UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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EFEITOS DA INFUSÃO DE *Heteropterys aphrodisiaca* (O. MACH) ASSOCIADA AO TREINAMENTO FÍSICO NO SISTEMA MUSCULOESQUELÉTICO DE RATOS WISTAR

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Juliana Castro Monteiro e aprovada pela Comissão Julgadora.

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Orientadora: Profa. Dra. Mary Anne Heidi Dolder

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Dedico esta tese

Aos meus pais,
Lucas e Marinete,
A quem devo tudo que sou!

“Senhor, vós me perscrutais e me conheceis,
Sabeis tudo de mim, quando me sento ou me levanto.
De longe penetrais meus pensamentos.
Quando ando e quando repouso, vós me vedes, observais todos os meus passos.
A palavra ainda me não chegou à língua, e já, Senhor, a conheceis toda.
Vós me cercais por trás e pela frente, e estendeis sobre mim a vossa mão.
Conhecimento assim maravilhoso me ultrapassa, ele é tão sublime que não posso atingi-lo.
Para onde irei, longe de vosso Espírito? Para onde fugir, apartado de vosso olhar?
Se subir até os céus, ali estareis; se descer à região dos mortos, lá vos encontrareis também.
Se tomar as asas da aurora, se me fixar nos confins do mar,
é ainda vossa mão que lá me levará, e vossa destra que me sustentará.
Se eu dissesse: Pelo menos as trevas me ocultarão, e a noite, como se fora luz, me há de envolver.
As próprias trevas não são escuras para vós,
A noite vos é transparente como o dia e a escuridão, clara como a luz.
Fostes vós que plasmastes as entranhas de meu corpo, vós me tecestes no seio de minha mãe.
Sede bendito por me haverdes feito de modo tão maravilhoso.
Pelas vossas obras tão extraordinárias, conheceis até o fundo a minha alma.
Nada de minha substância vos é oculto, quando fui formado ocultamente,
Quando fui tecido nas entranhas subterrâneas.
Cada uma de minhas ações vossos olhos viram, e todas elas foram escritas em vosso livro;
Cada dia de minha vida foi prefixado, desde antes que um só deles existisse.
Ó Deus, como são insondáveis para mim vossos desígnios! E quão imenso é o número deles!
Como contá-los? São mais numerosos que a areia do mar;
Se pudesse chegar ao fim, seria ainda com vossa ajuda.
Oxalá extermineis os ímpios, ó Deus, e que se apartem de mim os sanguinários!
Eles se revoltam insidiosamente contra vós, perfidamente se insurgem vossos inimigos.
Pois não hei de odiar, Senhor, aos que vos odeiam?
Aos que se levantam contra vós, não hei de abominá-los?
Eu os odeio com ódio mortal, eu os tenho em conta de meus próprios inimigos.
Perscrutai-me, Senhor, para conhecer meu coração; provai-me e conhecei meus pensamentos.
Vede se ando na senda do mal, e conduzi-me pelo caminho da eternidade.”

(Salmo 138)

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RESUMO

Este estudo investigou os efeitos da infusão de *Heteropterys aphrodisiaca* no músculo esquelético, tendão e osso de ratos submetidos ao treinamento de *endurance*. Ratos Wistar machos foram agrupados da seguinte maneira: CS-controle sedentário, HS-*H. aphrodisiaca* sedentário, CT-controle treinado, HT-*H. aphrodisiaca* treinado. O protocolo de treinamento consistiu de corrida em esteira motorizada, cinco vezes por semana, com aumento semanal de velocidade e duração. Os animais dos grupos controle receberam água, enquanto HS e HT receberam infusão de *H. aphrodisiaca*, diariamente, por gavagem durante 8 semanas, correspondente ao período de treinamento. O sangue foi coletado para dosagem de testosterona. O músculo extensor longo dos dedos (EDL), tendão calcanear e a tíbia foram congelados para análises histoquímicas, bioquímicas, biomecânicas e para *Western Blot*, ou preservados em fixador Karnovsky, sendo posteriormente processados para análises morfométricas e estereológicas utilizando microscopia de luz e eletrônica. O conteúdo de hidroxiprolina, a tensão máxima e o módulo de elasticidade aumentaram ($p<0,05$) nos tendões dos animais do grupo HT. A atividade da metalopeptidase-2 foi reduzida significativamente nos tendões dos animais do grupo HT. A região de compressão dos tendões dos animais do grupo HT apresentou intensa metacromasia, o que sugere aumento na concentração de glicosaminoglicanos nessa região do tendão. Foi observada intensa birrefringência nas regiões de tensão e compressão dos tendões dos animais do grupo HT, o que pode indicar maior nível de organização dos feixes de colágeno. Os níveis de testosterona plasmáticos e a concentração de receptores de andrógeno no músculo esquelético aumentaram significativamente nos animais do grupo HS. A área média de secção transversa das fibras musculares dos animais do grupo HT foi semelhante à área das fibras dos

animais sedentários, e aumentou significativamente quando comparados com os animais do grupo CT. A vascularização intramuscular e a densidade volumétrica de mitocôndria foram significativamente maiores nos animais do grupo HT. Nenhuma alteração foi observada nos tipos de fibras musculares para todos os grupos. Os animais do grupo HT mostraram significativo aumento de força e tensão no limite elástico durante o teste biomecânico da tíbia. O conteúdo de colágeno e os dados morfométricos da tíbia não foram alterados nos diferentes grupos. A força e tensão máxima, rigidez e módulo de elasticidade da tíbia foram semelhantes em todos os grupos experimentais. A microscopia eletrônica de varredura mostrou aumento de lacunas e canais de Havers nos ossos dos animais treinados, além disso, os ósteons estavam mais desorganizados quando comparados com os ossos dos animais sedentários. Essas alterações podem indicar que os ossos dos animais treinados estavam sendo remodelados. Portanto, com oito semanas de treinamento não foi possível verificar alterações nas medidas morfométricas, composição e nas propriedades mecânicas (rigidez e módulo de elasticidade) dos ossos dos animais treinados e/ou tratados com a infusão da planta. Por outro lado, a associação do treinamento de *endurance* com *H. aphrodisiaca* resultou em tendões mais resistentes para suportar as altas cargas geradas pelas contrações musculares repetidas, aumentou a área de secção transversa das fibras musculares, a densidade volumétrica de mitocôndrias e a vascularização do músculo, assim, sugerindo um aumento da capacidade de *endurance* dos animais.

ABSTRACT

This study investigated the effects of *Heteropterys aphrodisiaca* infusion on the skeletal muscle, tendon and bone of rats under endurance training. Male Wistar rats were grouped as follows: CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT–control trained, HT- *H. aphrodisiaca* trained. The training protocol consisted in running on a motorized treadmill, five times a week, with weekly increase in treadmill velocity and duration. Control groups received water while the HS and HT groups received *H. aphrodisiaca* infusion, daily, by gavage for the 8 weeks of training. The blood was collected for testosterone dosage. Extensor digitorum longus (EDL) muscle, Achilles tendons and tibiae were frozen for histochemical, Western Blotting, biochemical and biomechanical analysis or preserved in Karnovsky's fixative, then processed for morphological analysis by light microscopy and electron microscopy. Biomechanical analysis showed significant increase ($p<0.05$) in maximum stress and modulus of elasticity of the tendons of the HT animals. The metalloproteinase-2 activity was reduced in the HT tendons. The compression region of tendons of HT animals had a stronger and more intense metachromasy, which suggests increase in glycosaminoglycan concentration in this region of the tendon. The most intense birefringence was observed in both compression and tension regions of the tendon of HT animals, which may indicate a higher organizational level of collagen bundles. The tendon hydroxyproline content increased in the HT group. The plasma testosterone levels and muscle androgen receptor concentration increased significantly in HS animals. The EDL mean cross-section area of HT group was similar to sedentary groups and increased significantly when compared with CT group. The intramuscular vascularization and mitochondria volume density were significantly greater in the HT group compared with other groups. No alterations were

observed in the muscle fiber composition for all groups. The HT group showed significantly higher yield load and yield stress in the tibiae three-point bending test. The tibiae collagen content, morphometrical data were not significantly different for the four groups. The maximum load, stiffness, maximum stress and elastic modulus were statistically similar for all of the experimental groups. Scanning electron microscopy showed more lacunae and Havers canals in the bone of trained animals, moreover the osteons were more disorganized, when compared with sedentary groups. These alterations may indicate that the bone of trained animals was being remodeled. Possibly, the duration of training in this study was not sufficient to alter the bone morphometrical measurements, composition and mechanical properties (stiffness and modulus of elasticity) of the trained and treated animals. On the other hand, the association of endurance training with *H. aphrodisiaca* resulted in more resistant tendons to support high loads from repeated muscle contraction. Also, the association of *H. aphrodisiaca* and the exercise protocol increased the mean area of muscle fiber, mitochondrial volume density and muscular vascularization, suggesting an increase of the endurance capacity of these animals.

SUMÁRIO

1. INTRODUÇÃO

Há vários séculos as plantas vêm sendo empregadas em preparações tradicionais (chás, sucos, xaropes, cataplasmas, tinturas, ungüentos) pelas mais diversas culturas ao redor do mundo. Atualmente, vem crescendo o interesse científico por pesquisas com plantas usadas na medicina popular, acarretando em vários trabalhos acerca de seus princípios ativos puros (PITMAN, 1996; SRIVASTAVA et al., 2005).

Pesquisas de plantas medicinais brasileiras mostram-se cada vez mais necessárias, em razão do grande número de plantas com potencial medicinal existente na nossa flora e que são utilizadas comumente pela população como parte de uma cultura que é passada de geração a geração (GALVÃO, 1997). Estudá-las é sem dúvida um caminho difícil, pois seus efeitos medicinais preconizados através da tradição podem ou não serem confirmados, e até mesmo descobertas novas ações farmacológicas, antes desconhecidas ou então interpretadas erroneamente pela população.

1.1. *Heteropterys aphrodisiaca*

A planta *Heteropterys aphrodisiaca* foi descrita por Hoenhne (1920) como tendo propriedades afrodisíacas e estimulantes. Foi incluída na família Malpighiaceae por Othon X.B. Machado em 1949 (PIO CORRÊA, 1984). É uma planta arbustiva, de 0,6 a 2,0 metros de altura, encontrada principalmente nos cerrados de Mato Grosso, Goiás e norte de Minas Gerais. Historicamente a população usa as raízes desse arbusto como tônico, estimulante sexual, para o tratamento de debilidades do sistema nervoso e fortalecimento de músculos e ossos (PIO

CORRÊA, 1984; POTT & POTT, 1994; GUARIM NETO, 1996). Do ponto de vista etnobotânico, *H. aphrodisiaca* é um dos mais famosos afrodisíacos do centro-oeste brasileiro, sendo conhecida popularmente como nó-de-cachorro, raiz de Santo Antônio e cordão de São Francisco (POTT & POTT, 1994; GUARIM NETO, 1996).

A análise fitoquímica do vegetal revelou a presença de glicosídeos (flavônicos, cardiotônicos, antracênicos combinados e aromáticos simples), compostos antracênicos livres, polifenois, taninos e alcaloides (GALVÃO, 1997).

A administração do extrato hidroalcóolico desta espécie resultou em melhora da memória em ratos idosos, sem que se notassem efeito nefrotóxico nem hepatotóxico (GALVÃO et al., 2002). Outro estudo utilizando o mesmo extrato em ratos idosos mostrou aumento significativo de enzimas antioxidantes (superóxido dismutase e superóxido dismutase manganês dependente), além de redução nos níveis de lipoperoxidação em homogenatos de cérebro. Os autores sugerem que a ativação da defesa antioxidante pode ter refletido em melhora da atividade cerebral em ratos velhos e em recuperação da memória e atenção, funções cerebrais mais afetadas com o envelhecimento (MATTEI et al., 2001).

Ratos adultos tratados com diferentes concentrações do extrato de *H. aphrodisiaca* por 56 dias apresentaram massa corporal e testicular aumentadas, bem como aumento significativo na proporção do interstício do parênquima testicular. Essa diferença foi atribuída, principalmente, ao aumento na proporção de células de Leydig dos animais tratados, em relação aos animais controle e também em relação aos tratados com *Anemopaegma arvense* (vergateza), outra espécie considerada afrodisíaca no centro-oeste brasileiro (CHIEREGATTO, 2005).

