transgenic mice submitted to mesterolone or gum arabic (vehicle) orogastric administration. There was significant difference between Sed-M *vs.* Ex-M ($P < 0.05$). One-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

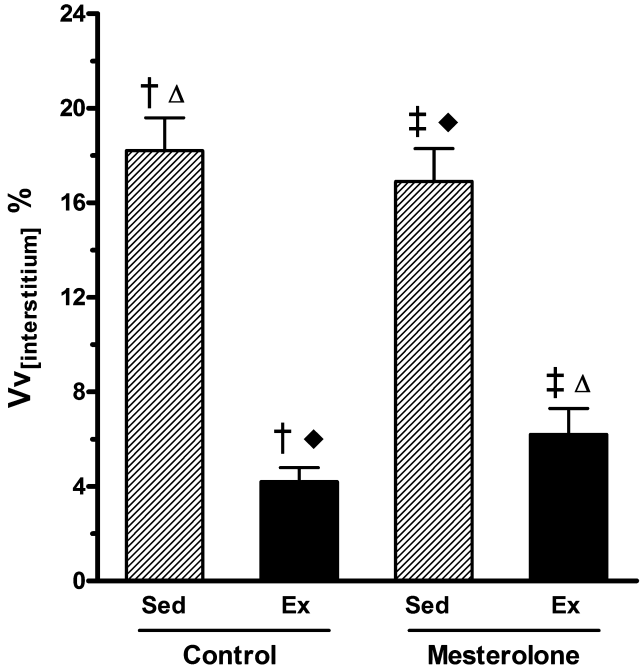

Figure 5 Volume density of left ventricle interstitium ($V_{v(int)}$) from exercised and sedentary CETP$^{+/-}$LDLr$^{-/+}$ transgenic mice treated orally with mesterolone and gum arabic (vehicle). There were significant differences among groups as follows: Sed-C *vs.* Ex-C and Sed-M *vs.* Ex-M ($P < 0.05$). One-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

indicated that a beneficial effect of the exercise training on the plasma lipids was sufficient to counteract the adverse mesterolone effect.

The plasma levels of HDL-c were not different between Sed-M and Sed-C, but were elevated in Ex-C ($P = 0.0002$) and Ex-M ($P < 0.001$), indicating the predominant effect of the exercise training in controlling HDL-c levels in mice administered mesterolone.

The plasma levels of LDL-c were increased in Sed-M as compared with Sed-C ($P < 0.01$) and in Sed-M as compared with Ex-M ($P = 0.03$), indicating the side effect of mesterolone and the benefit of exercise.

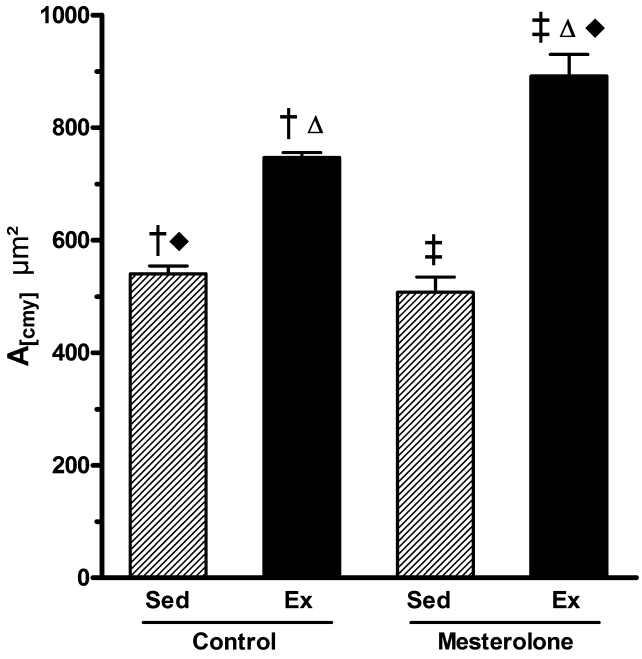

Figure 6 Left ventricle cardiomyocyte cross-sectional area ($A_{(cmy)}$) from exercised and sedentary $CETP^{+/-}LDLr^{-/+}$ transgenic mice treated with mesterolone or gum arabic (vehicle). There were significant differences among groups as follows: Sed-C < Ex-C, Ex-C < Ex-M and Ex-M > Sed-M ($P < 0.05$). One-way and two-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

The levels of VLDL-c were lower in Ex-C (approximately 40% reduced) and Ex-M (approximately 30% reduced) compared with Sed-C group ($P < 0.005$), and were decreased approximately by 40% in Ex-M compared with Sed-M ($P < 0.01$). Very low-density lipoprotein cholesterol did not differ between exercised groups.

Cardiac hypertrophy

Exercise alone (Ex-C) or mesterolone alone (Sed-M) promoted a trend for increasing left ventricle (LV) mass index in comparison with Sed-C but the differences were in the borderline of statistical significance ($P = 0.054$). In contrast, the LV mass index increased by 25% in Ex-M compared with Sed-M ($P = 0.03$). These results show that exercise associated with mesterolone had an additive effect on LV mass index (Figure 4).

Stereology

The volume density of the interstitium, $V_{v(int)}$, decreased by 65% in Ex-M and 80% in Ex-C when compared with their sedentary counterparts ($P < 0.05$). Exercise training *per se* decreased the $V_{v(int)}$ in control mice treated with vehicle whereas increased in the sedentary mice treated with mesterolone (Figure 5). These findings show that exercise training is able to reduce the cardiac interstitium (collagen fibre increment).

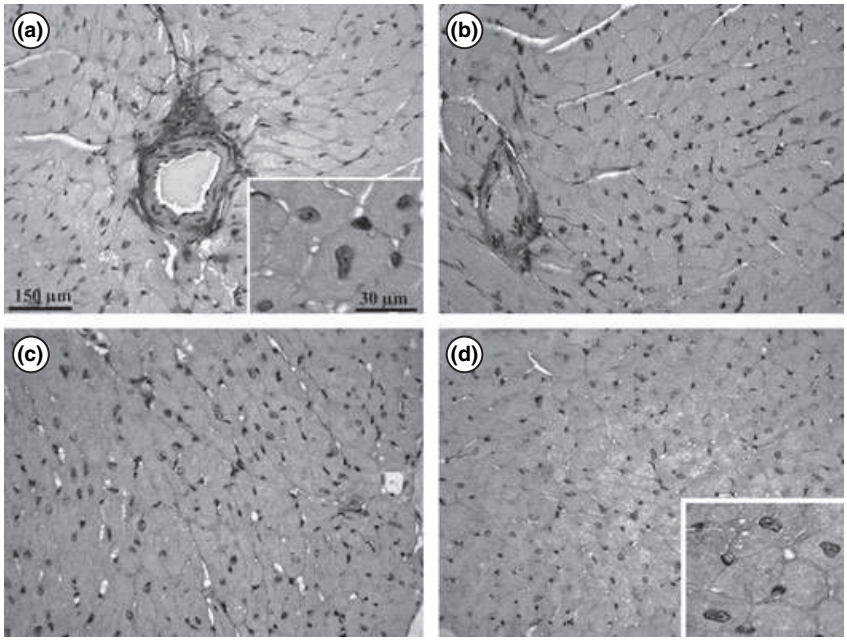

Figure 7 Photomicrographs showing the left ventricle myocardium in transgenic mice. (a) Ex-M: exercised mice treated with mesterolone; (b) Ex-C: exercised animals treated with gum arabic (vehicle); (c) Sed-M: sedentary animals treated with mesterolone; (d) Sed-C sedentary mice treated with vehicle. Note that qualitatively cardiomyocytes are higher in size in exercised (a, b) and smaller in sedentary (d, c) groups. The insets depict a high magnification of cardiomyocytes of Ex-M (a) and Se-C (d).

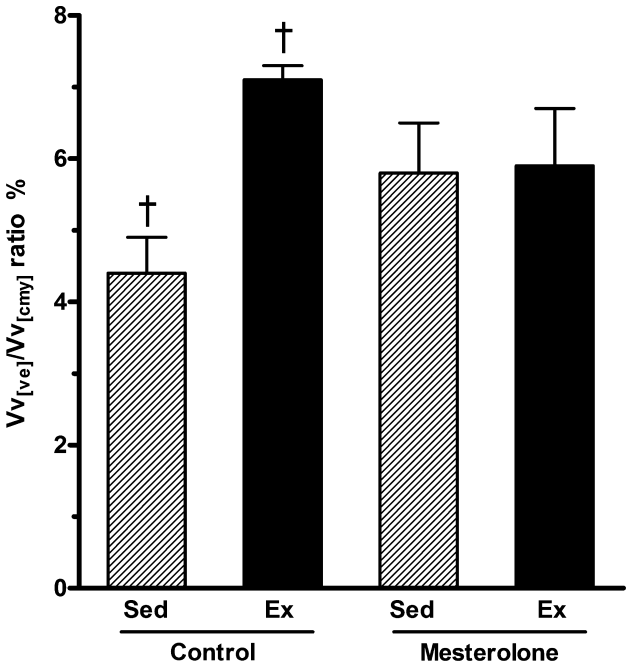

Figure 8 Myocardial vascularization in the left ventricle from exercised and sedentary $CETP^{+/-}LDLr^{-/+}$ transgenic mice treated with mesterolone and gum arabic (vehicle). There was significant difference between Sed-C *vs.* Ex-C ($P < 0.05$). One-way and two-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

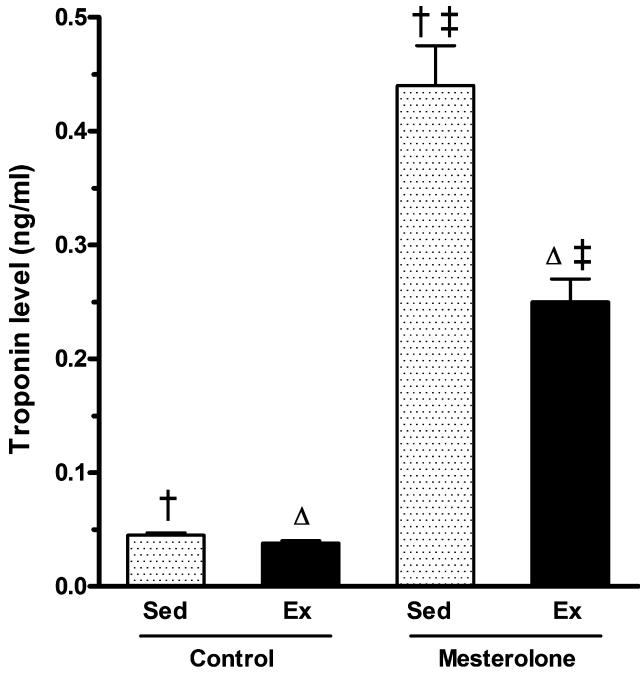

Figure 9 Troponin T (TnT) plasmatic level in the $CETP^{+/-}LDLr^{-/+}$ transgenic mice in control groups (Sed-C) and exercised (Ex-C) treated with gum arabic (vehicle) and the experimental groups sedentary (Sed-M) and exercised (Ex-M) treated with mesterolone. The same symbol over the bar indicates significant differences among groups ($P < 0.05$). One-way ANOVA followed by Tukey test. Data are expressed as mean ± SEM.