Em estudos prévios do nosso grupo de pesquisa também foi verificado aumento significativo de massa corporal, diâmetro e volume nuclear das células de Leydig de ratos Wistar

adultos tratados com infusão de *H. aphrodisiaca* por 56 dias (MONTEIRO et al., 2008). Embora a dosagem de testosterona não tenha sido realizada, esses animais possivelmente possuíam maiores níveis plasmáticos desse hormônio. Como não foi observado aumento no peso dos órgãos sexuais andrógeno-dependentes, foi inferido que a testosterona pode exercer efeito anabolizante nesses animais (MONTEIRO, 2007).

1.2- Músculo esquelético

As fibras musculares esqueléticas são formadas através da fusão dos mioblastos, produzindo células multinucleadas. A capacidade de contração das fibras está relacionada à organização repetitiva das suas unidades contráteis extremamente elaboradas, os sarcômeros. A força de contração de uma fibra muscular relaciona-se com a atividade ATPásica da porção globular da miosina (TALMADGE & ROY, 1993).

Os músculos esqueléticos são constituídos por diferentes tipos de fibras, sendo elas puras ou híbridas. Através da velocidade de hidrólise do ATP, as fibras musculares podem ser classificadas em lentas ou rápidas, expressando uma determinada isoforma de miosina (SCHIAFFINO & REGGIANI, 1994). A forma lenta é denominada de MHCI e as rápidas são representadas principalmente pelas MHCIIa, MHCIId, e MHCIIb (PETTE & STARON, 1997; STARON et al., 1999).

Em ratos, as fibras I, IIA, IID, e IIB são classificadas como puras e contêm cadeias pesadas de miosina dos tipos I (MHCI), IIA (MHCIIa), IID (MHCIId) e IIB (MHCIIb), respectivamente. As fibras híbridas IC, IIC, IIAD, IIDA, IIDB, e IIBD expressam duas ou mais cadeias de miosina dos tipos IC (MHCI > MHCIIa), IIC (MHCIIa > MHCI), IIAD (MHCIIa >

MHCIId), IIDA (MHCIId > MHCIIa), IIDB (MHCIId > MHCIIb) e IIBD (MHCIIb > MHCIId), respectivamente (STARON & PETTE , 1993; STARON et al., 1999; PETTE & STARON, 2000). A fibra do tipo IID (MHCIId) é classificada como IIX (MHCIIx) por outros pesquisadores (SCHIAFFINO et al., 1989; DE NARDI et al., 1993; SCHIAFFINO & REGGIANI, 1994; 1996).

Essa diversidade de fibras torna o músculo esquelético capaz de responder de diferentes formas a diversos estímulos, levando a alterações moleculares que têm início no interior da célula, através da expressão de genes específicos que regulam a produção dos tipos específicos de miosina (GONDRET et al., 2005). Esses genes permitirão, por sua vez, a transcrição de novas moléculas de RNA mensageiro e conseqüente tradução de proteínas específicas, culminando na expressão do fenótipo muscular adaptado à demanda (WILLOUGHBY & PELSUE, 2000). Os estímulos podem tanto ter um fator endógeno quanto exógeno. Entre os estímulos endógenos encontram-se: alterações na atividade neuromuscular, alterações nos níveis hormonais e idade. Nos exógenos observam-se aqueles fatores que agem sobre o organismo através de influências externas: estimulação elétrica, carga mecânica, ausência de carga, micro gravidade e exercício físico ativo (ROY et al., 1997; JAKUBIEC-PUKA et al., 1999; MANNION, 1999).

1.3- Tendões

Os tendões são de natureza conjuntiva, cuja função é transmitir forças entre os músculos e os ossos, sendo capazes de suportar altas forças de tensão. Além disso, exibem a capacidade de reparo e respondem ao exercício físico e à imobilização, modulando a sua composição (BENJAMIN & RALPHS, 1998).

O tendão calcanear (também chamado Tendão de Achilles) é o maior e mais resistente tendão do corpo humano, sendo responsável pela fixação dos músculos gastrocnêmio e sóleo no osso calcâneo (DANGELO & FATTINI, 2004).

O tendão é um tecido conjuntivo denso modelado, fibroso, composto essencialmente por fibras de colágeno do tipo I, altamente ordenadas em feixes. Cerca de 90 a 95% do seu componente celular são compostos de fibroblastos e fibrócitos. Os fibroblastos com numerosas organelas citoplasmáticas refletem sua alta atividade metabólica, característica típica de células que estão sintetizando grandes quantidades de componentes da matriz extracelular (MEC). Com o decorrer do tempo, os fibroblastos tornam-se alongados e se diferenciam em fibrócitos quiescentes, porém mantêm a capacidade de síntese, que pode ser reativada durante processos de reparo após lesões do tendão (HAYEM, 2001; ESQUISATO et al., 2003; SHARMA & MAFFULLI, 2005). Os fibroblastos sintetizam colágeno, proteoglicanos e proteínas não-colagênicas, que são os componentes da MEC, e juntamente com os fibrócitos localizam-se entre a MEC depositada por eles. Outros componentes celulares incluem: os condrócitos na região de inserção, as células sinoviais e as células vasculares (endoteliais).

A MEC dos tendões é composta principalmente por moléculas de colágeno tipo I, que representam cerca de 80 a 90% do peso seco do tecido e estão arranjados em fibras e feixes de fibras, dispondo-se paralelamente ao maior eixo do tendão (VIDAL, 1970; VIDAL & CARVALHO, 1990; LIN et al., 2004). Este tipo de colágeno é o principal responsável pela estabilidade estrutural e mecânica atribuída a esse tecido (EZURA et al., 2000). Junto ao colágeno tipo I, também podem ser encontrados o colágeno tipo II na região de compressão dos tendões, o tipo III, que está relacionado ao controle do diâmetro fibrilar e compõe fibrilas heterotípicas com os colágenos tipos I e V, e o tipo VI que é encontrado na parte mediana e na

entese do tendão calcanear, além dos tipos XII e XIV que participam na regulação do crescimento e associação fibrilar (BERENSON et al., 1996; WAGGETT et al., 1998; YOUNG et al., 2000).

Os proteoglicanos são constituídos de cadeias de glicosaminoglicanos (GAGs), as quais estão covalentemente ligadas a uma proteína central. Os GAGs são cadeias polissacarídicas não ramificadas, compostas de unidades repetidas de dissacarídeos. Em função da alta concentração de cargas negativas dos glicosaminoglicanos que exercem pressão de entumescimento na matriz colagênica, aos proteoglicanos são atribuídas as propriedades de resistência à compressão (SCOTT et al., 1997). Além disso, devido à variabilidade da proteína e das diferentes classes de GAGs, estas moléculas desempenham outras funções nos tecidos conjuntivos (IOZZO & MURDOCH, 1996). O colágeno fibrilar e os proteoglicanos estão associados formando fibrilas. Os pequenos proteoglicanos estão envolvidos com a montagem das fibras e fibrilas colagênicas, podendo tanto influenciar na organização destas por alteração do padrão nas quais se formam, quanto no tamanho final da fibra do colágeno (KUC & SCOTT, 1997). Os grandes proteoglicanos são representados principalmente pelo agrecam e o versicam, entre os pequenos destacam-se o decorim, biglicam e fibromodulim.

Na MEC de tendões, além de colágeno e proteoglicanos, também estão presentes as glicoproteínas não colagênicas e diversas pequenas moléculas. Entre aquelas, destacam-se glicoproteínas adesivas como a fibronectina e a trombospondina, que participam em processos de reparo e regeneração dos tendões (JOZSA et al., 1991).

A capacidade que os tendões apresentam de resistir aos vários tipos de estresse mecânico ao qual estão submetidos, está diretamente relacionada com a organização estrutural de sua MEC (ESQUISATTO et al., 2003). As propriedades biomecânicas dos tendões são determinadas tanto

pela associação entre o colágeno e os outros elementos da matriz extracelular, como também, pelo diâmetro e orientação de suas fibras e feixes (VIDAL & CARVALHO, 1990).

A carga mecânica leva a uma adaptação fisiológica do tecido, que responde ao estresse alterando sua organização estrutural, de modo a atender à nova demanda mecânica (CHIQUET, 1999; COVIZI et al., 2001). Essa alteração e a própria manutenção da estrutura tecidual são parcialmente dependentes da estimulação mecânica (COVIZI et al., 2001). O estímulo mecânico é importante para a homeostase da MEC nos tecidos conjuntivos, pois afeta a expressão de proteínas específicas da MEC. Sendo assim, deve existir um mecanismo de *feedback* pelo qual as células que são atingidas, via substrato, por sinais desencadeados pelo estresse mecânico, respondam alterando a expressão de proteínas e, conseqüentemente, remodelando a MEC para melhor adaptação às necessidades biomecânicas. Entretanto, a quantidade e a composição da MEC são controladas não apenas pelo tipo e magnitude do estresse mecânico ao qual o tecido está submetido, mas também por programas celulares endógenos e fatores de crescimento (CHIQUET, 1999).

A curva de tensão-deformação de um tendão é caracterizada por três regiões distintas. Inicialmente ocorre realinhamento das fibras colágenas que se encontram em um padrão ondulatório, denominado *crimp* (KISNER & COLBY, 1998). Nesta região, uma quantidade mínima de força produz uma grande deformação. Após essa fase, o tecido entra na região elástica, onde a deformação apresentada aumenta linearmente com a força aplicada (ENGLES, 2001). A inclinação da curva nessa região representa a rigidez do tecido, também chamada de módulo elástico e está diretamente relacionado com a resistência do material à deformação (LATASH & ZATSIORK, 1993). Na fase elástica, o tecido retorna a sua forma original com a retirada da carga (ENGLES, 2001; NORDIN et al., 2003). Quando o tendão excede seu limite

elástico, ele entra no limite plástico da curva, e o tecido torna-se permanentemente deformado, não sendo capaz de se recuperar após a retirada da força, ocorrendo assim, falha tecidual (NORDIN et al., 2003). Quando o músculo contrai, o tendão torna-se tenso e aumenta seu comprimento linearmente até um limiar, no qual o alongamento ocorre mais devagar, sendo um risco para a injúria. Esse limiar varia de 20 a 50% do comprimento do tendão (NORDIN et al., 2003).

1.4- Ossos

Os ossos caracterizam-se em uma forma rígida de tecido conjuntivo, que tem como função proteger órgãos internos, proporcionar os movimentos do corpo, assim como sua locomoção, fixar músculos e facilitar suas ações e movimentos, constituindo um sistema de alavancas que amplia as forças geradas na contração muscular (RESTRÖM, 1993; JUNQUEIRA & CARNEIRO, 2008). Em adição, os ossos funcionam também como depósito de cálcio, fosfato e outros íons, armazenando-os ou liberando-os de maneira controlada para manter constante a concentração desses importantes íons nos líquidos corporais (BUCKWALTER et al., 1996).

Os ossos são compostos por diferentes tipos celulares: osteoclastos, osteoblastos e osteócitos. Os osteoclastos são células grandes e multinucleadas que reabsorvem o tecido ósseo, participando dos processos de remodelação óssea. Os osteoblastos dispõem-se nas superfícies ósseas e são responsáveis por sintetizar a parte orgânica da matriz óssea e regular a sua mineralização. Uma vez aprisionado pela matriz recém-sintetizada, o osteoblasto diferencia em osteócito. Os osteócitos, tipo celular mais abundante do osso, estão localizados dentro de lacunas, das quais partem canalículos, onde os prolongamentos dos osteócitos fazem contato com

prolongamentos de células vizinhas através de junções comunicantes. Os osteócitos são responsáveis pela manutenção da matriz óssea e funcionam como mecanosensores dos ossos (MARKS & ODGREN, 2002; JUNQUEIRA & CARNEIRO, 2008).

A matriz óssea é composta por substâncias orgânicas, 35% em volume e 25% em peso, e inorgânicas, 36% em volume e 65% em peso do osso. O restante é ocupado por água e células (BUCKWALTER et al., 1996; MARKS & ODGREN, 2002). A porção orgânica da matriz é formada em sua grande maioria por colágeno tipo I, que representa 90% de seus constituintes, sendo o restante da MEC constituído por glicosaminoglicanos e proteoglicanos (MARKS & ODGREN, 2002). A porção inorgânica contém sais minerais, como os compostos de cálcio e fósforo que formam os cristais de hidroxiapatita, conferindo dureza ao osso, entre outros componentes, como magnésio, carboneto e sódio (MARKS & ODGREN, 2002; JUNQUEIRA & CARNEIRO, 2008).

As fibras colágenas do osso têm grande força de resistência à tração, enquanto os sais de cálcio conferem rigidez, e os glicosaminoglicanos exibem grande resistência à força compressiva (BAILEY et al., 1999). Essas propriedades combinadas ao grau de coesão e ao arranjo das fibras colágenas e dos cristais de cálcio resultam em uma estrutura dotada de extrema resistência a forças de tensão e compressão.