Figures 6 and 7 show the cardiomyocyte area measurements and morphology. Compared with Sed-C mice, the largest cardiomyocytes were observed in the Ex-M group ($A_{(cmy)}$ where there was a 65% area increase ($P < 0.01$), followed by the Ex-C group (with 40% increase, $P < 0.02$) [differences were significant between Sed-C *vs.* Ex-C; Sed-C *vs.* Ex-M; Sed-M *vs.* Ex-M and Ex-C *vs.* Ex-M ($P < 0.05$) (Figure 6)]. These findings indicated that exercise training *per se* causes cardiomyocyte hypertrophy and exercise training combined with administration of mesterolone enhances this hypertrophy. This was confirmed by the two-way ANOVA ($P = 0.03$; Figure 6) and histological observations (Figure 7).

The intramyocardial vascularization (Figure 8) was significantly greater in the Ex-C group compared with the Sed-C group (by 40%, $P < 0.05$). However, no statistical differences were observed in intramyocardial blood vessels of Ex-M and Sed-M groups (Figure 8). Thus, mesterolone treatment blunted the benefit gained by exercise.

The plasma levels of TnT were lowest in the Ex-C group with no significant difference with that of the Sed-C (Figure 9). The highest value was achieved in the Sed-M group (0.44 ng/ml). In Ex-M mice, the TnT value was 0.25 ng/ml, indicating a 40% reduction in TnT release from cardiomyocytes promoted by treadmill running ($P < 0.001$).

Discussion

In this study, transgenic mice with a human lipaemic phenotype ($CETP^{+/-}LDLr^{-/+}$) were used to evaluate the effects of mesterolone treatment combined with or without an aerobic exercise training protocol, on the cardiovascular system, specifically on BP, plasma lipoprotein profile and cardiac remodelling. The use of testosterone or AASs is a well-recognized risk factor for hypertension, disturbances in lipid profile and accelerated coronary artery disease (Alen & Rahkila 1984; Ganten *et al.* 1989; Hurley *et al.* 1984; Rockhold 1993). In the present study, compared with vehicle, mesterolone-treated sedentary mice showed significant elevation of the TC, TG, LDL-c and VLDL-c plasma levels. On the other hand, the exercise training programme applied to these mice increased the levels of HDL-c and decreased VLDL-c (the

precursor of LDL-c) and TG significantly, both in mice treated with vehicle or mesterolone. The significant difference in the BM gain and BP produced both by mesterolone and exercise *per se* taking as reference Sed-C is likely not relevant from the physiological point of view.

Regular and moderate exercise training have been associated with a reduced risk of CVD, partially because of an improvement in the lipoprotein profile (LaRosa 1992). However, the amount of exercise training required to obtain benefits is debatable (Blair *et al.* 2004). In general, only high-intensity exercise training or long-term endurance exercise programme are able to improve overall lipoprotein profile, including elevation of HDL-c and reduction in TG levels in humans (Seals *et al.* 1984; Kraus *et al.* 2002; Halverstadt *et al.* 2007). On the other hand, studies on the exercise-mediated improvement of lipoprotein profile in mice and rats should be observed with prudence if the aim is to compare with humans, mainly because differences exist in the expression of proteins involved in transport and tissue uptake of lipoproteins. In this regard, the animal model studied here presented a lipid profile akin to the humans and so lipoprotein response to exercise training is expected to be more feasible in comparison with that in humans. The exercise programme (6 weeks, 5 times/week and 60 min/day) with velocity of 17.4 m/min in the last 3 weeks is considered high aerobic to animals and comparable to long-term and high-endurance exercise in humans, considering the life span of both.

Clinical reports of misuse of AASs by athletes and studies in experimental animals have shown the occurrence of deleterious structural myocardial alterations and heart hypertrophy (Bauman *et al.* 1988; Ganten *et al.* 1989; Nieminen *et al.* 1996) (for review see Hartgens & Kuipers 2004), including irreversible changes in the myocardium, such as concentric LV hypertrophy, even after discontinuing the AAS intake (Urhausen *et al.* 2004). Treatment of rats with supraphysiological doses of AASs induced pathological myocardial hypertrophy, and when combined with exercise, these steroids reduced the beneficial effects of exercise on LV hypertrophy and cardiac circulation (Tagarakis *et al.* 2000a,b; Woodiwiss *et al.* 2000).

In the current study, mesterolone alone (Sed-M) promoted only slight changes in the cardiac structure, i.e. a borderline trend towards LV hypertrophy ($P = 0.054$). On the other hand, the exercise training induced favourable cardiac remodelling either in mice treated with mesterolone or vehicle. This is evidenced by a marked reduction in the cardiac interstitium (more prominent in Ex-C) and enlargement of cardiomyocyte size. Increased levels of circulating TnT, a marker of myocardium lesion, confirm the deleterious effect of mesterolone in sedentary mice and exercise training attenuated this adverse effect of mesterolone on the cardiac integrity. In addition, the exercise training enhanced significantly the cardiac vascularization in the Ex-C and, to a lesser extent, in Ex-M groups (compared with Sed-C). This probably represents a response to the increased myocardial oxygen demand imposed by physical activity. Similar effects of exercise training were observed in a previous study in ovariectomized rats (Marques *et al.* 2006). This increase in cardiac vascularization may be of importance in the case of ischaemic events. Mesterolone treatment of exercised mice suppressed the amelioration of the benefits of physiologic hypertrophy, the improvement of the myocardial circulation and part of the reduction in interstitium induced by the exercise training programme.

Therefore, our findings show the strong adverse effects of mesterolone on plasma lipid and lipoprotein metabolism, which were prevented or attenuated by exercise training in this murine model. On the other hand, our data also show that exercise *per se* induces beneficial changes in cardiac remodelling and myocardial vascular supply, all of which are totally or partially impaired by the mesterolone use.

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Quadro geral demonstrativo dos resultados

4. RESUMO DOS RESULTADOS

- **Mesterolona (Sed-C *vs.* Sed-M):**

- ↑ massa corporal
- ↑ massa muscular TA
- ↑ % significativamente das fibras IC, IIAC, IIDA, IID/X e ↓ % da fibra IIA no SOL
- ↑ significativamente a área das fibras I, IIA e IID/X no SOL; I e IIDA, IID/X no TA e I, IIA, IIDA, IID/X e IIB do GAS
- ↑ síntese NOS I e NOS III
- Tendência hipertensão arterial e ao perfil aterogênico

- **Exercício (Sed-C *vs*. Ex-C):**

- ↓ massa corporal
- ↑ massa muscular SOL e GAS
- ↑ % significativamente das fibras IID/X no SOL; IIA e IIDA no TA; ↑ IID/X e ↓ % IIDA e IIDB no GAS
- ↑ significativamente a área das fibras I, IIA, IIDA, IID/X no SOL; I, IIA, IIDA, IID/X, IIB no TA e I, IIA, IIDA, IID/X e IIB do GAS
- ↑ síntese NOS I e NOS III
- Induziu favoravelmente remodelamento cardíaco, ↓ perfil aterogênico e hipertensivo
- ↑ conteúdo de hidroxiprolina

- **Exercício e Mesterolona (Sed-M *vs*. Ex-M; Ex-C *vs.* Ex-M):**

- ↑ massa muscular SOL
- ↑ % significativamente das fibras I, IIA, IIDA no TA; ↑ IIB, ↓ % IIDA e IIDB no GAS

Quadro geral demonstrativo dos resultados

- ↑ significativamente a área das fibras I, IIA, IIDA e IID/X no SOL
- ↑ significativamente a área de todas as fibras do TA
- ↑ significativamente a área das fibras I, IIA, IIDA, IID/X e IIB no GAS
- Promoveu ação hipertrófica das fibras intra e extra fusais
- Ativou as células satélites
- Promoveu a angiogênese e a biogênese mitocôndrias
- Favoreceu o surgimento das fibras com núcleo central (regeneração)
- ↑ síntese NOS I (TA e GAS) e NOS III (TA)
- Modificações patogênicas: tendência à hipertensão arterial e ao perfil aterogênico
- ↑ conteúdo de hidroxiprolina, ↑ diâmetro e da área das fibrilas dos tendões.