1.5- Exercícios de *endurance* e o efeito sobre o sistema musculoesquelético

O treinamento físico compreende respostas fisiológicas e adaptações funcionais que são necessárias para o melhora de resistência muscular, força, potência e velocidade, levando a maior tolerância à atividade física (COFFEY & HAWLEY, 2007). O termo exercício de *endurance*

pode ser traduzido como exercício aeróbico, que compreende exercícios realizados durante extensos períodos (10 a 40 minutos), com intensa atividade muscular envolvendo centenas de contrações repetidas que aumentam a captação de oxigênio para os músculos (KNUTTGEN, 2007). Nesse trabalho, optou-se por usar o termo *endurance* por ser mais específico ao tipo de treinamento físico utilizado.

Aerobic exercise involves exercise performed for extended periods (e.g., 10-40 minutes) with large muscle activity involving hundred of consecutive repetitions that challenge the delivery of oxygen to the active muscles

O exercício físico é um dos fatores externos de grande importância na alteração do comportamento das fibras musculares, permitindo a transição das fibras tanto no sentido de lento para rápido (I=>IIA=>IID=>IIB) quanto de rápido para lento (IIB=>IID=>IIA=>I), sendo que essa mudança não se dá sempre entre os extremos, mas para aquelas fibras que melhor respondem metabolicamente ao estímulo aplicado (GOLDSPINK, 1998; WILLIAMSON et al., 2000; PETTE & STARON, 2001; WILLIAMSON et al., 2001; PARCELL et al., 2003). O exercício de *endurance* promove a transição de fibras de contração rápida para um fenótipo de fibras mais lentas, mas isso não significa mudança do tipo II para o tipo I. Nesse caso, pode ocorrer uma mudança dentro dos subtipos de fibras do tipo II, ou seja, uma transformação no sentido de IIB→IID→IIA, sendo que o tipo de alteração depende do músculo analisado (OKUMOTO et al., 1996; SEENE et al., 2005; FONTANA, 2008). Além disso, o treinamento de *endurance* resulta em aumento de densidade volumétrica de mitocôndrias e das enzimas mitocondriais relacionadas com a oxidação aeróbica de substratos, além de aumento de vascularização e captação máxima de oxigênio. Essas alterações aumentam a capacidade aeróbica

do músculo, o que contribui para a melhora do seu desempenho (INASHIMA et al., 2003; SEENE et al., 2005).

A tensão gerada pela contração muscular durante o exercício físico é transmitida por interações entre a matriz e a célula, resultando em adaptações simultâneas e remodelamento das células do tecido muscular (MILLER et al., 2005). Entretanto, a hipertrofia das fibras musculares somente será plenamente funcional se ocorrer também remodelação do tecido conjuntivo que o circunda, incluindo os tendões que ligam os músculos aos ossos. MILLER e colaboradores (2005) relatam aumento de 100% na síntese de colágeno em tendões humanos após 60 minutos de exercícios intensos e que a síntese elevada de colágeno se manteve por três dias após o exercício.

Em ratos treinados em esteira, por 4, 8 e 16 semanas, ocorreu aumento na área de secção transversa acompanhada de diminuição das propriedades biomecânicas, bem como alterações histológicas do tendão supraespinhoso, sendo que uma maior quantidade de células com morfologia arredondada foi encontrada nos tendões dos animais treinados quando comparados com o grupo controle (HUANG, 2003). O treinamento de *endurance* também leva a redução do número de ligações cruzadas do tipo piridinolina (CURWIN et al., 1988) e alterações no metabolismo dos componentes da MEC (HARVEY et al., 1982) em tendões de aves submetidas a um programa de corrida em esteiras por 8 semanas.

Além do aumento da síntese de colágeno na MEC de tendões e músculo esquelético, também é observado aumento na degradação de proteínas. A atividade de metalopeptidases da matriz (MMP) aumenta imediatamente após exercícios de *endurance* (KOSKINEN et al., 2004). A degradação do colágeno é iniciada extracelularmente pelas MMPs, principalmente pela MMP-2 e MMP-9 (KJAER, 2004). As MMPs compreendem uma família de enzimas que desempenham

papel central na renovação e remodelamento da MEC, uma vez que são capazes de hidrolisar as principais proteínas e alterar as funções biológicas das macromoléculas presentes neste local. As MMPs são enzimas zinco e cálcio dependentes que são sintetizadas como zimogênios nos tecidos conjuntivos. Sob condições normais, as MMPs estão presentes em baixos níveis, usualmente na forma latente e são responsáveis pela renovação fisiológica dos tecidos (PENDER & MACDONALD, 2004). Entretanto, em muitas condições patológicas, existe um desequilíbrio entre a síntese e degradação da matriz, levando a degradação do tecido. Após a injúria, a proteólise é requerida para a remoção da matriz danificada e para ajudar na síntese de tecido sadio. Portanto, aumento nos níveis de atividade das MMPs indica degradação da matriz, bem como reparação do tecido, sendo este fenômeno necessário para o processo de remodelamento em tecidos que estão em processo de reparo (RILEY et al., 2002; RILEY, 2005).

Com relação ao tecido ósseo, é sabido que este é dinamicamente responsivo à demanda funcional imposta pelo exercício, o que gera alterações de sua massa e resistência (CARTER et al., 1996). O estresse contínuo provocado pelo exercício físico resulta em adaptações morfológicas, tais como o aumento da espessura cortical, maior conteúdo ósseo na inserção musculotendínea, aumento do conteúdo colagênico e de *cross-links*, remodelamento de fibrilas de colágeno e aumento de mineralização (KRAHL et al., 1994; BAILEY et al., 1999; WARNER et al, 2006). Tais alterações levam a melhoria das propriedades mecânicas dos ossos (ISAKSSON et al., 2009). A adaptação do osso dependerá, portanto, da magnitude da carga e da freqüência de aplicação, as quais, sendo regularmente repetidas, desencadeiam efeitos osteogênicos (SNOW et al., 2001).

1.6- Exercícios de *endurance* e o efeito sobre o sistema endócrino

Muitos autores têm investigado o efeito do treinamento físico no sistema endócrino. Enquanto alguns demonstram que o treinamento de *endurance* não altera os níveis de testosterona total em atletas (LÚCIA et al., 1996; SMILIOS et al., 2003), outros mostram claramente que há variação hormonal com o treinamento (WHEELER, et al., 1984; DUCLOS et al., 1996; TREMBLAY et al., 2005). Esses resultados contraditórios podem ser explicados por uma reação dependente de muitos fatores, como intensidade, duração e tipo de exercício, freqüência de estímulo, além do nível de treinamento dos atletas (TREMBLAY et al., 2004).

Segundo NINDL e colaboradores (2001), alterações nos níveis hormonais após o exercício provavelmente servem para mediar a energia necessária durante o processo de recuperação e regeneração dos tecidos lesados em decorrência das repetidas contrações musculares. Isto é, as baixas concentrações de testosterona, em conjunto com elevados níveis de cortisol (normalmente aumentados com o exercício), levam ao aumento da atividade lipolítica e catabolismo de proteínas. Imediatamente após o estresse imposto por alto volume de contrações musculares, o corpo está em um fluxo de energia catabólico em vez de anabólico (NINDL et al., 2001).

Muitos fatores parecem influenciar a resposta da testosterona sérica total em relação ao exercício de *endurance*. A magnitude de elevação dos níveis de testosterona durante o treinamento de *endurance* tem sido relacionada com os músculos envolvidos e o tipo, intensidade, volume e exercício realizado, além do *status* nutricional e da experiência no treinamento (KRAEMER & RATAMESS, 2005). Na perspectiva de um atleta, tais aumentos podem melhorar seu desempenho, uma vez que a testosterona age aumentando a quantidade de

massa muscular e, consequentemente, de força muscular, diminuindo o acúmulo de gordura (TREMBLAY et al., 2004).

1.7- Os Esteróides anabólico-androgênicos (EAA) e seus mecanismos de ação

Os hormônios esteróides são produzidos pelas gônadas (ovário e testículo) e pelo córtex da glândula adrenal. Os esteróides anabolizantes ou esteróides anabólico-androgênicos (EAA) são os hormônios sexuais masculinos promotores e mantenedores das características sexuais associadas à masculinidade. Durante a puberdade, a quantidade crescente de testosterona faz o pênis, o escroto e os testículos aumentarem de volume. Além disso, a testosterona determina o desenvolvimento das características sexuais secundárias que distinguem o homem da mulher, como por exemplo, distribuição dos pêlos pelo corpo, calvície, voz, espessura da pele, desenvolvimento muscular, crescimento ósseo e a retenção de cálcio nos mesmos (Da SILVA et al., 2002; GEBARA et al., 2002; GUYTON & HALL, 2002; DOHLE et al., 2003).

No homem, as células de Leydig constituem praticamente a única fonte de testosterona. A secreção testicular de testosterona é regulada pelo hormônio luteinizante (LH), produzido pela hipófise anterior que, por sua vez, é controlada pelo hormônio liberador de gonadotrofinas (GnRH) secretado pelo hipotálamo intermitentemente durante alguns minutos, uma vez a cada 1 a 3 horas. A secreção de LH também é cíclica, seguindo a liberação pulsátil do GnRH (GUYTON & HALL, 2002).

Os EAA incluem a testosterona e seus derivados, entretanto, BAHRKE & YESALIS (2004) e KAM & YARROW (2005) definem os esteróides anabolizantes como derivados sintéticos da testosterona que possuem atividade anabólica superior à atividade androgênica.

Estudos comprovam que dentre os efeitos colaterais conhecidos dos anabolizantes sintéticos estão: atrofia testicular, ginecomastia, hiperplasia prostática, disfunção tireoidiana e do córtex da adrenal, hipertensão, alterações do metabolismo glicêmico e lipídico, acidente vascular cerebral, distúrbios hepáticos, cardiovasculares e alterações de humor (GUYTON & HALL, 2002).

Os EAA ligam-se a receptores citoplasmáticos protéicos, formando um complexo receptor-hormônio, o qual migra para o núcleo e se fixa em regiões promotoras no DNA, promovendo a transcrição gênica e a síntese de proteínas, as quais modulam o metabolismo celular dependente de andrógeno. A resposta em diferentes tecidos varia de acordo com a concentração de receptores androgênicos nas células e com a atividade das enzimas 5-alfa-redutase ou aromatases (BAHRKE & YESALIS, 2004; KAM & YARROW, 2005).

Os EAA melhoram o desempenho atlético por acelerar o crescimento da musculatura através do aumento da síntese protéica muscular, maior retenção de nitrogênio, inibição do catabolismo protéico e estimulação da eritropoiese. Além disso, é atribuído aos EAA o aumento da agressividade e da motivação (Da SILVA et al., 2002). URBAN e colaboradores (1995) mostraram que os andrógenos podem aumentar a síntese protéica, através da estimulação intramuscular da expressão do gene para IGF-1 (*insulin-like growth factor*). Além disso, GONZÁLEZ e colaboradores (2000) demonstraram que o decanoato de nandrolona, um andrógeno anabólico semi-sintético, promove o aumento da expressão da "proteína de choque térmico", a HSP, em fibras musculares de contração rápida, o que contribuiria para o aumento da tolerância do músculo esquelético ao treinamento de alta intensidade. Essa proteína é usualmente sintetizada em resposta ao estresse, inclusive aquele causado pelo exercício físico (ANTUNES-NETO et al., 2006).

O uso de EAA causa hipertrofia muscular associada a um aumento na área de secção transversal das fibras do tipo I e II, aumento quantitativo mionuclear, e possivelmente formação de novas fibras (hiperplasia), além de aumento na expressão de receptores androgênicos (KADI, 2000; KAM & YARROW, 2005). CATLIN (2001) afirma que a testosterona age diretamente na expressão do gene da proteína motora (miosina) nos músculos de animais, uma vez que esta causa aumento no diâmetro das fibras musculares devido à elevação no número de miofilamentos e miofibrilas, além de induzir mudanças na estrutura das isoformas da miosina de cadeia pesada.

Os EAA causam alterações morfológicas, bioquímicas e biomecânicas em tendões de humanos e ratos. O tratamento com EAA compromete a síntese de colágeno ocasionando alterações estruturais que resultam em aumento de rigidez do tendão e perda de elasticidade, o que o deixa mais propenso a ruptura (KARPAKKA et al.,1992; MILLES et al., 1992; INHOFE et al.,1995; EVANS, 2004). Alterações no ângulo dos *crimp* e comprimento das fibrilas de colágeno foram descritas por WOOD et al. (1988), quando tendões de ratos foram analisados em microscopia de polarização. MARQUETI e colaboradores (2006) mostraram que EAA associados (Decadurabolin e Durateston) comprometem a remodelação de tendões de ratos exercitados e sedentários, por diminuir a atividade de metalopeptidades da MEC. Além disso, MICHNA (1987) descreve alterações ultra-estruturais em tendões de camundongos pré-tratados com esteróide.

A principal ação da testosterona no esqueleto é a redução da reabsorção óssea. A maior parte dessa ação é indireta, via aromatização da testosterona a estrógeno. Assim como o estrógeno, a testosterona aumenta o período de atividade dos osteoclastos e osteoblastos, interferindo na via apoptótica (RIGGS et al., 2002). A testosterona também tem um modesto efeito na proliferação dos osteoblastos. Ambos os efeitos da testosterona (estimulação da

proliferação e inibição da apoptose) contribuem para aumento da formação óssea (RIGGS et al., 2002). Assim, com a administração prolongada de testosterona, os ossos crescem consideravelmente em espessura e também ocorre deposição de quantidade substancial de sais de cálcio (GUYTON & HALL, 2002).

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3. OBJETIVOS

3.1- Gerais

Investigar os efeitos da infusão de *Heteropterys aphrodisiaca* associada ao treinamento físico em ratos Wistar machos adultos.

3.2- Específicos

- Avaliar as possíveis alterações nos níveis de testosterona plasmática e na concentração de receptores de andrógenos no músculo esquelético;
- Investigar através de métodos histoquímicos e morfológicos (estrutura e ultra-estrutura) os efeitos da infusão e do treinamento físico no músculo extensor longo dos dedos (EDL);
- Investigar, através de métodos bioquímicos, biomecânicos e morfológicos, os efeitos da infusão e treinamento físico no tendão calcanear (tendão de Achilles);
- Identificar as alterações morfológicas, biomecânicas e bioquímicas do osso tibial.

4. CAPÍTULOS

Esta tese foi confeccionada de acordo com a portaria CPG/01/2008-UNICAMP que regulamenta o formato alternativo para a tese de doutorado e permite a inserção de artigos científicos publicados, submetidos ou em fase final de redação, de autoria ou co-autoria do candidato.

O exemplar de tese está composto por 3 artigos, sendo dois submetidos e um em fase final de submissão, conforme descrito abaixo:

4.1- Artigo 1- More resistant tendons obtained from the association of *Heteropterys aphrodisiaca* and endurance training (submetido a Connective Tissue Research)

4.2- Artigo 2- Plasticity of skeletal muscle after endurance exercise and *Heteropterys aphrodisiaca* administration

4.3- Artigo 3- Does *Heteropterys aphrodisiaca* administration and endurance training alter bones of mature rats? (submetido a Brazilian Journal of Morphological Science)

ARTIGO 1

MORE RESISTANT TENDONS RESULTING FROM THE ASSOCIATION OF *Heteropterys aphrodisiaca* AND ENDURANCE TRAINING

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ABSTRACT

This study investigated the effects of *Heteropterys aphrodisiaca* infusion on the tendon properties and extracellular matrix of rats under endurance training. Wistar rats were grouped as follows: CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT–control trained, HT- *H. aphrodisiaca* trained. The training protocol consisted in running on a motorized treadmill, five times a week, with weekly increase in treadmill velocity and duration. Control groups received water while the HS and HT groups received *H. aphrodisiaca* infusion, daily, by gavage for the 8 weeks of training. Achilles tendons were frozen for biochemical and biomechanical analysis or preserved in Karnovsky's fixative, then processed for histomorphological analysis by light

microscopy. Biomechanical analysis showed significant increase in maximum stress and modulus of elasticity of the tendons of the HT animals. The MMP-2 activity was reduced in the HT group. The compression region of tendons of HT animals had a stronger and more intense metachromasy, which suggests increase in glycosaminoglycan concentration in this region of the tendon. The most intense birefringence was observed in both compression and tension regions of the tendon of HT animals**,** which may indicate a higher organizational level of collagen bundles. The hydroxyproline content increased in the HT group. The association of endurance training with *H. aphrodisiaca* resulted in more organized collagen bundles and more resistant tendons to support high loads from intense muscle contraction. Despite the clear anabolic effects of *Heteropterys aphrodisiaca* and the endurance exercise association, no side effects were observed, such as those found for synthetic anabolic androgenic steroids.

Keywords: Achilles tendon, biomechanics, endurance exercise, extracellular matrix, *H. aphrodisiaca*.

INTRODUCTION

Heteropterys aphrodisiaca O. Mach. (Malpighiaceae), also known as “nó-de-cachorro”, “nó-de-porco” and “cordão-de-São-Francisco”, was described by Hoehne (1920) as a plant with stimulant and aphrodisiac properties. Also, popular medicine uses *H. aphrodisiaca* root infusion as a tonic or stimulant and for the treatment of nervous debility, nervous breakdown and for muscle and bone weakness. This plant is found mainly in the “Cerrado” regions of Mato Grosso and Goiás States (Brazil) (Pio Corrêa 1984; Pott and Pott 1994).

Previous studies with *H. aphrodisiaca* suggested that the root extract could increase corporal and testicular weight, as well as Leydig cell volume within rat testis (Chieregatto 2005; Monteiro et al. 2008). In the male, Leydig cells produce testosterone and regulate muscle protein metabolism, erythropoiesis, plasma lipids, bone metabolism and cognitive functions (Kam and Yarrom 2005). Since testosterone regulates growth, structure and functions of accessory sex organs (Creasy 2001) and the treatment with *H. aphrodisiaca* infusion showed no alterations of these organs’ weights, Chieregatto (2005) related the corporal weight gain to the anabolic property of this hormone on the skeletal muscle mass.

Higher doses of anabolic androgenic steroids (AAS), when combined with a training program, appears to increase muscle strength and mass (Bahrke and Yesalis 2004). However, some reports have mentioned that tendons do not follow the same rate of protein gain achieved by the muscle bulk, and then high intensity and frequency of training might lead to tendon ruptures (Bach et al. 1987; Battista et al. 2003). Some studies using animals suggested that steroids modify the crimp pattern of collagen and biomechanical properties of tendon (Wood et al. 1988). Steroid use appears to cause collagen dysplasia in the flexor digitorum tendon of mice

(Michna 1986), increasing tendon stiffness and diminishing both elongation and energy absorption (Miles et al. 1992; Inhofe et al. 1995). Therefore, such tendons are more likely to fail during certain activities. In addition, AAS treatment can impair tissue remodeling in tendons of animals undergoing physical exercise by down regulating matrix metalloproteinase (MMP) activity, thus increasing the potential for tendon injury (Marqueti et al. 2006). In human beings, morphological changes at the muscle-tendon junction, as well as tendon ruptures were found when anabolic steroids were associated with exercise (März and Novotný 2008). High intensity loads during exercise seem to play a role in the process that allows the deleterious effects of steroids to be manifested. In the above studies, the groups that had been treated with both steroids and exercise suffered the worst damage.

The response of tendons to exercise and some treatments may be analyzed regarding structural, chemical and mechanical aspects. However, most studies have been limited to analyzing only one or two of these aspects (Buchanan and Marsh 2002). The present study aggregates biochemical, structural and biomechanical data in order to precisely investigate the effects of *H. aphrodisiaca* infusion on the tendon properties under endurance training. This study is part of a comprehensive study dealing with the effects of *H. aphrodisiaca* administration and intensive endurance exercise in some biological systems.

MATERIALS AND METHODS

Animals

Adult Wistar rats, 90 days old, were obtained from the Center for Biological Investigation - CEMIB (State University of Campinas, Campinas, SP, Brazil). The rats were housed, three per

cage, under standard conditions with 12hrL:12hrD. Animals were provided with commercial rat feed and water *ad libitum*. The Institutional Committee for Ethics in Animal Care and Use of this University approved the experimental protocol (process n° 1233-1).

Medicinal Plant

H. aphrodisiaca roots were collected in February 2007, in Mato Grosso State, Brazil. The species was identified by comparison with the voucher herbarium specimen of the plant at the Herbarium Federal University of Mato Grosso, Brazil (number 23928). The roots were dried at room temperature. The dried roots were crushed and powdered using a grinding mill. The infusion was routinely prepared by pouring 100mL of boiling water over 25 g of powdered roots, which was allowed to steep for 4 hours, then filtered using filter paper. The yield was an infusion of 68.66 mg of dry extract (6.866% w/v) and a yield of 6.832% (w/w) in terms of initial crude dry weight of plant material. The infusion was prepared every four days and it was stored in the refrigerator. The doses of *H. aphrodisiaca* were selected according to previous studies (Monteiro et al. 2008).

Study groups and experimental protocol

Forty-eight male rats were divided into four groups (n=12/group): two sedentary (CS and HS) and two submitted to involuntary running on a motorized treadmill (CT and HT). All groups received either 0.5mL of distilled water (CS and CT, control groups) or *H. aphrodisiaca* infusion (HS and HT). The water and infusion (104 mg/Kg) was administered daily by gavage, during the 8 weeks of training or sedentary period. Trained rats (group CT and HT) were allowed to adapt to treadmill running for a 3 week period, prior to the beginning of the experimental protocol, which

consisted of low to moderate level exercise carried out daily for 5 days a week (Table 1). After adaptation, trained rats were subjected to 8 weeks of intensive aerobic exercise training (treadmill running), also on a weekly cycle of 5 consecutive exercising days followed by a two days rest, as scheduled in Table 1 (adapted from Moraska et al. 2000; Smolka et al. 2000; Demirel et al. 2001; Fontana et al. 2008). This program is a form of endurance training and does not compare with power training (Fontana et al., 2008). Forty-eight hours after the last training, the rats were anesthetized with xylazine chloride (Anasedan, Vetbrands, São Paulo, Brazil) and ketamine chloride (Cetamin, Syntec, Cotia, Brazil) (5 and 80 mg/Kg body weight, respectively). The right and left tendons were dissected and frozen for biochemical, biomechanical and zymographical analysis or preserved in Karnovsky's fixative for morphological analysis.

Light Microscopy Analysis

Tendon samples (n=4/group) were immersed in Karnovsky's fixative for 24 h and then processed for inclusion in paraffin (Histosec, Merck). Longitudinal serial sections, 7 μm thick, were stained with toluidine blue (0.025%) in McIlvaine buffer (0.03 M citric acid, 0.04 M sodium phosphate, dibasic - pH 4.0) and analyzed by polarized and conventional light microscopy. The organization of collagen bundles was examined with a Nikon E800 microscope, connected with Cool Snap Pro-Color camera (Media Cibernetica)**.** To assess birefringence, the analyzer and the polarizer were crossed and the material was positioned at an angle of 45° relative to the polarizer. Proteoglycans were detected in extracellular matrix tissue sections stained with Toluidine Blue (pH 4.0) and observed under an Olympus BX 41 light microscope.

Extraction procedures

The tendons (n=4/group) 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 (Heinegård and Sommarin 1987) at 4°C for 24 h. The mixture was then centrifuged (20,000 xg, 4°C, 30 min), the supernatant precipitated in acetate-ethanol and used in the biochemical analyses.

Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Zingales (1984), using gradient gels (4–16%). The tendon extracts (50 μL) from all experimental groups were precipitated with 100 μL of 50 mM acetate buffer, pH 7.4, and 9 volumes of absolute ethanol, for 24 hr at 4^{o}C. These samples were analyzed by SDS-PAGE. Gels were stained with Comassie Brilliant Blue R-250. The relative molecular masses were estimated by comparisons with protein standard molecular mass markers.

Quantitative analysis

The quantities of proteins in the extract of GuHCl were measured by the Bradford method (1976), using bovine serum albumin (BSA) (1mg/mL) as a standard. The readings were performed in a micro-plate reader at 595 nm. Sulfated glycosaminoglycans (GAG) of GuHCl extracts were quantified by the dimethylmetilene blue method (DMMB) (Farndale et al. 1986) using chondroitin sulphate (1mg/mL) as standard, and the readings were performed in a micro-plate reader at 526 nm. To quantify hydroxyproline, tendon fragments (n=4) were dehydrated in

acetone for 48 h and, subsequently, for another 24 hours in a mixture of chloroform and ethanol, at a ratio of 2:1. The tendon fragments were then hydrolyzed in 6 N HCl (10 mg of tissue/mL), for 18 h at 120^{o}C, and the hydrolysate was neutralized with 6N NaOH. After, the samples were treated with chloramine T solution and perchloric acid/aldehyde, as described by Stegemann and Stalder (1967). After incubation for 15 min at 60^{o}C, the material was cooled and the absorbance was measured at 550nm in a spectrophotometer, Ultrospec 2100 (Pro Amersham Biosciences, England). The amount of hydroxyproline in the sample was calculated by comparison with a standard curve of hydroxyproline, and expressed as mg/g of wet tissue.