Síntese dos resultados e Conclusões

5. SÍNTESE DOS RESULTADS E CONCLUSÕES

- Com relação à resposta adaptativa dos músculos SOL, TA e GAS, os resultados mostraram que o maior impacto ocorreu em relação à fibra tipo I do SOL e na IIB do TA e GAS, respectivamente fibra resistente e não resistente à fadiga. O **SOL** foi o músculo que mais mostrou alteração adaptativa na composição de suas fibras em resposta à mesterolona, aumentando em 4 vezes a fibra IC, em três vezes a fibra IIAC, mais que dobrou as IIDA e reduziu à metade as fibras IIA, indicando que as fibras IIA foram recrutadas em favor do aumento das fibras IC (fibras oxidativas de contração lenta), IIAC (fibra intermediária, de contração lenta e metabolismo oxidativo-glicolítico) e IID/X (fibra de contração rápida e metabolismo glicolítico), aumento este tanto menor quanto maior o metabolismo glicolítico e a fatigabilidade da fibra. Entretanto, a fibra IID/X foi a mais afetada pela ação do exercício sendo 8x a mais nos Ex-C, enquanto as demais fibras não alteraram significativamente a sua proporção no SOL. A ação da mesterolona por si aumentou três vezes a proporção das fibras IID/X. Em relação ao **TA**, o exercício aumentou em quase 2x a proporção de fibras IIA e IIDA, enquanto a associação de mesterolona e exercício elevaram em 2x as fibras IIA e 3x as fibras IIDA, indicando que no tibial anterior a mesterolona potenciou o efeito do exercício, e que as alterações adaptativas sofridas pelo TA foram por conta de recrutamento de fibras glicolíticas, de contração rápida, fatigáveis, em favor de um perfil mais oxidativo do músculo evidenciado pela diminuição de IID/X e IIDB (não significativamente) em favor de aumento significativo da IIA e IIDA Diferentemente, no **GAS** o exercício promoveu a redução em quase 3x das fibras IIDA, em 2x as fibras IIDB e quase dobrou a proporção de fibras IID/X, indicando uma adaptação do gastrocnêmio para um perfil de contração mais rápida e metabolismo glicolítico. Por outro lado, a mesterolona em animais exercitados (Ex-M) minimizou os efeitos do exercício para as fibras IIDA (2x), porém potenciou-os para as fibras IIDB, que passaram a ter a sua proporção reduzida em mais de 3x. As fibras IIB aumentaram em mais de 1x a proporção nos animais Ex-M.

Concluindo, os resultados indicam que o exercício em animais tratados com mesterolona levam o SOL e o GAS para um fenótipo mais glicolítico com fibras de contração rápida e mais facilmente fatigável, enquanto dirige o TA para um perfil mais oxidativo e mais resistente à fadiga, enquanto não altera o SOL. Entretanto, a mesterolona per se em animais sedentários altera o SOL e não altera os outros músculos.

- Em relação ao aumento da massa muscular, os resultados mostraram que o efeito trófico da M no peso do músculo foi maior no tibial anterior, enquanto o causado pelo exercício aeróbico foi similar nos três músculos. Enquanto o exercício aeróbico se contrapôs ao efeito miotrófico da mesterolona nos três músculos, a mesterolona potenciou o efeito do exercício físico, exceto em relação ao TA. O aumento do peso dos músculos deveu-se à hipertrofia das fibras musculares e não à hiperplasia, isto é, à aquisição de novas miofibras, como demonstrado pela contagem do número de fibras musculares nos músculos após os tratamentos. A hipertrofia das fibras foi diferenciada dependendo do músculo considerado e a mesma fibra teve modulação diferenciada em resposta ao estímulo e dependendo do músculo em que se encontrava. A ação hipertrófica da M foi observada para as fibras I (33.7%), IIA (18.5%) e IID/X (22.6%) do SOL, como evidenciado pela medida da área seccional transversa das mesmas. Além disso, as fibras I (14%), IIDA (65%), IID/X (30%) do TA e as I (35%), IIA (18.5%), IIDA (18.2%) IID/X (26.8%) e IIB (12.5%) do GAS sofreram significativa hipertrofia por ação da administração de mesterolona. Em relação ao efeito do exercício por si, foi demonstrado que este causou hipertrofia significativa nas fibras I (101%), IIA (84%), IIDA (37.5%) e IID/X (56%) no SOL; nas I (76.2%), IIA (63%), IIDA (61%) e IID (58%), no TA; e nas fibras I (65%), IIA (35.3%), IIDA (26.5%), IID/X (19%) e IIB (20%) no GAS. Já o exercício + mesterolona (Sed-M vs. Ex-M) promoveu aumento significativo no tamanho das fibras I (61%), IIA (63.5%), IIDA (36%) e IID/X (32%) no SOL; nas fibras I (86%), IIA (62%), IIDA (19%), IID/X (34.5%) e IIDB (37.2%) no TA; e nas fibras I (47%), IIA (31%), IIDA (37.5%), IID/X (34.6%), e IIB (16%) no GAS. O efeito da mesterolona em animais exercitados (Ex-C vs. Ex-M) foi observado nas fibras I (7%) do SOL; IIDA

(22%) e IIDB (20%) do TA; IIA (16%), IIDA (28.3%), IID/X (43.3%) e IIB (8.7%) no GAS.

O efeito hipertrófico causado pela mesterolona foi em geral mais moderado do que o causado pelo exercicio. Isto refletiu-se tanto na intensidade da hipertrofia, quanto no número de tipos de fibras que hipertrofiaram. A ação miotrófica da mesterolona teve como alvo principal as fibras oxidativas no SOL, e as fibras glicolíticas no TA e GAS. Em geral, as fibras que se hipertrofiaram eram fibras puras, com exceção da fibra IIDA que foi afetada nos três músculos estudados tanto pelo estímulo da mesterolona, do exercício e de ambos combinados. O exercício modulou diferentemente os efeitos da mesterolona, dependendo do músculo e da fibra.

- Em relação à ultra estrutura, foi verificado no SOL que tanto o exercício como a M causaram hipertrofia das fibras intra e extrafusais, promoveram o aumento do número e da densidade das mitocôndrias por área de fibra, aumento da área das mitocôncrias, causaram freqüente dano mitocondrial, aumento do número de capilares/fibras, aparecimento de "split fibers" e de células satélites com sinal de ativação. Além disso, grupos de pequenas células musculares com núcleo centrais, principalmente encontradas nos grupos tratados com mesterolona, aparentemente derivadas do splitting de fibras diferenciadas ou das células satélites ativadas eram comuns, sugerindo regeneração tecidual.

O fato de haver aumento da densidade e área das mitocôndrias e de vasos por fibra pela ação do exercício + mesterolona, sugere a aquisição de um perfil mais oxidativo ao sóleo. Estes resultados conferem com os obtidos através da tipagem que mostrou que nos animais Ex-M, para o sóleo, o exercício se contrapôs ao efeito da mesterolona que havia promovido o sóleo para um perfil mais rápido glicolítico, tornando não significativa essa tendência.

- Com o intuito de avaliar o envolvimento do óxido nítrico sob ação da administração de M, da atividade de alta resistência promovida pelo intenso exercício

aeróbico e pela associação dos dois, a expressão da NOS I e da NOS III foi avaliada nos tres músculos. Os resultados mostraram que há uma relação direta entre o metabolismo e a força de contração do músculo em relação à expressão dos dois tipos de NOS, quanto mais glicolítico e quanto maior a força de contração, maior a expressão da NOS I e III. A M promoveu significativo aumento da expressão de ambas as NOS nos 3 músculos estudados,

exceto para o GAS onde não houve alteração da NOS I. A ação da mesterolona foi mais acentuada para o TA para ambas as enzimas. O exercício promoveu aumento significativo da expressão das NOS I e III no SOL, e da NOS III no GAS. Enquanto o exercício nos animais tratados com mesterolona (Sed-M vs. Ex-M) promoveu aumento significativo da NOS I (GAS) e NOS III (TA), a mesterolona nos animais exercitados (Ex-C vs Ex-M) promoveu aumento da NOS I no SOL, TA e GAS, e da NOS III no SOL e TA. A atividade das NOS I e III alterou-se diferencialmente nos três músculos pela atividade do exercício, da M ou ambos.

O óxido nítrico tem sido descrito como tendo papel na modulação do metabolismo de carboidratos do músculo esquelético em resposta ao exercício. O NO tem sido relacionado ao desenvolvimento de força, e a força está diretamente relacionada ao aumento da área seccional transversa da fibra (hipertrofia). Os dados mostrando que a expressão das NOSs I e III é maior no TA corroboram os dados da literatura. Não há na literatura nenhum relato sôbre a ação de EAA na produção de NO, portanto este é um estudo pioneiro tanto quanto sabemos. Nossos resultados mostrando aumento da expressão das NOS I e III no sóleo correlacionam-se positivamente com o fato deste músculo ter adquirido um perfil mais glicolítico e suas fibras terem sido as que tiveram maior grau de hipertrofia (aquisição de força e atividade contrátil mais rápida). É possivel que o aumento da vascularização vista no sóleo responda pelo aumento significativo da NOS III nesse músculo.

- Como parte do sistema músculo-esquelético, os tendões de Achilles foram avaliados indiretamente quanto à síntese de colágeno através da dosagem de hidroxiprolina e diretamente pela evidenciação de bandas alpha1 e alpha 2 através de

SDS-PAGE, e do diâmetro das fibrilas de colágeno e da soma das áreas das fibrilas contidas em uma área-teste, além da análise ultraestrutural dos fibroblastos. Os resultados mostraram que a mesterolona mais exercício físico foram capazes de aumentar o conteúdo de hidroxiprolina, as bandas de colágeno, o diâmetro e área das fibrilas colágenas, além do que ao microscópio eletrônico os fibroblastos mostraram-se hipertrofiados com aparência de estarem engajados ativamente em processos de síntese. Nos animais Ex-M, entretanto, apesar da significativa hipertrofia das fibrilas, o seu número era muito menor dentro da área-teste do que nos animais exercitados ou nos animais tratados com mesterolona. A mesterolona e a mesterolona + o exercício mostraram também o aparecimento de bandas proeminentes de proteínas não colagênicas. Por outro lado, o exercício abole essas bandas, mas evidencia cadeias beta, não evidenciáveis nos tendões dos grupos M- e Ex-M.

Os resultados sugerem que a mesterolona associada ao exercício físico tem efeito aditivo sôbre o aumento da hidroxiprolina, na síntese de colágeno e aumento do diâmetro das fibrilas. O aumento do diâmetro das fibrilas em razão da associação mesterolona + exercício, porém com menor número delas dentro da área-teste sugere que pode ter havido aumento do número de ligações cruzadas entre o colágeno e/ou fibrilas indicando maior agregação. Por outro lado, o maior número de fibrilas dentro da área teste nos tendões dos animais Ex-C e Sed-M e o maior tamanho delas (não significativo) em relação aos Sed-C pode sugerir fibrilogênese. O desparecimento das bandas de proteínas não colagênicas por efeito do exercício pode refletir a ação de proteases provavelmente envolvidas na remodelação do tendão provocada por intensa ação mecânica.