Gelatin Zymography

Tendon fragments (n=4/group) were incubated in 0.3mL of extraction buffer (10 mM cacodylic acid, pH 5.0, 0.15 M NaCl, 1 μM $ZnCl_2$, 20 mM $CaCl_2$, 1.5 mM NaN_3, 0.01% Triton X-100 [v/v]), at 4°C for 24 hours. After this period, the solution was centrifuged for 10 minutes (20,000 xg at 4°C). Samples were dried and resuspended in the same extraction buffer (pH 5.0). Zymography assays were performed on 10% polyacrylamide electrophoresis gels containing 0.1% gelatin, using 8 μg and 50 μg of protein per sample to indentify MMP-2 and MMP-9, respectively. After electrophoresis, the gels were washed with 2.5% Triton X-100 at room temperature and incubated overnight in a solution of 50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$ and 0.02% NaN_3 at 37°C for 20 hours. Finally, the gels were stained with Coomassie Brilliant Blue. The protein bands corresponding to gelatinolytic activity were observed after washing the gels with a solution containing 30% methanol and 10% acetic acid. The gel was evaluated by band densitometry using the Scion Image program. Each sample was analyzed individually and the experiments were repeated three times.

Biomechanical Test

After euthanasia, the right paw with the gastrocnemius and soleus muscles and Achilles tendon were excised. Five tendons of each group were used for the mechanical test. They were kept in physiological solution until the test to prevent drying of the fibers. For the test, the specimens were clamped in a mechanical support with the myotendinous junction and the phalanges at opposite extremities, as employed by Nakagaki and colleagues (2007). The clamp-to-clamp distance was maintained at 7 mm for all tests. Each tendon was submitted to a pre-conditioning test, with 10 cycles of loading-unloading from 0 to 0.5mm, at a speed of 20mm/min (Toyama and Yasuda 2000), then submitted to the uniaxial tensile test. During the test, the tendon was subjected to a gradual increase in load at a constant displacement rate of 20mm/min using a load cell of 1kN, until the tendon broke (Toyama and Yasuda 2000). The results were used to calculate the material properties (maximum stress, strain at maximum stress and modulus of elasticity or modulus of Young) of the tendons in each group (Gupte et al. 2002). The maximum stress was obtained from the relation between load (N) and cross-section area (CSA) (mm^2) and expressed in MPa (megapascal). The CSA was determined according to Goodship and Birch (2005). Each tendon had its shape cast using an alginate dental impression paste (Avagel) manufactured by Dentsply. The CSA mould obtained was cut transversally and photographed. These images were then analyzed with the software Image Pro-Plus. The strain was calculated using the formula $\varepsilon = \Delta L/L_0$, where $\Delta L = L - L_0$ (L= final length and L_0= initial length). Strain was calculated based on clamp-to-clamp displacement. The elastic modulus of each group was measured in the interval corresponding to the most linear region of the stress-strain curve for each

sample. The experiments were done with a machine designed specifically for testing the mechanical properties of materials (MTS, model TETSTAR II, designed by the Laboratory of Mechanical Properties, School of Mechanical Engineering, UNICAMP).

Statistical analysis

The Statistica software (v 8.0) (Tulsa, OK, USA) was used for the statistical analysis. All data were presented as mean ± standard deviations (S.D.), and a value of $p<0.05$ was considered significant. The statistical comparison among the control and treated groups was determined using one-way ANOVA followed by the post hoc test of Tukey. In addition, two-way ANOVA was used, when appropriate, to determine how *H. aphrodisiaca* treatment and/or exercise training affected the results, and whether there was interaction between these two conditions.

RESULTS

Biochemistry analyses

Analysis in SDS-PAGE of the Guanidinium chloride extract showed the presence of faint bands of collagen and noncollagenous proteins (NCP) in extracts of animals of the CT group, compared with other groups. This result showed that exercise alone markedly reduced the presence of collagen and NCP. However *H. aphrodisiaca* treatment in sedentary rats maintained the bands displayed in CS, and also additional ones. Furthermore, the exercise plus *H. aphrodisiaca* (HT group) accentuated the appearance of NCP (Fig1).

The NCP levels were significantly decreased ($p<0.05$) in animals of the control trained group (CT) when compared to other groups (Fig2a), confirming the SDS-PAGE analysis. Two-

way ANOVA showed interaction between training and the treatment with plant infusion for tendon protein content (p= 0.0008). The sulfated glycosaminoglycan levels were statistically similar among all groups (Fig2b).

Hydroxyproline is an indicator of collagen concentration in tissues. Its content was the highest in HT group (Fig2c). Two-way ANOVA showed that there was interaction between endurance training and treatment with *H. aphrodisiaca* for hydroxyproline levels in the tendon (p= 0.005).

Zymography Analysis

The zymography (Fig3 a,b) data demonstrated a tendency towards reduction of activity of the MMP-2 isoforms for the CT and HT groups (Fig3c), but the difference between them was not statistically significant. The total MMP-2 activity was much reduced in the HT group (Fig3e), when compared with other groups. MMP-9 activity was not different for any of the experimental groups (Fig3 d).

Morphology

To detect proteoglycans within the compression region of the tendon, the sections of the different experimental groups were stained with Toluidine Blue (Fig 4). Although metachromasy was observed in the territorial matrix of the compression region of all groups, the trained tendons (Fig4 c, d) had a larger and more intensely stained area compared with sedentary rats (CS and HS). This pattern suggested an increase in the proteoglycan concentration in this region (Fig4 a, b).

The analysis of trained tendons with polarized light microscopy showed intense birefringence in both of the compression (Fig5 e, g) and tension regions (Fig5 f, h), indicating highly organized collagen bundles. However, the HT group showed the most intense birefringence in both compression and tension regions of the tendon (Fig5 g, h). Both sedentary groups (Fig5 a-d) had the same morphological pattern, despite the fact that collagen bundles were not as well aligned as observed in the trained ones.

Biomechanical Parameters

Biomechanical analysis showed significant increase in maximum stress (Fig6 b) and modulus of elasticity (Fig6c) of the tendon of animals trained and treated with *H. aphrodisiaca* (HT). Two-way ANOVA analysis showed that there was interaction between training and treatment with plant infusion only for the maximum stress ($p= 0.006$). The strain (Fig6 a) was similar in all groups, with no significant difference among them.

DISCUSSION

In this study, we were able to identify biochemical, biomechanical and morphological alterations on the Achilles tendon of rats resulting from endurance training and treatment with *H. aphrodisiaca* infusion.

The implications of endurance training (Viidik et al. 1969; Woo et al. 1980; Vilarta and Vidal 1989; Huang et al. 2004) and AAS treatment (Wood et al. 1988; Miles et al. 1992; Marqueti et al. 2006) on tendon properties have been studied by several investigators. The use of AAS produced a stiffer tendon that failed with less elongation in Wistar rats. The exercise did not

significantly alter the tendon elongation in athlete animals. However, the combination of exercise and steroids significantly increased the stiffness and decreased the elongation, as well as the energy that the tendon could absorb at tendon failure (Miles et al. 1992). In all cases the maximum load that the tendon could withstand did not seem to be affected. However, the use of steroids in the presence of exercise increased the cross-sectional area and reduced the flexibility of the tendon (Miles et al. 1992). Wood et al. (1988), in similar study, noted alterations in the collagen content and the toe-limit strain in rats that had been treated with anabolic steroids and exercise.

In the present study, the maximum stress and modulus of elasticity were higher in tendons of trained and treated rats, which also exhibited higher hydroxyproline content. These data showed that HT animals have more resistant tendons**,** differing from other studies where the combination of AAS and exercise did not improve the tendon's biomechanical properties (Wood et al. 1988; Miles et al. 1992). Moreover, the interaction between strenuous exercise and *H. aphrodisiaca* promoted significant increase in the material properties (maximum stress and modulus of elasticity) and of collagen content**,** resulting in stronger tendons able to support intense muscular contraction**.** Tendons may show a more prompt response to the number of cycles of loading, than to the magnitude of the load (Buchanan and Marsh 2002). Simonsen et al. (1995) found that a strength-training regimen (high force with few loading cycles) did not stimulate increase in strength of the Achilles tendon of rats; however, low-force endurance training (e.g. swimming) resulted in stronger tendons. They suggested that the tendons may respond to the number of muscle contractions that occur during training rather than the absolute tension exerted by the muscle. In this case, increasing tendon mechanical resistance observed during endurance training and *H. aphrodisiaca* treatment might not represent a requirement for

increased strength, but rather a mechanism to prevent tendon damage due to mechanical fatigue. This biomechanical behavior could be due to the increase of the collagen content, to the fiber orientation and to the interaction between collagen and ground substance (Woo et al. 1999). There is a relationship between mechanical properties and collagen content (Buchanan and Marsh 2002), since more rigid tissues have either more collagen per area or collagen fibers with larger diameters (Woo et al. 1999).

The biomechanical results corroborate the results obtained by polarized microscopy. The organizational aspect of the fibers is better understood when the slides are analyzed under polarized microscopy, due to the birefringence properties of collagen bundles. This observation is important because it shows micro-morphological details hidden within these bundles. In the present study, this technique revealed high birefringent brightness due to the condensation and highly tidy fiber array in the trained group. Besides, in the trained group that also received the plant infusion (HT) the results were even more prominent, showing brighter collagen fibers, possibly indicating highly compacted bundles. It seems possible that this increase in collagen compaction rate is associated to an increase in the amount of cross-links (Viidik 1978; Frank et al. 1998). The lower birefringence found in sedentary animals reflects the lower organization of collagen bundles in these groups.

Some observations in the compression region showed that, in HT animals, there was an increase in the round cell population (stereological data not shown), as well as in metachromasy intensity, which indicates a greater proteoglycan accumulation due to the increased compressive forces during endurance exercise. However, this result was not confirmed by the GAG dosage. It is important to say that microscopical analysis of sections stained with toluidine blue are limited to a specific region of the tendon where there is greater accumulation of proteoglycans due to the

presence of localized compressive forces, as was observed in tendons of rats (Covizzi et al. 2001) and pigs (Feitosa et al. 2002). Nevertheless, the proteoglycans, mainly the low weight ones, are distributed all over the tendon, associated to the collagen fibers (Cribb and Scott 1995), probably regulating collagen fibrillogenesis (Vogel and Meyers 1999).

Analysis of the SDS-PAGE results showed faint bands of collagen and other proteins (14-67 kDa), as well as lower concentrations of total protein content in CT animals. A plausible explanation is that the temperature increases enormously within the tendon during an intense endurance protocol (Birch et al. 1997), because these tendons play a specialized role by acting as elastic energy stores. The stored strain energy, however, is not entirely recoverable; some 5-10% is released as heat (Riemersma and Schamhardt 1985). Therefore, if the blood supply is not enough to dissipate the energy generated, a rise in temperature would be expected to occur. This temperature increase in the tendon core, generated by high-speed locomotion, does not persist for a period long enough to cause tendon cell death *in vivo*. However, repeated exposure to short periods of hyperthermia (daily exercise) may compromise cell function, resulting in reduced synthetic ability or changes in cell metabolism, which could alter matrix composition (Birch et al. 1997).

The degradation of collagen, as well as of a great number of other EMC compounds, is initiated by metalloproteinases (MMPs). An increase in net MMP activity is likely to indicate matrix degradation and accelerated remodeling (Koskinen et al. 2004; Riley 2005). Increased MMPs activity was verified in human tendons after acute running exercise (Koskinen et al., 2004) and in exercised rats (jumping in water) (Marqueti et al. 2006). In the present study, the zymography showed that the MMP-2 activity in CT animals was similar to the sedentary groups, differing from other studies in which the exercise increased the MMPs levels in tendons

(Koskinen et al. 2004; Marqueti et al. 2006). It is noteworthy that the MMP activity in the above studies was analyzed after only 3 days and 6 weeks of training, respectively.

In the present study, the animals were sacrificed after 11 weeks of training, and therefore the MMP activity was evaluated after a long period of training. The biomechanical and morphological data demonstrated that the tendons of treated and trained animals had undergone adaptation to the increased demand. Thus, we suggest that there was a period of increased MMP activity to permit tissue remodeling, followed by a period of reduced activity when the tendons were already adapted to the load required. In this case, the mechanical load did not represent a stimulus for the synthesis of pro-MMPs and their consequent activation. Also, the MMP-9 activity did not alter in the exercised animals, which confirms the tendons' adaptation, in that high activity of this protein is associated with the presence of immune cells during the initial inflammatory process (Clutterbuck et al. 2008), which occurs in response to tissue damage caused by exercise. In human tendons, protein synthesis and degradation were chronically elevated 4 weeks after the beginning of the training period, whereas protein synthesis remained high throughout a 12-week training cycle, while the degradation was slowly reduced. This suggests that there was probably an early period in the exercise program when collagen turnover in tendons was increased in order to restructure and readapt the tendon to the increased loading pattern (Langberg et al. 2001).

Marqueti et al. (2006) affirmed that the MMPs activity strongly decreased in AAS-treated animals. The inhibition could be due to a decrease in MMP synthesis or inhibition of activation of latent pro-MMPs. Also, the exercise itself was not enough to compensate the inhibition of MMP activity induced by AAS treatment. In the present investigation the plant infusion alone did not inhibit MMP activity, and the data obtained suggests that the infusion associated to exercise

could up regulate the MMPs' activity, considering that in the HT group the MMP-2 activity was reduced compared to all other groups, and that in this group the tendons were more resistant, according to the biomechanical results, in relation to the CT group. Therefore, we suggest that remodeling was more efficient in the HT group. However, further research is necessary to evaluate the effect of the plant infusion on MMP activity in exercised animals.

According to the results found in the present study, it can be concluded that endurance training associated with *H. aphrodisiaca* infusion increases the material properties of tendons. The treadmill protocol used did not modify tendon strength by itself. Rather, the association resulted in more resistant tendons, due to the increase in collagen molecules and probable corresponding increase in the cross-links between them, which is inferred by the higher molecular organization of the collagen observed in this study. Despite the clearly anabolic effects of *H. aphrodisiaca* and associated endurance training, no side effects were observed, such as those found after synthetic AAS use: collagen dysplasia, greater stiffness, reduction of strain, impaired tissue remodeling and others. Therefore, *H. aphrodisiaca* associated with endurance training contributed to more efficient remodeling of the extracellular matrix, resulting in more resistant tendons to support high loads from intense muscle contraction. These findings suggest that the *H. aphrodisiaca* infusion is a potential aid to optimize tendon remodeling in athletes, where the disparity of the faster physiologic muscle adjustment in relation to the tendon often leads to lesions, because the tendons may not resist the tension produced by the stronger muscles.

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TABLE

Table 1- Exercise protocol for treadmill running

Event	Week	Velocity (m/min)	Duration (min)
Treadmill adaptation	1	10.68	5
	2	12.42	7.5
	3	14.16	10
Training	1	14.16	20
	2	19.62	30
	3	19.62	40
	4-8	22.92	45

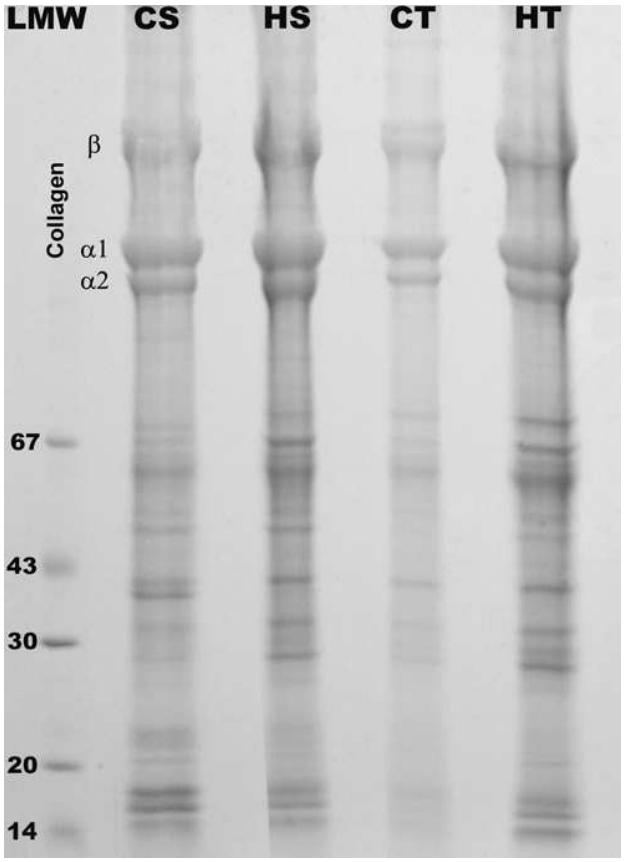

Fig1: Analysis in SDS-PAGE showed faint bands of collagen and other proteins in control trained animals (CT). Note that *H. aphrodisiaca* treatment (HS) maintained the bands displayed in control sedentary animals (CS) and also exhibited other NCP bands. Also, in treated and exercised animals (HT) the appearance of NCP was accentuated. LMW- Low molecular weight standard.

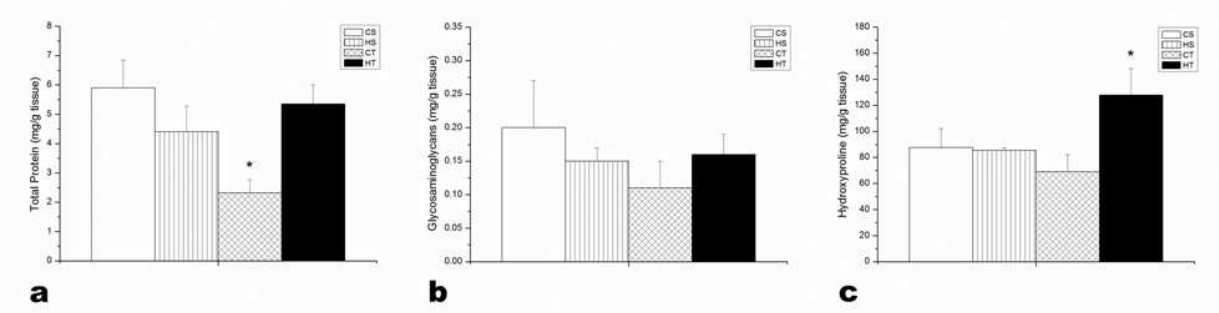

Fig2: Quantifications of noncollagenous protein (NCP), sulfated glycosaminoglycans and hydroxyproline in the Achilles tendon of the different groups. The NCP levels (a) were significantly decreased with exercise alone, whereas *H. aphrodisiaca* treatment did not alter protein levels. The sulfated glycosaminoglycan levels (b) were statistically similar in the four groups. Observe the remarkable effect of training plus *H. aphrodisiaca* treatment on the hydroxyproline content (c). CS-control sedentary; HS- *H. aphrodisiaca* sedentary; CT- control trained; HT- *H. aphrodisiaca* trained. The columns are the mean $\pm$ SD. * The differences were significant for $p<0.05$ (ANOVA) compared with other groups, by Tukey test.

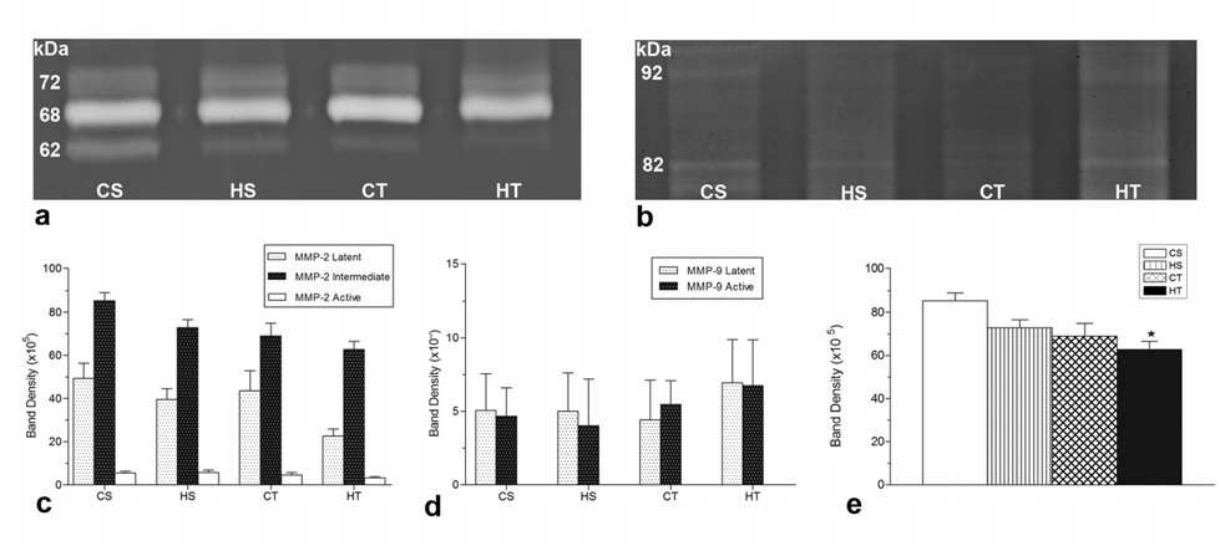

Fig3: Zymography of Achilles tendon extracts. Each lane represents a sample of one animal. a, b- Gelatin zymography gel used to quantify activities of MMP-2 and MMP-9, respectively; c, d and e- Densitometry analysis of MMP-2, MMP-9 and total MMP-2, respectively. CS-control sedentary; HS- *H. aphrodisiaca* sedentary; CT- control trained; HT- *H. aphrodisiaca* trained. In HT group the MMP-2 activity was reduced when compared with CS. MMPs activity was similar in both HS and CS groups, indicating the *H. aphrodisiaca* treatment did not inhibit the MMP activity. MMP-9 latent (92 kDa), MMP-9 active (82 kDa), MMP-2 latent (72 kDa), MMP-2 intermediate (68 kDa), MMP-2 active (62 kDa). The columns are the mean $\pm$ SD. * The differences were significant for $p<0.05$ (ANOVA) compared with CS group, by the Tukey test.

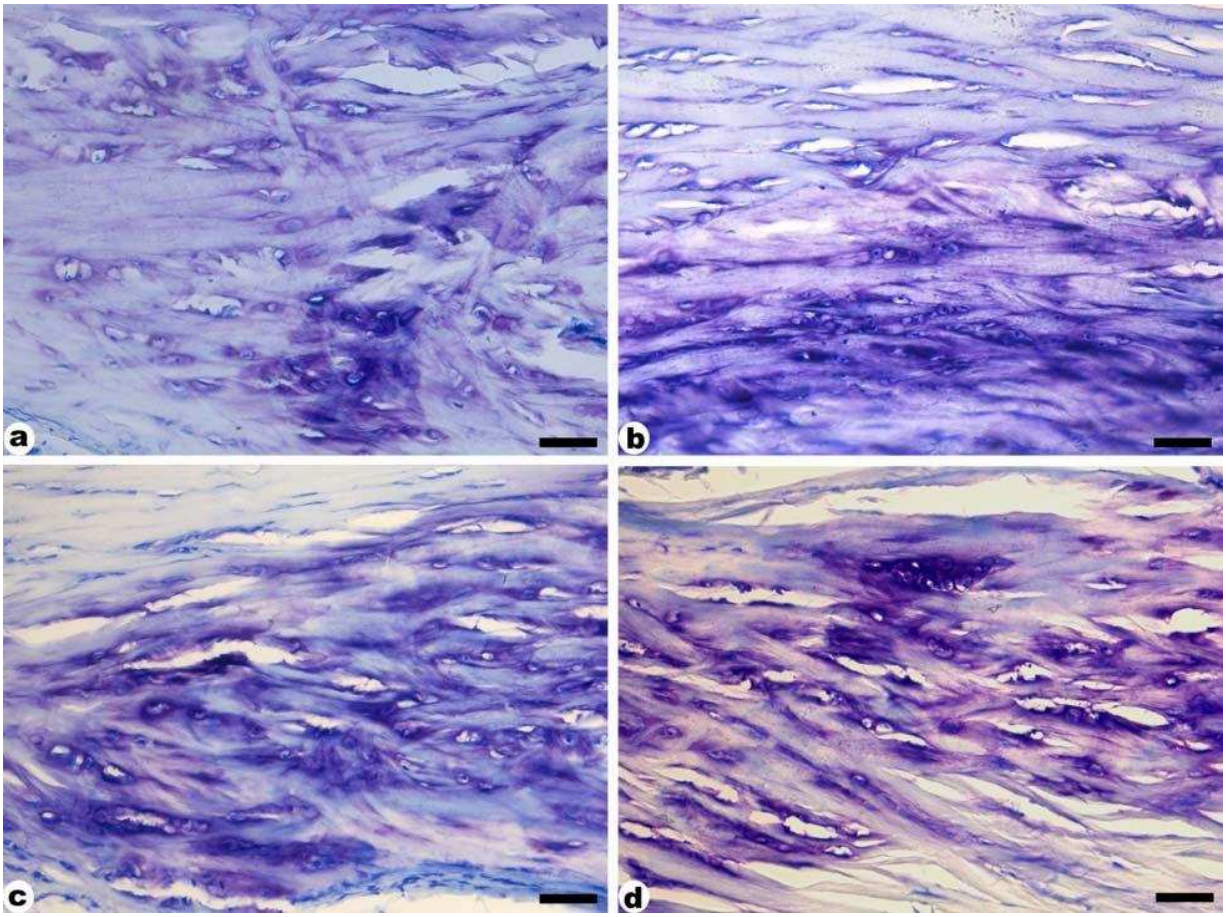

Fig4: Longitudinal sections of tendons stained with Toluidine Blue. a- control sedentary; b- *H. aphrodisiaca* sedentary; c- control trained; d- *H. aphrodisiaca* trained. Observe intense metachromasy in trained groups (c, d). The treatment with *H. aphrodisiaca* accentuated the metachromasy in sedentary (b) as well as trained (d) rats, compared with the controls. Scale bar= 25 μm.