- Com o objetivo de avaliar os efeitos de esteróides androgênicos anabolizantes no remodelamento e dano cardíaco e no perfil lipídico dos camundongos transgênicos, o mesmo modelo animal/protocolo foi usado. Análises morfológicas e estereológicas para determinação da hipertrofia ventricular esquerda, da área seccional transversa dos cardiomiócitos, da densidade de volume dos capilares intramiocardiais e do interstício cardíaco, dos níveis plasmáticos de troponina T, e dos lípides/lipoproteínas (triglicérides,

colesterol total, LDL-c, HDL-c, VLDL) foram feitos para avaliação de alterações do perfil lipídico. Os resultados mostraram que a mesterolona causou aumento dos níveis plasmáticos do colesterol total, triglicérides, LDL-c e VLDL. O exercício aboliu muito dos efeitos ao diminuir LDL-c, VLDL e triglicérides e aumentar HDL-c. O exercício promoveu efeitos cardíacos benéficos, tais como hipertrofia fisiológica dos cardiomiócitos, aumento da circulação miocárdica e diminuição do interstício. Porém, a mesterolona aboliu os efeitos benéficos e elevou os níveis de troponina T.

Os resultados mostraram o indesejável remodelamento e dano cardíaco, a indução de perfil pró-aterogênico nos animais tratados com mesterolona, e que o exercício neutraliza alguns desses efeitos. Os efeitos negativos do remodelamento cardíaco e a aquisição de perfil pró-aterogênico aliado à propensão de hipertensão e aumento de pesos provocados pela mesterolona são fatores de risco para o desenvolvimento de doenças cardiovasculares.

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Anexos

7. ANEXOS

7.1. Anexo 1 – Resolução do formato alternativo para defesa da tese de doutorado

DELIBERAÇÃO CCPG – 001/98

Dispõe a respeito do formato das teses de Mestrado e de Doutourado aprovadas pela UNICAMP

Tendo em vista a possibilidade, segundo parecer PG Nº 1985-96, das teses de mestrado e de doutourado terem um formato alternativo àquele já bem estabelecido, a CCPG resolve:

Artigo 1: Todas as teses (alternativas) de mestrado e de doutorado da UNICAMP terão, a partir de janeiro de 1999, o seguinte formato padrão:

I) Capa com formato único, dando visibilidade ao nível (mestrado ou doutorado), e à Universidade

II) Primeira folha interna dando visibilidade ao nível (mestrado ou doutorado) à Universidade, à Unidade em que foi defendida e à banca examinadora, ressaltando o nome do orientador e co-orientador. No seu verso deve constar a ficha catalográfica.

III) Segunda folha interna onde conste o Resumo em português e o Abstract em inglês.

IV) Introdução Geral

V) Capítulos

VI) Conclusão Geral

VII) Referências Bibliográficas

VIII) Apêndices (se necessário)

Artigo 2: A critério do orientador, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

Parágrafo único: Os veículos de divulgação deverão ser expressamente indicados.

Artigo 3: A PRPG providenciará o projeto gráfico das capas bem como a impressão de um

7.2. Anexo 2: Certificado do Comitê de Ética em Pesquisa Animal

Universidade Estadual de Campinas
Instituto de Biologia

UNICAMP

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

C E R T I F I C A D O

Certificamos que o Protocolo nº **700-1**, sobre **"INFLUÊNCIA DOS ESTERÓIDES ANABÓLICO-ANDROGÊNICO (EAA) ASSOCIADOS AO EXERCÍCIO FÍSICO SOBRE OS MÚSCULOS DE CAMUNDONGOS QUE EXPRESSAM PROTEÍNA DE TRANSFERÊNCIA DE ÉSTER DE COLESTERIL (CETP) COM DEFICIÊNCIA PARCIAL DO RECEPTOR DE LDL-COLESTEROL"** sob a responsabilidade de **Profa. Dra. Maria Alice Cruz-Höfling/Karina Fontana** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 01 de Julho de 2004.

C E R T I F I C A T E

We certify that the protocol nº **700-1**, entitled "_____", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on July 1st, 2004.

Campinas, 1º. e Julho de 2004.

Profa. Dra. Liana Verinaud
Presidente

Fátima Alonso
Secretária

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Capítulo VI

Capítulo VI:

Para alcançarmos os nossos objetivos, foram avaliados: estado de supra-organização da cromatina, conteúdo de DNA e fragmentação (TUNEL), área e perímetro dos hepatócitos e a atividade das transaminases hepáticas (AST e ALT).

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Histology and Histopathology

Cellular and Molecular Biology

Hepatocyte nuclear phenotype: the cross-talk between anabolic androgenic steroids and exercise in transgenic mice

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Summary. The growing and indiscriminate use of high doses of anabolic androgenic steroid (AAS) among youth and athletes has raised serious concerns about its hepatotoxic effects. Herein, the influence of AAS in the nuclear phenotype of hepatocytes was investigated in sedentary and trained mice heterozygous for the human CETP (cholesteryl ester transfer protein) transgene and for LDL-receptor null allele ($CETP^{+/-}LDLr^{+/-}$) by image analysis. Five groups were assayed comprising treadmill exercised (Ex) and sedentary (Sed) mice, administered mesterolone (AAS) or gum arabic (GA) and a sedentary blank control: G1(SedAAS), G2(SedGA), G3(ExAAS), G4(ExGA), and G5(SedBL). To assess nuclear phenotypes, the state of chromatin supraorganization, DNA content and fragmentation (TUNEL assay), area and perimeter of hepatocytes were determined in Feulgen-stained liver imprints. In addition, the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) hepatic transaminases were measured. SedAAS-G1 showed the lowest chromatin condensation and highest Feulgen-DNA content, polyploid nuclei frequency, nuclear area and perimeter, suggesting gene activation. Contrarily, ExAAS-G3 showed a highest chromatin condensation, and a significant decrease of Feulgen-DNA content and decreased frequency of polyploid nuclei, which suggest gene silencing. Image analysis of the nuclear phenotype offered a coherent descriptive picture of the changing patterns of chromatin organization, which were shown to be congruent with the levels of Feulgen-DNA content, geometric nuclear parameters and hepatocyte activity. In this study, the image analysis permitted the monitoring of the nuclear response to mesterolone and physical exercise action in liver cells, the molecular mechanism of which is in prospect.

Key words: Chromatin supraorganization, DNA content, Hepatoxicity, Image analysis, Mesterolone

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Introduction

Androgenic-anabolic steroids (AAS) are synthetic derivatives of testosterone, which were chemically modified to maximize anabolic and minimize undesirable androgenic effects (Haupt and Rovere, 1984). Pharmacologic and suprapharmacologic doses of AAS and/or testosterone have been successfully used for the treatment of patients with hypogonadism, age-related sarcopenia, HIV-related muscle wasting (Vermeulen, 2001) and protection against ageing-associated decline of cognitive functions (Resnick and Maki, 2001).

During the past few decades, the use of AAS has exceeded the medical recommendations, becoming

Abbreviations: AAS, anabolic androgenic steroids or mesterolone; ALT, alanine aminotransferase; Apo-A1, apolipoprotein A1; AST, aspartate aminotransferase; A.U., arbitrary units; CETP, cholesteryl ester transfer protein; Ex, treadmill exercised mice; GA, gum arabic; HDL-C, high-density lipoprotein cholesterol; IOD, integrated optical density; LDL-C, low-density lipoprotein cholesterol; LDL-r, LDL receptor gene; OD, optical density or average absorbance; SDtd, standard deviation of gray average in pixels per nucleus; Sed, sedentary mice; TC, total cholesterol; TG, triglycerides; VLDL, very low density lipoproteins cholesterol.

popular among youth and athletes for their aesthetic body muscle building effects and performance-enhancing properties (Haupt and Rovere, 1984). The abuse of AAS has been associated with the occurrence of premature cardiovascular diseases. These events can negatively be mediated by the appearance of an atherogenic lipoprotein profile which has been associated with AAS self-administration (Taggart et al., 1982; Haffner et al., 1983; Applebaum-Bowden et al., 1987; Glazer, 1991; Hartgens et al., 2004). Besides, epidemiological data have shown the close relationship between AAS use and the high incidence of hepatic disorders, including benign and malignant hepatocellular tumors, cholestatic jaundice, peliosis hepatis, and sub-cellular morphological alterations (Goldman, 1985; Ishak and Zimmerman, 1987; Creagh et al., 1988; Soe et al., 1992; Cabasso, 1994; Kosaka et al., 1996).

Biochemically, the major actions of AAS occur through hormone binding to intracellular receptors in target tissue. This hormone-receptor complex then translocates to chromatin binding sites and binds to its target gene promoter, inducing gene transcription and subsequent synthesis of mRNA (Bahrke and Yesalis, 2004; Wang et al., 2005). The liver expresses estrogen and androgen receptors and experimentally both of those receptors have been implicated in stimulating hepatocyte proliferation, altering the cell cycle, and possibly acting as liver tumor inducers or promoters (Giannitrapani et al., 2006). The increase of lean body mass, muscle size and strength caused by the anabolic effect of AAS results from increased protein metabolism and collagen synthesis (Griggs et al., 1989; Falanga et al., 1999; Bahrke and Yesalis, 2004). It has been reported that dynamic changes to patterns of chromatin condensation are associated with altered cell activity and the cell cycle (Mello and Russo, 1990; Vidal et al., 1998; Leitch, 2000; Maria et al., 2000; Aldrovani et al., 2006; Moraes et al., 2005). Hitherto, a description of accidental alterations provoked by AAS abuse in the chromatin general *status* of cells in the target tissues was not found in current literature.

In a recent study, we showed that the androgenic-anabolic steroid mesterolone caused adverse cardiac remodeling and serious atherogenic lipoprotein profile in sedentary transgenic mice which express the human cholesteryl ester transfer protein (CETP) and are a knockout for the low-density lipoprotein receptor (LDL-r) (Fontana et al., 2008). In contrast, in mesterolone administered-treadmill exercised mice, the unfavorable cardiac remodeling and lipoprotein profile was markedly blunted. In this murine transgenic model, the expression of the CETP gene and reduction of the LDL receptor gene (LDLr) expression, drive its lipemic phenotype closer to humans, but apart from the wild type mice which show very low levels of low-density lipoprotein (LDL) and high levels of high-density lipoprotein (HDL) (Cazita et al., 2003; Casquero et al., 2006).

Given the hypothesis that the spatial organization of the interphase nucleus may represent one of the fundamental control mechanisms in gene expression (Park and De Boni, 1999), the study of nuclear phenotype is an initial step for monitoring transcriptional activity or gene silencing caused by AAS abuse.