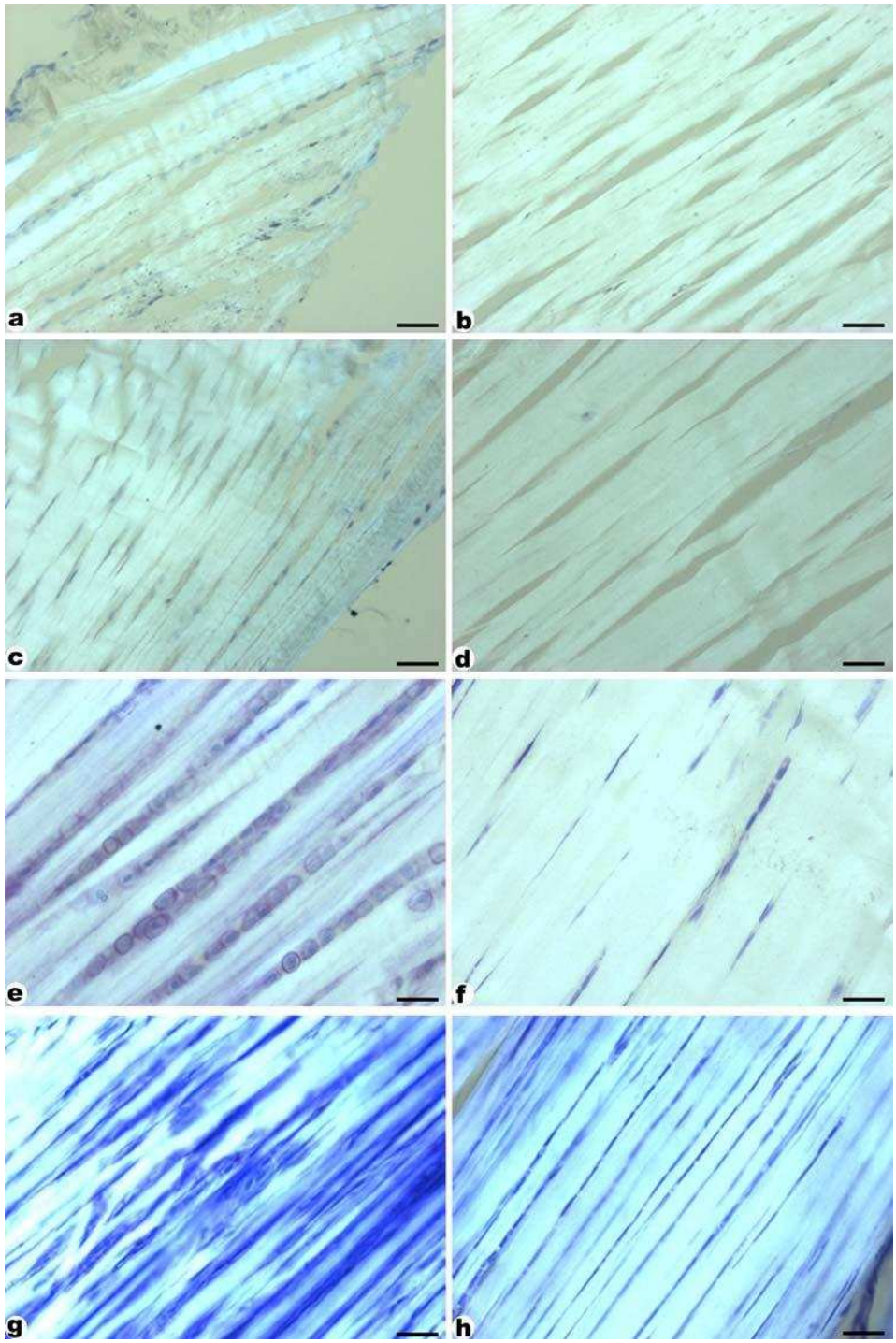
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Fig5: Polarized light microscopy of Toluidine Blue-stained sections. a, b- correspond to control sedentary; c, d- *H. aphrodisiaca* sedentary; e, f- control trained; and g, h- *H. aphrodisiaca* trained. The left column corresponds to the compression region and right column corresponds to the tension region. Observe a larger organization of the collagen bundles in tendons of trained (e-h) versus sedentary (a-d) animals. However, the most intense birefringence was found in tendons of trained rats and also those treated with the plant infusion (g, h). The stronger birefringence is due to the aggregation and longitudinal organization of the collagen bundles. Scale bar= 30 µm.

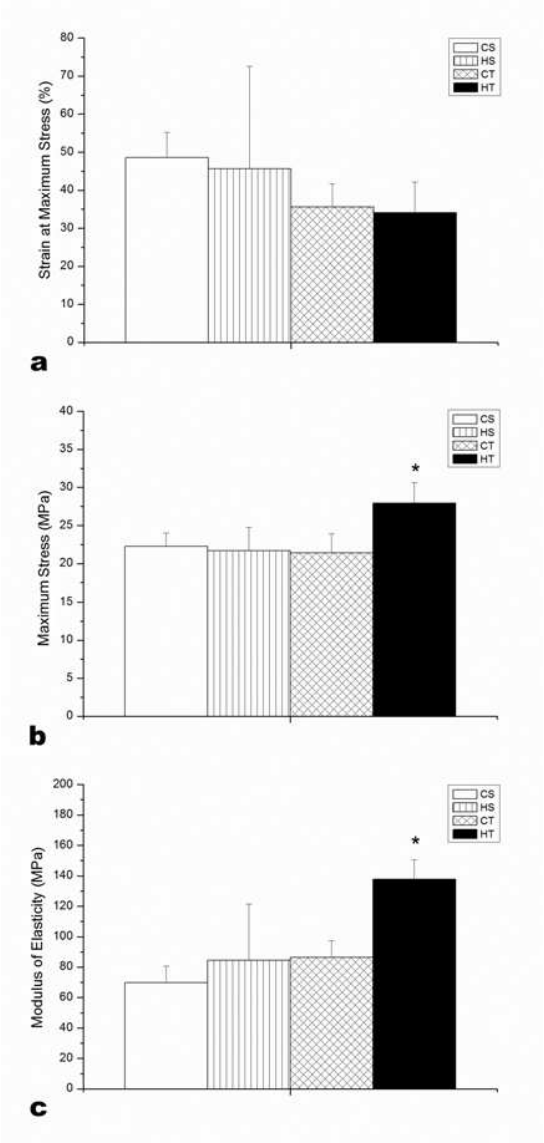

Fig6: Biomechanical properties of Achilles tendons from control and *H. aphrodisiaca* sedentary and trained rats. The maximum stress (b) and modulus of elasticity (c), but not strain (a), were higher in *H. aphrodisiaca* trained rats. CS- control sedentary; HS- *H. aphrodisiaca* sedentary; CT- control trained; HT- *H. aphrodisiaca* trained. The columns are the mean $\pm$ SD. * The differences were significant for $p<0.05$ (ANOVA) compared with other groups, by the Tukey test.

ARTIGO 2

PLASTICITY OF SKELETAL MUSCLE AFTER ENDURANCE TRAINING AND *Heteropterys aphrodisiaca* INFUSION ADMINISTRATION

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ABSTRACT

The aim of this study was to evaluate the effect of the *H. aphrodisiaca* infusion on the skeletal muscle of sedentary and aerobically exercised rats. Male rats were divided into four groups (n=6): CS and CT- control sedentary and trained (distilled water), HS and HT- *H. aphrodisiaca* sedentary and trained (plant infusion, 104 mg/day). The training protocol consisted in running on a motorized treadmill, 5 times a week, with weekly increase in treadmill velocity and duration, consisting in 3 weeks of adaptation and 8 weeks of training. The blood was collected for testosterone dosage. The extensor digitorum longus (EDL) muscles were either frozen in liquid nitrogen for histochemical and Western blotting analysis or fixed in Karnovsky for ultrastructural analysis. The main fiber types (I, IIA, IID and IIB) were identified by m-ATPase (myofibrillar

adenosine triphosphatase) technique, after incubation in pH 4.2, 4.5 and 10.6. To measure capillary, muscular fiber and mitochondrial volume density we employed the point-sampling technique of classical stereology. The testosterone levels and androgen receptor concentration increased significantly in HS animals. No alterations were observed in the fiber composition for all groups. The EDL mean cross-section area of the HT group was similar to the sedentary groups and increased significantly when compared with the CT group. Intramuscular vascularization and mitochondria volume density were significantly greater in the HT group compared with other groups. The training protocol used in this study did not alter the muscle morphology of control animals; however, the association of *H. aphrodisiaca* and this exercise protocol resulted in increased mean area of muscle fiber, mitochondrial volume density and muscular vascularization, suggesting an increase of the endurance capacity of these animals.

Keywords: EDL muscle, testosterone, androgen receptor, fiber types, capillary density, mitochondrial density

INTRODUCTION

Skeletal muscle is an extremely adaptable organ, demonstrating impressive structural and functional plasticity in response to alterations in metabolic and functional demand (Hepple, 2000). The muscle fiber types, which can be delineated according to various parameters, for example, myofibrillar protein isoforms, metabolic enzyme profiles, and structural and contractile properties, respond to altered functional demands and a variety of signals by changing their phenotypic profiles. Their phenotypic profiles are affected by innervations/neuromuscular activity, mechanical loading/unloading, exercise training, hormones, and aging (Pette & Staron, 2001).

It is well established that increased contractile activity, as occurs in endurance training, promotes a transition from type II to type I muscle fiber types, which happens at the expense of the type II fiber population (Thayer et al., 2000). Moreover, the endurance exercise results in an increase of mitochondrial density, capillary supply, changes in key metabolic enzymes, and increased maximal oxygen uptake (Seene et al., 2005). In males, an acute bout of endurance exercise increases circulating testosterone and dehydroepiandrosterone (Galbo et al., 1977; Tremblay et al., 2005) and regular exercise can influence the resting hormone profile (Hackney et al., 1998).

Heteropterys aphrodisiaca O. Mach. (Malpighiaceae) is found mainly in the “Cerrado” regions of Mato Grosso and Goiás States (Brazil), and is known as “nó-de-cachorro”, “nó-de-porco” and “cordão-de-São-Francisco” (Pio Corrêa 1984; Pott and Pott 1994). It was described by Hoehne, in 1920, as a plant with stimulant and aphrodisiac properties. *H. aphrodisiaca* root infusion has been used by Brazilian popular medicine as a tonic or stimulant and for the treatment

of nervous debility, nervous breakdown, for muscle and bone weakness (Pio Corrêa 1984; Pott and Pott 1994).

Previous studies with *H. aphrodisiaca* suggested that the root extract could increase corporal and testicular weight, as well as Leydig cell volume within rat testis (Chieregatto 2005; Monteiro et al., 2008). Moreover, the administration of the *H. aphrodisiaca* extract to old rats provoked increases in the antioxidant enzymes in brain homogenates, reflecting in improvement of cerebral activity and recovery of memory and attention span (Mattei et al., 2000; Galvão et al., 2002).