This is the first study that utilizes image analysis to investigate the nuclear phenotype of hepatocytes in transgenic $CETP^{+/-}LDLr^{+/-}$ mice. The aspect of chromatin supraorganization, DNA content, DNA fragmentation, area and perimeter of hepatocytes nuclei were determined. In addition, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), that are commonly used as markers of inflammation and/or injury, were measured in liver cells homogenate. This is a descriptive study aimed at correlating the alterations of the nuclear phenotype of hepatocyte in transgenic mice, sedentary or submitted to aerobic exercise, to which the anabolic-androgenic steroid mesterolone or gum arabic (vehicle) were administered orally. The utilization of the image analysis method can be prognostic about ongoing transcriptional activities.

Materials and methods

Drugs and chemicals

Mesterolone (1alpha-methyl-5alpha-androstan-17beta-ol-3-one) is a non-17 alpha-alkylated derivative of testosterone, whose commercial name is “Proviron”, a registered trademark of Schering (Schering do Brazil, São Paulo, SP, Brazil). Gum arabic, also called gum acacia, is taken from *Acacia trees* and purchased from Sigma (St. Louis, MO, USA).

Animals

The study protocols were approved by the university’s Committee for Ethics in Animal Experimentation (CEEA/UNICAMP) and the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1996) were followed. The transgenic mice used in this study have been cross-bred and maintained in the Department of Physiology and Biophysics, Institute of Biology, UNICAMP for ten years. Mice heterozygous for the human CETP transgene and for LDL-receptor null allele ($CETP^{+/-}LDLr^{+/-}$) have been described elsewhere (Cazita et al., 2003; Casquero et al., 2006). The mice were housed in a temperature-controlled room (22±1°C), humidity of 55-65%, 12h light/dark cycle and had free access to water and standard chow (Nuvilab®, Colombo, PR, Brazil).

Experimental Protocol

Male heterozygous mice ($CETP^{+/-}LDLr^{+/-}$), 2 months old (~22 g body weight) were used. The animals were divided into five groups (n=12/group). Group 1 (G1) – sedentary plus mesterolone (SedAAS); Group 2 (G2) – sedentary plus gum arabic (vehicle) (SedGA); Group 3 (G3) – trained in treadmill plus mesterolone

(ExAAS); Group 4 (G4) – trained in treadmill plus vehicle (ExGA); Group 5 (G5) – intact sedentary (blank control) (SedBL). G3 and G4 animals had a week of adaptation, in which a low level of exercise, consisting of treadmill running, was done daily for 5 days (15 m/min during 20 min/day). After the week of adaptation, trained mice were subjected to a 6 weeks of treadmill running, 5 days a week, 60 min/day, as scheduled in Table 1. The animals received mesterolone (AAS) (2 µg/g body weight) or gum arabic (GA) (2 µg/g body weight) by orogastric tube during the last 3 weeks (three days a week: Monday, Wednesday and Friday) of the training or sedentary period (Table 1). In this study, gum arabic was used as vehicle for AAS administration given its proabsorptive effect in the small intestine (Codipilly et al., 2006).

At the end of the experimental period, overnight fasted mice were anesthetized with a 1:1 mixture of ketamine chloride (Dopalen®, 100 mg/kg of animal) and xylazine chloride (Anasedan®, 10 mg/kg of animal) (2 ml/mg body weight, i.p.). Both anesthetics were purchased from Vetbrands (Jacareí, SP, Brazil). Livers were removed and parts were stored for liver imprints and determination of nuclear phenotype. Other parts of dissected livers were processed for histology, and DNA fragmentation analysis.

Nuclear phenotypes

Small pieces of liver tissues were dissected out from six animals of each group, placed in cold physiologic solution (0.9% NaCl), and were used to prepare imprints by making direct touch of a surface of the selected livers tissue onto glass slides. The prepared imprints were fixed in a mixture of absolute ethanol – glacial acetic acid (3:1, v/v) for 1 min, rinsed in 70% ethanol for 5 min and then air dried (Moraes et al., 2005).

Imprints were processed according to Feulgen reaction to detect nuclear DNA. Acid hydrolysis was done with 4 M HCl at 25°C for 90 min. Next, the imprints were stained with Schiff's reagent for 40 min, rinsed in sulfurous water (mixture of distilled water, 10% sodium metabissulfite and 1 M HCl; 18:1:1; v/v/v) and distilled water, air-dried, cleared in xylene and mounted with a coverslip in natural Canada balsam (nD=1.54) (Vetec, Rio de Janeiro, RJ, Brazil). Nuclei stained with the Feulgen reaction were analyzed using Olympus microscopy (Olympus American Inc., NY, USA) equipped with 40x UplanFl objective, optovar 2, 0.90 condenser and 546 nm wavelength. The images were captured using a Q-color 3 digital video camera (Olympus American Inc., NY, USA), transmitted to a Pentium 4 computer and analyzed using Image-Pro-Plus 5.01 software for Windows (Media Cybernetics, Inc., NY, USA). The parameters analyzed were optical density (OD = average absorbance), standard deviation of gray average in pixels per nucleus (SDtd of OD), Feulgen-DNA values (integrated optical density, IOD, in arbitrary units, A.U.), nuclear area (μm^2) and nuclear perimeter (µm). For each of the experimental groups 460 hepatocytes nuclei were analyzed. Fifty (50) nuclei of lymphocytes from the blank control (SedBL-G5) group were also analyzed as reference for establishing the classes of hepatocytes ploidy (Feulgen-DNA values), given lymphocytes typical diploid standard 2C-DNA content (Hayday et al., 1985).

DNA fragmentation

After sacrifice of the mice and removal of the liver, some pieces of tissue were destined for examination of DNA fragmentation associated with programmed cell death. Fragments of livers of the five groups (n = 6 animals/group) were fixed in 4% paraformaldehyde and embedded in paraffin. Sections 5 µm thick were dewaxed, submitted to a descendent ethanol series and washed in distilled water to examine DNA fragmentation using the TUNEL POD assay, as described by the manufacturer (Roche/Amersham). Since UTP was labeled with fluorescein, anti-fluorescein antibodies coupled to peroxidase were incubated with 3-3'-diaminobenzidine (DAB, Sigma Chemical, St. Louis, MO, USA) for 10 min at room temperature (25°C). Counter-staining was done with hematoxylin (Merck KGaA, Darmstadt, Germany), followed by rinsing in distilled water. The preparations were then air dried and mounted in natural Canada balsam. Endogenous peroxidase was blocked with 3% H_2O_2 in absolute methanol for 30 min at room temperature. A negative control was done by excluding the TdT enzyme from the reaction. As positive controls, preparations incubated with DNase I (3000 U/mL in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ 1 mg/mL BSA, for 10 min at 25°C) were also used.

Measurement of enzymatic activity

At the end of the experimental protocol, all mouse groups (n=6) were anesthetized as described above, and the livers were removed, rinsed repeatedly with large volumes of physiological saline, and homogenized by hand in five volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 0.15 M KCl and 1 mM EDTA, and the crude homogenate was centrifuged

Table 1. Exercise (6 weeks) and AAS or vehicle administration (3 weeks) protocol.

Weeks	Velocity (m/min)	Duration (min)
1	12.42	20
2	14.70	30
3	16.68	45
4 - 6 (*)	17.04	60

(*) AAS or gum arabic (vehicle) administered orally by gavage at 10:00 a.m. (Monday, Wednesday and Friday) in the last three weeks.

at 9000g for 20 min. Ultracentrifugation to pellet the microsomal fraction was performed at 105 000g for 1 h. Supernatant was stored as cytosolic fraction. All procedures were carried out at 0-4°C. The activities of glutamic oxalacetic transaminase (GOT - AST) and glutamic-pyruvic transaminase (GPT - ALT) were measured in the supernatant of homogenized livers according to the instructions of the manufacturer contained in commercially available kits (Laborlab no 02600, Guarulhos, SP, Brazil).

Statistical analysis

All calculations and statistical analyses were done using the Minitab 12™ software (State College, PA, USA) and involved frequency histograms, marginal and regression plots, sort manipulation and Analysis of Variance (Anova), Kruskal-Wallis and Mann-Whitney post-tests. Differences were considered statistically significant at P-values < 0.05.

Results

Nuclear phenotypes

Color digital images of Feulgen-positive hepatocyte nuclei were converted for gray levels and used for establishing nuclear phenotypes. After the conversion, nuclei of the population of each mouse group showed variations in the level of gray intensity, reflecting differences in chromatin packaging, and chromocenters (areas of more condensed chromatin). Only a digital image of the control sample is displayed (Fig. 1A) because differences of nuclear phenotypes among the groups studied can only be discernible by computer-aided image analysis, and not visually. Fig. 1B displays hepatocyte nuclei after image treatment that allows us to visualize in detail areas of more condensed chromatin/chromocenters.

Image analysis of preparations stained with Feulgen reaction yielded the values listed in Tables 2 and 3.

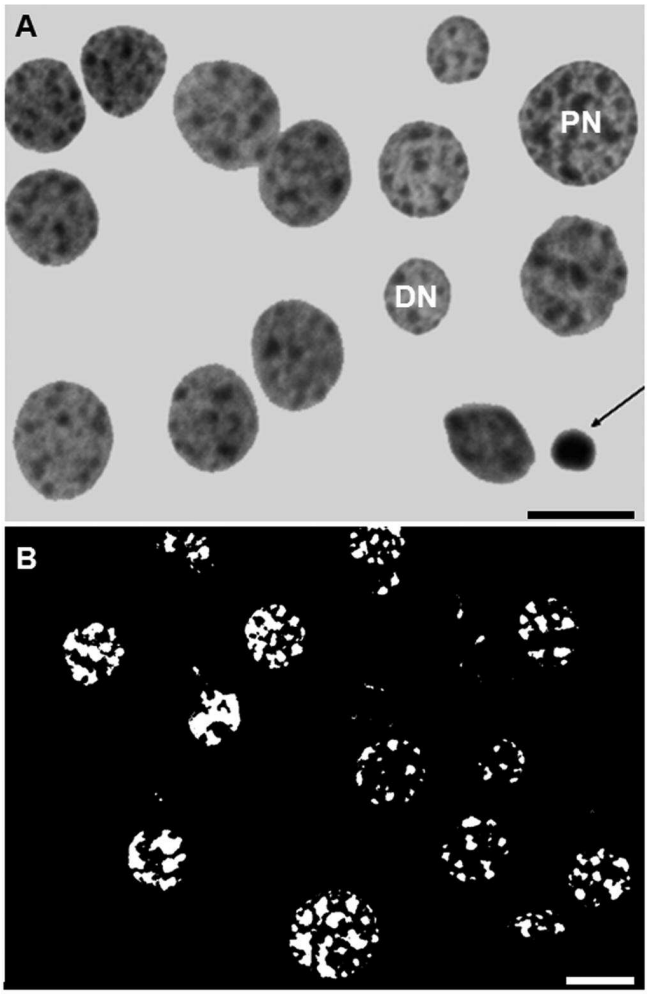

Fig. 1. A. Digital image of blank hepatocyte nuclei stained with the Feulgen reaction. The image was converted for the gray levels using Image-Pro-Plus software. PN, polyploid nucleus; DN, diploid nucleus. The arrow indicates a lymphocyte that was used as control for ploidy. **B.** Hepatocyte nuclei after image treatment for visualization of more condensed chromatin. Bars: 1.25 µm.

Table 2. Evaluation of densitometric and textural parameters by image analysis of Feulgen-stained hepatocyte nuclei obtained from liver imprints of $CETP^{+/-}/LDLr^{+/-}$ transgenic mice.

Experimental groups	IOD		OD		SDtd	
	η	Z	η	Z	η	Z
G1-Sedentary treated with mesterolone	37.28^{a}	6.82	0.3082^{a}	-16.14	0.0380^{a}	-23.14
G2-Sedentary treated with gum arabic	32.00^{c}	-2.53	0.3902^{b}	-2.90	0.0799	8.76
G3-Trained treated with mesterolone	34.51^{e}	0.46	0.4157^{c}	19.22	0.0959	20.07
G4-Trained treated with gum arabic	20.82^{d}	-11.19	0.3324	-11.72	0.0904	15.61
G5-Control (intact sedentary)	36.00^{b}	6.45	0.3389^{d}	-2.35	0.0432^{b}	-21.30

Statistical tests, Kruskal-Wallis; η, median; Z-value, indicates how the mean rank for each group differs from the mean rank for all groups; IOD, integrated optical density (Feulgen-DNA values); OD, optical density (absorbance); SDtd, standard deviation of the gray average, in pixels, per nucleus; number of measurements, 460. All comparing values show significant statistical difference ($p<0.05$) IOD: G1 (a) vs. G5 (b); G2 (c) vs. G5 (b); G3 (e) vs. G5 (b); G4 (d) vs. G5 (b); OD: G1 (a) vs. G2 (b); G2 (b) vs. G3 (c); G1 (a) vs. G5 (d); SDtd: G1 (a) vs. G5 (b).

Table 3. Evaluation of geometric parameters by image analysis of Feulgen-stained hepatocyte nuclei obtained from liver imprints of CETP$^{+/-}$/LDLr$^{+/-}$ transgenic mice.

Experimental groups	Area		Perimeter	
	η	Z	η	Z
G1-Sedentary treated with mesterolone	122.45	16.98	44.7 a	12.33
G2-Sedentary treated with gum arabic	75.23	2.41	34.39	- 6.96
G3-Trained treated with mesterolone	75.63	1.96	34.66 c	- 8.81
G4-Trained treated with gum arabic	71.66	3.28	35.46 d	- 3.80
G5-Control (intact sedentary)	99.85	11.62	41.24 b	7.24

Statistical tests, Kruskall-Wallis;η, arithmetic median; Z-value, indicates how the mean rank for each group differs from the mean rank for all groups; number of measurements, 460. All comparing values show significant statistical difference ($p<0.05$). Perimeter: G1 (a) vs. G5 (b); G3 (c) vs. G4 (d).

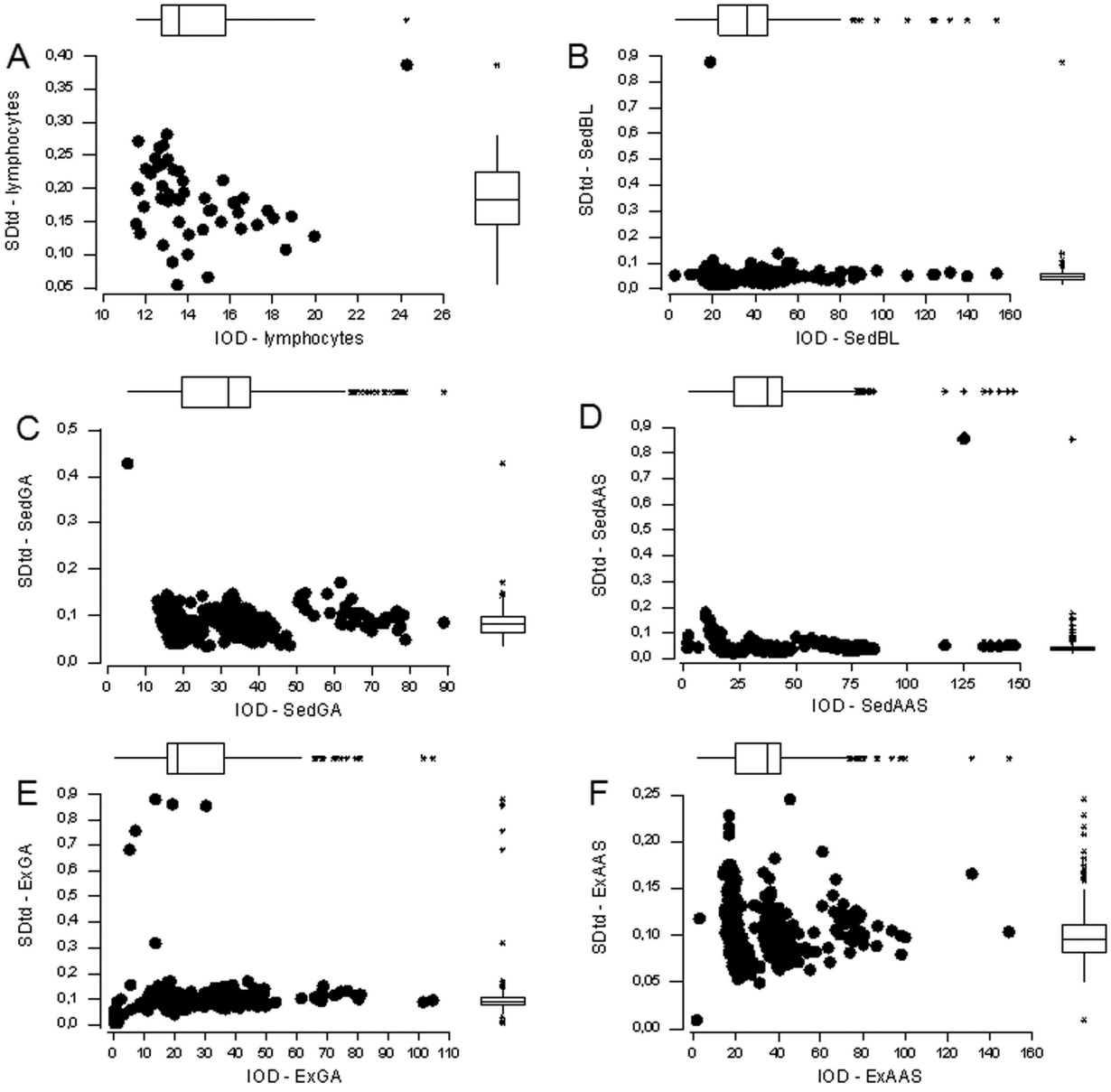

Fig. 2. Marginal plot of SDtd in function of IOD. SedBL, sedentary blank controls; SedGA, sedentary plus gum arabic; SedAAS, sedentary plus mesterolone; ExGA, trained in treadmill plus gum arabic; ExAAS, trained in treadmill plus mesterolone.

Hepatocyte nuclei from sedentary animals treated with mesterolone (SedAAS-G1) showed the lowest values of OD (chromatin condensation for nuclei populations) and SDtd (chromatin diffuseness index per individual nuclei) compared to the other mice groups (Table 2). In contrast, ExAAS-G3 showed the highest OD and SDtd values. No significant difference was observed between SDtd values of ExAAS-G3 and ExGA-G4 groups ($P<0.05$ level). OD and SDtd are correlated by the fact that the nuclei image is saved in bytes containing the total values of the gray

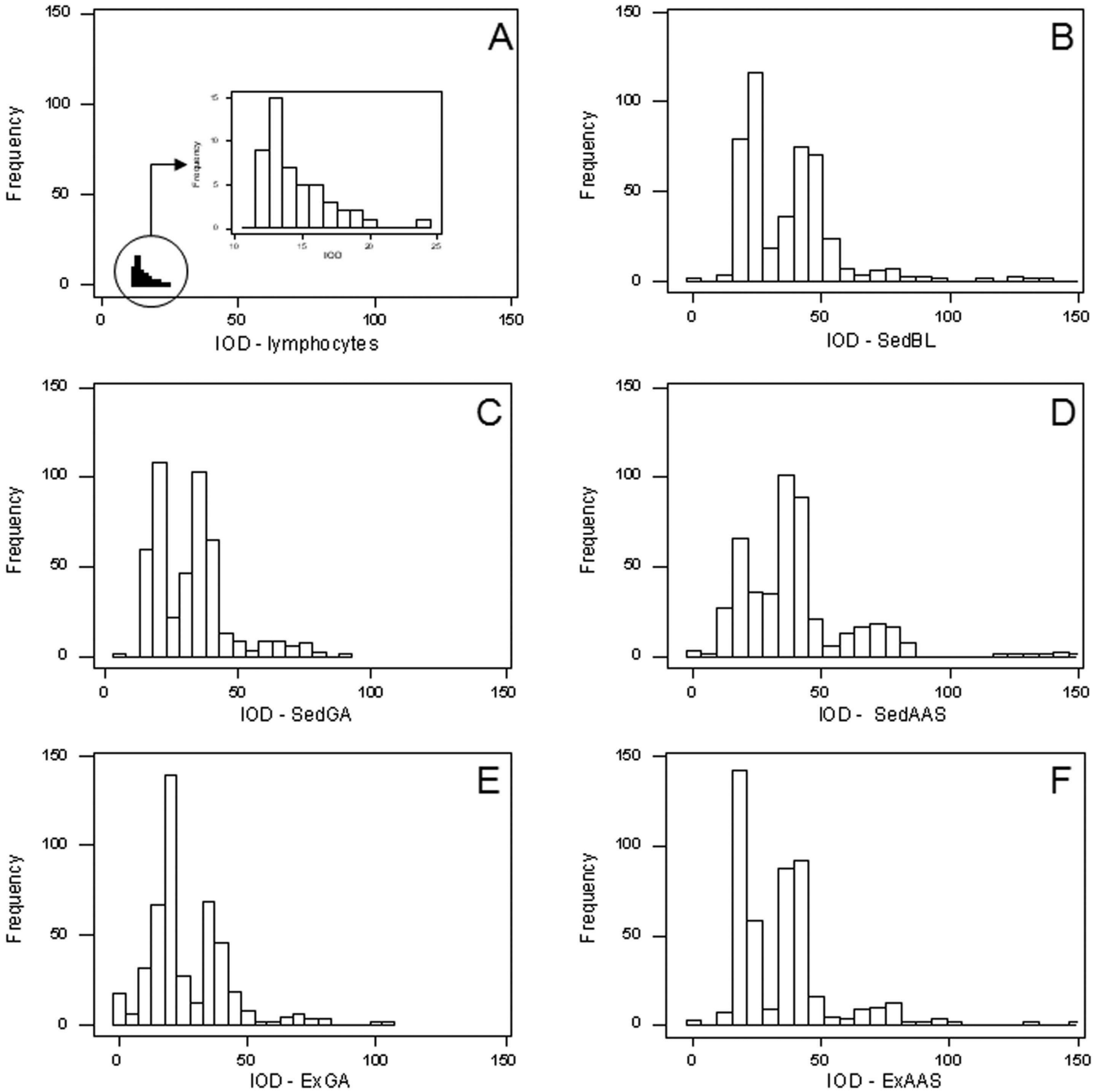

Fig. 3. Frequency histograms of the Feulgen-DNA values obtained for the mice groups studied. IOD values from hepatocytes were compared to IOD values obtained from lymphocytes, which typically showed a single peak indicating that the content of DNA is 2C. SedBL, sedentary blank controls; SedGA, sedentary plus gum arabic; SedAAS, sedentary plus mesterolone; ExGA, trained in treadmill plus gum arabic; ExAAS, trained in treadmill plus mesterolone.

intensity levels that are transformed in OD.

Graphics of SDtd in function of IOD (IOD is calculated as OD x nuclear area, and OD is correlated with SDtd as previously mentioned) were constructed (Fig. 2), showing that the nuclei with the highest IOD values possess the smallest contrast between condensed and descondensed chromatin (SDtd values). All experimental groups, except SedAAS-G1, presented higher spreading of IOD and SDtd values than intact sedentary SedBL-G5. The ExAAS-G3 group displayed the highest spreading of SDtd values (Fig. 2F).

IOD median values, listed in Table 2, show the Feulgen-DNA content. All mice groups, except SedAAS-G1 which showed the highest value, showed hepatocyte nuclei with lower IOD values than the intact sedentary SedBL-G5 group. The lowest IOD median value was observed in treadmill trained gum arabic-treated animals (ExGA-G4), followed by sedentary gum arabic-treated mice (SedGA-G2) and treadmill trained mesterolone-treated mice (ExAAS-G3), respectively. Significant differences were observed between IOD median values from hepatocyte nuclei of all groups in comparison with the blank control mice (SedBL-G5), including SedAAS-G1 vs. SedBL-G5 ($P_{0.02}$ *level*).

The distribution of the hepatocytes' IOD values was analyzed by frequency histograms to show levels of ploidy. IOD values from hepatocyte nuclei were established by comparison with IOD values from lymphocyte nuclei, which typically showed a single peak relative to 2C-DNA content or diploid nuclei. Lymphocyte IOD values were distributed in the interval of 11.5–25 A.U. (Fig. 3A), indicating that these Feulgen-DNA values represent diploid nuclei in different phases of the cell cycle. Hepatocytes from all groups (G1 to G5) showed multimodal distribution of the IOD values (Fig. 3 B-F), indicating the presence of nuclei with different

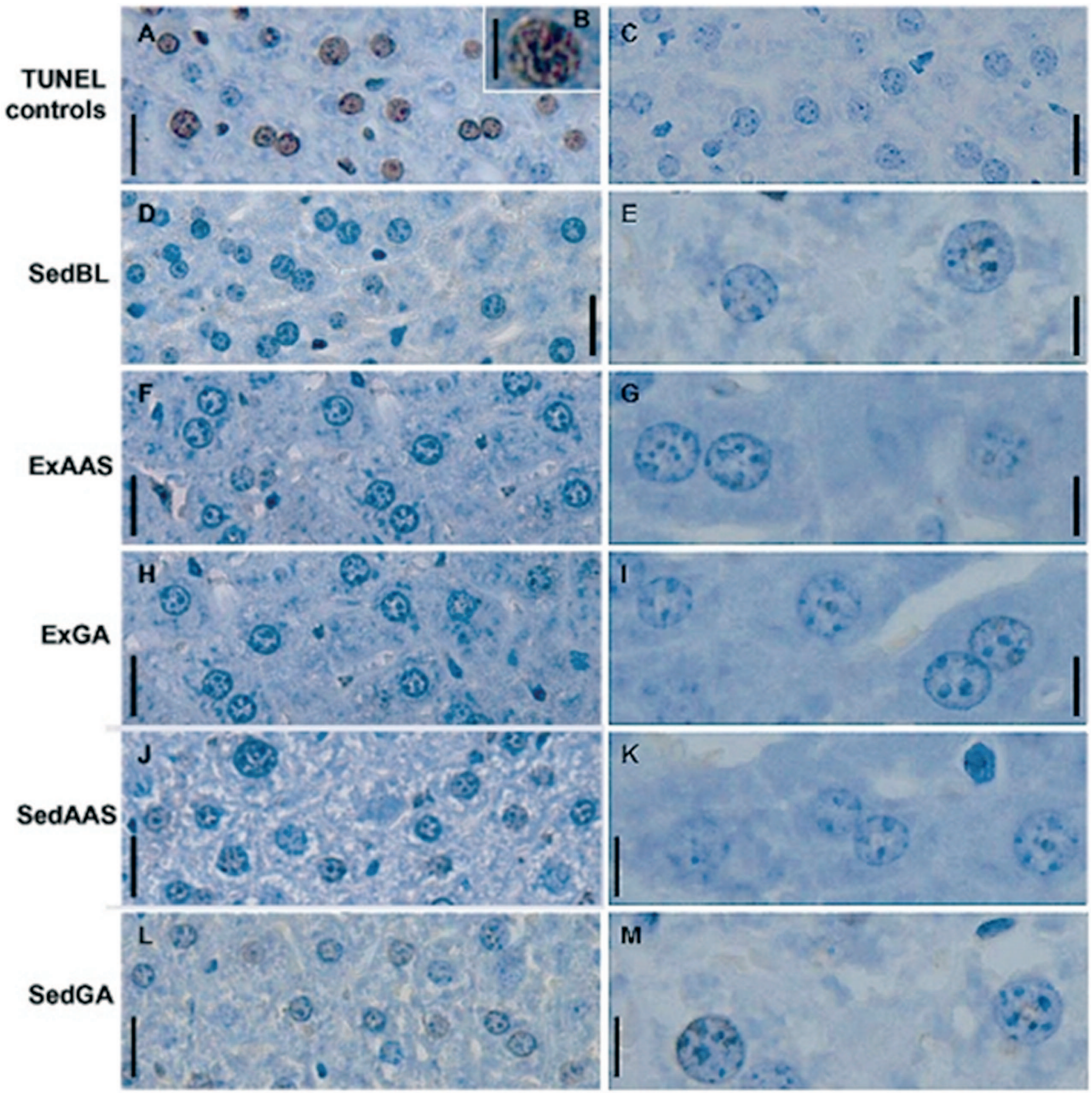

Fig. 4. Hepatocyte nuclei subjected to the TUNEL assay. **A and B,** hepatocytes subject to treatment with DNaseI (positive controls); **C,** negative control; **D and E,** Sedentary blank controls (SedBL); **F and G,** trained in treadmill plus mesterolone (ExAAS); **H and I**, trained in treadmill plus gum arabic (ExGA); J and K, sedentary plus mesterolone (SedAAS); **L and M,** sedentary plus gum arabic (SedGA). Bars: A, C, D, F, H, J, L, 25 µm; B, E, G, I, K, M, 10 µm.

ploidy levels. Those with IOD values between 11.5–25 A.U. were diploids, whereas those with IOD ≥ 25.1 A.U. were polyploids (25.1–50 A.U. = 4C-DNA, 50–99.9 A.U. = 8C-DNA and ≥ 100 = 16C-DNA content). A sort manipulation (Minitab 12™ software) of the IOD values allowed us to calculate the number of polyploid nuclei present in each hepatocyte population studied (n = 460): nuclei with IOD values ≥ 25.1 A.U (polyploids) were 330 in SedAAS-G1; 271 in SedGA-G2; 297 in ExAAS-G3; 184 in ExGA-G4; and 309 in SedBL-G5. Interestingly, all hepatocyte populations showed some nuclei with smaller IOD values than the minimum value obtained for lymphocytes (11.5 A.U.). Nuclei with IOD values < 11.5 A.U. was for G1= 6; G2= 1; G3= 2; G4= 34; and G5= 3 nuclei.

Hepatocyte nuclei from sedentary mice treated with mesterolone (SedAAS-G1) showed higher values for geometric parameters (nuclear area and perimeter) in comparison to hepatocyte nuclei of all other groups. The values of nuclear area and perimeter obtained for each sample are displayed in the Table 3.

The correlations between nuclear areas and Feulgen-DNA values were obtained using Minitab 12™ software and were expressed as a quadratic linear regression (R2). For all mice groups, except ExAAS-G3, there were no significant differences in the correlation coefficients when compared to the intact sedentary SedBL-G5 group (Table 4).

DNA fragmentation

DNA fragmentation typical of programmed cell death and/or apoptosis was not detected by the TUNEL assay in the hepatocytes studied (Fig. 4D-M) and in the negative controls (Fig. 4C); a positive response was obtained in hepatocytes incubated with DNAse I (Fig. 4A, B).

Transaminases

AST and ALT activities were detected in all samples, determined six weeks after beginning the experiments, and showed no significant difference among the groups studied (P>0.05) (Fig. 5). However, there was a trend to increase AST in SedAAS, with exercise counteracting this effect (see ExAAS).

Table 4. Correlation coeficient (R2), in percentage, between nuclear area and Feulgen-DNA content (integrated optical density, IOD).

Mice	R^2 (%)
G1- Sedentary treated with androgenic	64.5
G2-Sedentary treated with gum arabic	81.8
G3-Trained treated with androgenic	87.4
G4-Trained treated with gum arabic	83.2
G5-Control (intact sedentary)	90.1

For all mice groups, except G3, there were no significant differences in correlation coefficients when compared to the intact sedentary G5 group. (P<0.05): G1 VS. G5; G2 VS. G5; G4 VS. G5.

Discussion

The indiscriminate self-administration of anabolic-androgenic steroids has been associated with relevant hepatocellular disorders and development of an atherogenic profile in drug abusers (Goldman, 1985, Ishak and Zimmerman, 1987; Creagh et al., 1988; Soe et al., 1992; Cabasso, 1994; Kosaka et al., 1996). Herein, the computer-aided image analysis of the state of chromatin supraorganization, texture, DNA content/fragmentation, and geometric parameters in interphase nucleus of hepatocytes in intact, sedentary or exercised $CETP^{+/-}LDLr^{+/-}$ transgenic mice treated with mesterolone or vehicle showed interesting differences. This heterozygous transgenic mouse has a lipemic profile akin to humans, and as such, is useful for studies aiming to reproduce clinical research. Hepatic CETP mediates the plasma cholesteryl ester transfer from high-density lipoprotein (HDL) to apolipoprotein B (apoB)-containing lipoproteins (very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) in exchange

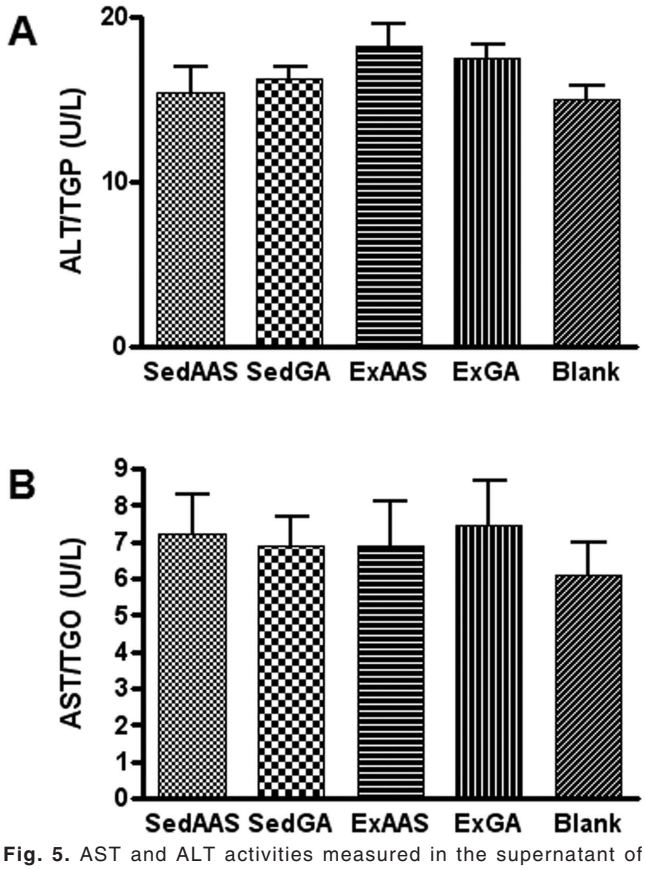

Fig. 5. AST and ALT activities measured in the supernatant of homogenized livers.

for triacylglycerol (Bruce et al., 1998). In humans, CETP mRNA is predominantly expressed in the liver and is secreted by hepatocytes. An increase in plasma CETP and hepatic CETP mRNA in transgenic mice expressing the human CETP gene can be induced by environmental conditions, such as alcohol, smoking, obesity, dietary alterations and drugs such as cholestyramine and statins (Jiang et al., 1992; Harada et al., 2006).

In our murine model, the six weeks of exercise (5 times/week) and the three weeks of mesterolone oral administration represent a considerable time-interval in terms of duration/intensity of the physical exercise, and period of drug abuse in human life cycle (Bronson and Matherne, 1997). Hence, this model can be useful for studies of complications of anabolic steroid misuse.

The present results showed that the lowest absorbance values (OD) were observed in sedentary mice treated with mesterolone (SedAAS-G1), and were compatible with chromatin unpackaging. In addition, this was substantiated by the least contrast between the higher and lower packaging states of chromatin (SDtd) found in the same group. Recent study in our laboratory with this lipemic mice strain, and using the same protocol as for the present study, showed that sedentary animals treated with mesterolone (SedAAS-G1) was the group which presented the highest plasmatic levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and very low-density lipoproteins (VLDL), while high-density lipoprotein (HDL) levels were slightly below that of SedGA (Fontana et al., 2008). A simultaneous decrease in OD and SDtd values in hepatocyte, nuclei has been associated with the recovery of transcriptional activities after refeeding of a 48 h-fasted inbred strain A/Uni mice (Cassia et al., 1991; Moraes et al., 2005). In contrast, the highest spreading of IOD (OD x area) and SDtd values observed in ExAAS-G3 is both an indication of increased packaging of certain chromatin areas, as well as of increased contrast between condensed and uncondensed chromatin. Since in exercised-vehicle mice (ExGA-G4) the level of chromatin condensation was higher than in SedAAS, but lower than in ExAAS, such chromatin remodeling may show that physical exercise counteracted the effects of AAS. The opponent (modulatory) action of exercise against mesterolone was also seen in the lipoprotein profile (Fontana et al., 2008). ExAAS showed a significant decrease of TG, LDL and VLDL, and more importantly, an increase of HDL in relation to SedAAS-G1. The lowering in plasmatic levels of lipid and lipoproteins in ExAAS is in agreement with the highest OD (chromatin condensation) and SDtd (chromatin diffuseness) values obtained for the group. Taken together, the chromatin remodeling seen by image analysis suggest an upregulation of transcriptional activity in SedAAS-G1 and a synthesis inhibition of some lipoproteins in ExAAS, or an antagonic effect of mesterolone vs. exercise. However, it could not be ignored that the control of DNA condensation is mediated via protein-chromatin interactions and influenced by epigenetic phenomena such as DNA methylation (Keshet et al., 1986) and histone acetylation (Houben et al., 1996). These epigenetic factors may be involved with transcriptional activity or gene silencing as suggested by the nuclear phenotype of hepatocytes in the current study. There are much molecular evidences to suggest that AAS acts by activating genes related with the synthesis of liver enzymes (Labrie et al., 2005). In the present study, it is possible that genes involved with lipid metabolism could have been also affected. However, we did not find differences in plasmatic levels of liver transaminases AST and ALT. There was not a consensus about transaminases changes due to AAS use in pertinent literature (see Glazer, 1991 for a review), and this could be attributed to differences in protocol, the kind of AAS and the animal studied.

In accordance with the results of OD and SDtd, the median values of IOD (Feulgen-DNA content) corroborate the hypothesis of highest gene activity in sedentary mice treated with mesterolone (SedAAS-G1). The G1-group showed the highest elevation of the Feulgen-DNA content in relation to all other groups, including the blank control mice (SedBL-G5). Note that nuclei with the highest IOD values were shown to possess the smallest contrast between condensed and uncondensed chromatin (SDtd values). It was also the SeAAS-G1 group which exhibited the highest perimeter and area of the interphase nucleus. This was positively correlated with the higher Feulgen-DNA content measured in SedAAS.

One phenomenon that can explain the present results is the polyploidization. The increase in IOD values triggered by AAS administration to sedentary mice (SedAAS-G1-group) is apparently associated with the highest frequency of polyploid nuclei in the G1 hepatocytes population. This would also be responsible for the larger values of the geometric parameters (nuclear area and perimeter) detected for the nuclei of the G1-group. Seventy percent of the G1 nuclei were polyploids. The hypothesis is feasible since there was a strict positive correlation between IOD median values and geometric nuclei parameters, with values indicating G1 > G5 > G3 > G2 > G4 (in which decreased nuclear area and perimeter are in conformity with the smaller Feulgen-DNA content), and the percentage of polyploids nuclei, where (G1 = 330:70%) > (G5 = 309:67%) > (G3 = 297:64%) > (G2 = 271:58%) > (G4 = 184:40%). Polyploidy results from incomplete mitotic cycles, and are generally related to increased physiological demands (Nicolini, 1980; Aldrovani et al., 2006). On other the hand, in terms of relative diploid hepatocytes frequency the results showed G4 > G2 > G3 groups, which was inversely proportional to IOD median values, i.e., G4 < G2 < G3. Another fact to be considered was that hepatocyte nuclei showing smaller IOD values than lymphocytes (11.5 A.U.) were observed in all groups studied. This may be indicative of a certain degree of DNA fragmentation and loss, as happens in apoptosis or programmed cell death (Maria et al., 2000), or due to

DNA hydrolysis during processing. We believe that the DNA/apurinic acid fragmentation is the most plausible explanation in the present case, since no positive response to the TUNEL assay has been revealed in the hepatocytes studied.

In conclusion, the present study is pioneering in demonstrating that the chronic use of mesterolone affects dynamically the status of chromatin condensation and texture, geometric parameters and Feulgen-DNA values in hepatocyte nuclei of sedentary and trained mice. Mesterolone induced in sedentary mice the highest Feulgen-DNA content, which we credited both to the higher number of polyploidy cells and unpackaging of the chromatin associated to the highest increase of the nuclear size. The assessment of nuclear phenotype by image analysis can give clues to the mechanisms underlying the effects of AAS and its interplay with exercise effects in transcriptional activation or repression of eukaryotic genes. This study using a human-like lipemic mouse strain is part of a comprehensive study aimed to further understand the effects of AAS abuse.
We believe that this study will allow us to continue investigations to understand the mechanisms by which gene alterations and/or epigenetic factors provoked by the use of AAS may be linked with hepatocellular dysfunction.

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