The aim of this study was to evaluate the effect of the *H. aphrodisiaca* infusion on the skeletal muscle of sedentary and aerobically exercised rats. We hypothesized that the high-intensity endurance training could affect the muscle phenotype, and that the plant infusion could have a modulating role on these effects. To investigate this possibility, we analyzed the plasma testosterone, the concentration of androgen receptor in muscle, fiber composition, cross-section area of fibers, capillary density and mitochondrial density of the extensor digitorum longus muscle (EDL).

MATERIAL AND METHODS

Animals

Adult Wistar rats, 90 days old, were obtained from the Center for Biological Investigation - CEMIB (State University of Campinas, Campinas, SP, Brazil). The rats were housed, three per cage, under standard conditions with 12hrL:12hrD. Animals were provided with commercial rat

feed and water *ad libitum*. The Institutional Committee for Ethics in Animal Care and Use of this University approved the experimental protocol (process n° 1233-1).

Medicinal Plant

H. aphrodisiaca roots were collected in February 2007, in Mato Grosso State, Brazil. The species was identified by comparison with the voucher herbarium specimen of the plant at the Herbarium Federal University of Mato Grosso, Brazil (number 23928). The dried roots were crushed and powdered using a grinding mill. The infusion was routinely prepared by pouring 100mL of boiling water over 25 g of powdered roots, which was allowed to steep for 4 hours, then filtered using filter paper. The infusion was prepared every four days and it was stored in the refrigerator. The yield was an infusion of 68.66 mg of dry extract (6.866% w/v) and a yield of 6.832% (w/w) in terms of initial crude dry weight of plant material. The doses of *H. aphrodisiaca* were selected according to previous studies (Monteiro et al., 2008). The animals were weighed weekly to adjust the dose of infusion.

Study groups and experimental protocol

Twenty-four male rats were divided into four groups (n=6/group): control sedentary (CS); *H. aphrodisiaca* sedentary (HS); control trained (CT); *H. aphrodisiaca* trained (HT). The HS and HT received *H. aphrodisiaca* infusion by gavage (104 mg/animal) daily, during the 8 weeks of training or sedentary period, whereas the control groups (CS and CT) received 0.5mL of distilled water. Trained rats (CT and HT groups) were allowed to adapt to treadmill running for a 3 week period, prior to the beginning of the experimental protocol, which consisted of low to moderate level exercise carried out daily for 5 days a week (Table 1). After adaptation, trained rats were

subjected to 8 weeks of intensive aerobic exercise training (treadmill running), also on a weekly cycle of 5 consecutive exercising days followed by a two days rest, as scheduled in Table 1 (adapted from Moraska et al. 2000; Smolka et al. 2000; Demirel et al. 2001; Fontana et al. 2008). This program is a form of endurance training and does not compare with power training (Fontana et al., 2008).

Surgical procedures

Forty-eight hours after the last training, the rats were anesthetized with xylazine chloride (Anasedan, Vetbrands, São Paulo, Brazil) and ketamine chloride (Cetamin, Syntec, Cotia, Brazil) (5 and 80 mg/Kg body weight, respectively). The blood was collected by heart puncture. The extensor digitorum longus muscles (EDL) were dissected, weighed and either frozen for histochemical, biochemical and Western blotting analysis or preserved in Karnovsky's fixative for stereological analysis.

Hormonal Analysis

The blood samples were centrifuged at 5000 rpm for 10 minutos at 4°C. The plasma was collected and the total testosterone was measured by chemi-luminescence in the Alvet Laboratory (Sorocaba, SP, Brazil).

Western Blotting

Muscle samples were weighed and homogenized for 1 min with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in 200 µL of a lysis buffer containing 150 mM NaCl, 1% Triton X-100, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1mM Hepes, pH 7.6, 2 mM

sodium vanadate, 0.2mM PMSF, 2 mg/ml leupeptin, 2 mg/ml aprotinin (Zambuzzi et al., 2008), and centrifuged at 10,000xg for 10 min. Protein concentration in the supernatant was determined using the Bradford's reagent (Bio-Rad Laboratories, Hercules, CA) and 50 μg of protein were resolved by SDS–PAGE using 10% gels under reducing conditions. After electrophoresis, proteins were electro-transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences), which were subsequently blocked with TBS-T containing 5% non-fat milk and probed with the antibody anti-androgen receptor (Cat. 06-680; Millipore) diluted at 1:500 in TBS-T containing 1% non-fat milk, followed by HRP-conjugated goat anti-rabbit IgG (Cat. A-6154; SigmaChemical Co.). The bands were developed using enhanced chemioluminescent substrate (Santa Cruz Biotechnology) and Kodak X-Omat films. The gels were stained with Coomassie Blue and the band corresponding to the molecular weight of tubulin (55 kDa) was quantified to verify the quantity of protein loaded in the gels. Quantification of the AR bands was made by measuring the optical densities using the Scion Image software version 4.0.

Determination of fiber type and Histomorphometry

The middle portion of the muscle was separated, oriented in a mixture of gum tragacanth (Sigma-G1128) and Tissue-Tek embedding compound (EMS, cat. 62550-01), immediately frozen in isopentane cooled to -156°C in liquid nitrogen, and stored at -70°C until ready for use. Transverse 12 μm sections were obtained in a cryostat, collected on coverslips and stored frozen at -40°C until all samples were processed. Fiber types were identified using the myofibrillar adenosine triphosphatase (mATPase) histochemistry (Staron and Pette, 1993) following incubation at pH 4.2, 4.5 (Brooke and Kaiser, 1970) and 10.6 (Guth and Samaha, 1970) (Figure

1). Three fields of each muscle section, obtained at pH 4.5, were selected, according to Esteva et al. (2008). These fields were photographed and mounted as a plate. This plate was used as a guide to identify the pure fiber types (I, IIA, IIB and IID) from sections obtained at pH 4.2, 4.5 and 10.6. Image Pro-Plus software version 6.0 (Media Cybernetics, Inc.) was used to calculate the cross-sectional areas of muscle fibers using a BX 50 Olympus light microscope. The same three fields used for fiber-type determination were used and all muscle fibers encompassed in these fields were evaluated.

Stereology

Capillaries were identified on the same cross sectional fields used for fiber-type determination. The number of capillaries per unit area (capillary density, CD=capillaries/mm^2) was measured to give an indication of the number of capillaries present in a standard area. The capillary volumetric and fiber density was obtained by the stereological methods described by Weibel (1979). The stereological analysis used a test-system with 112-test-points and a known area. The volume densities of the structures were estimated as $Vv[structure] = Pp[structure]/P_T$, where Pp is the number of points that hit the structure and P_T is the total number of test-points contained in the area surrounded by the frame. Muscular vascularization was calculated by ratio of capillary volume density and fiber volume density (Mandarim-de-Lacerda, 2003). Mitochondrial number and volume was studied using transmission electron microscopy (TEM) of the muscle fiber. We obtained photomicrographs from different randomly chosen fibers at a magnification of 10,000X. The images were digitally processed, the mitochondrial number was counted and the volume density was calculated by the point-sampling technique of classical stereology (Weibel, 1979), as described above. For mitochondrial analysis, we used a test-system

with 140-test-points. The results were expressed as the ratio of mitochondrial volume density and mean fiber cross-section area.

Statistical analyses

The Statistica software (v 8.0) (Tulsa, OK, USA) was used for the statistical analysis. All data were presented as mean ± SD and a value of $p<0.05$ was considered significant. The statistical comparison among the control and treated groups was determined using one-way ANOVA followed by the post hoc test of Duncan.

RESULTS

Biometry and Hormonal analysis

There was an increase of body mass in all groups during the treatment, but the trained groups of animals gained less mass than the sedentary animals (Table 2). The EDL weight was not altered between groups (Table 2). The sedentary animals treated with *H. aphrodisiaca* (HS) showed significantly higher testosterone levels (Table 2) when compared with other groups.

Western Blotting

The Western blotting analysis showed a significant increase in the AR protein concentration in EDL muscle of HS group, when compared with other groups (Figure 2).

Fiber type and histomorphometry

There was no significant difference in the muscle fiber types among the experimental groups (Figure 3A), however we observed a trend towards the transformation of the IIB→ IIA fiber types in the trained group. Treatment with the plant infusion did not alter the fiber size, but the association of the infusion and endurance training had a positive effect, since animals of the HT group showed the highest mean cross-sectional area of muscle fibers (Figure 3B).

Stereology

The capillary volume density increased in the HT group ($p<0,05$). No alterations were observed in the capillary and mitochondria number and the fiber volume density (data not shown). The intramuscular vascularization increased in trained animals; however it was significantly higher in HT group compared with the CS (Figure 4A). The mitochondria volume density was significantly greater in HT group compared with other groups (Figure 4B).

DISCUSSION

This study was undertaken to verify the effect of *Heteropterys aphrodisiaca* in adult male Wistar rats submitted to physical training. Thus, we proposed to evaluate possible alterations of testosterone dosage, in the expression of androgen receptors, in the types of fibers and ultrastructure of fibers of the extensor digitorium longus muscle of animals submitted or not to physical training and treatment with the plant infusion.

Many authors have investigated the effect of exercise on the endocrine system. While some demonstrated that endurance training does not lead to lowered total testosterone levels in

athletes (Lucia et al., 1996; Smilios et al., 2003), others have shown clearly that hormone levels vary during training (Wheeler, et al., 1991; Kern et al., 1995; Tremblay et al., 2005). These contradictory results can be explained by the dependence of such reactions to many different factors such as intensity, duration and type of exercises, as well as the level of the athletes' training (Tremblay et al., 2004).

In this study, we found that the administration of the infusion of *H. aphrodisiaca* significantly increased testosterone concentration in sedentary animals. However, in association with physical exercise the use of the infusion did not alter testosterone levels, as also occurred in control trained animals. Therefore our results are in agreement with the results of Lucia (1996) e Duclos (1996) and collaborators, as observed for athletes under endurance exercise.

It is known that the expression of AR can increase in skeletal muscle of animals in response to the effort of exercise (Inoue et al., 1993; Deschenes et al., 1994; Kadi et al., 2000), and in response to increased levels of circulating androgen (Antonio et al., 1999). In this study, the concentration of AR increased in animals that received infusion but were not exercised, in response to the higher levels of testosterone. However, the increase of hormone and androgen receptors did not increase the muscle mass of these animals. The increase of testosterone and androgen receptors in the sedentary animals treated with the infusion appears to have had an androgenic effect on the reproductive organs of these animals, since an increase of the prostate and seminal vesicle was found, as well as a higher spermatogenic yield (Gomes et al., 2009).

Changes in the cross section area of muscle fibers demonstrate the synthesis of contractile proteins, including myosin. It also indicates that new myofibrils are added to the fiber with the synthesis of these proteins (Booth et al., 1998). Analysis of the cross section of the EDL showed

that some of the control trained animals had a smaller average area, but in comparison with the sedentary animals, this reduced area is not significant.

Therefore, the endurance training without infusion treatment did not cause alterations in the cross section area of the rats' muscle fibers, thus differing from data presented by Trappe (2006) and Harber (2004) and collaborators that relate a reduction of muscle fiber diameters after endurance training in athletes. These differences can be explained by the type of exercise and duration of the training period, as well as the differences in species studied. On the other hand the association of the plant infusion and treadmill exercise resulted in a significant increase of the average cross section area of the muscle fibers, when compared to the control trained animals.

Testosterone directly stimulates muscle growth by affecting the rate of protein synthesis, protein breakdown, and net gain or loss of muscle protein. These actions that are reportedly mediated by the androgen receptor, which acts as a nuclear transcription factor (Hartgens and Kuipers, 2004). In addition, an indirect action mediated by locally produced autocrine and paracrine growth factors cannot be ruled out. Locally produced insulin-like growth factor 1 (IGF-1) was found to increase following acute muscle damage (Bamman et al., 2001) or chronic aerobic exercise (Hambrecht et al., 2005), with no changes in circulating IGF-1 (Bamman et al., 2001; Hambrecht et al., 2005). On the other hand, the increase of serum IGF-1 with exogenous administration of growth hormone or IGF-1 does not appear to stimulate myofiber hypertrophy in the absence of mechanical loading (Bamman et al., 1998). A sustained local over expression of IGF-1 promotes myofiber regeneration and hypertrophy through various pathways and has been shown to increase levels of myogenic regulatory factors and contractile protein mRNAs. IGF-1 also seems essential to mediate the loading-induced hypertrophy of skeletal muscle (Musaro et al., 2001; Olesen et al., 2006). Therefore, we believe that other sutdies are necessary to determine

which molecules initiate muscle hypertrophy in trained animals treated with the plant infusion, since the increase of plasma testosterone did not alter muscle fiber morphometry

It has been proposed that changes in the isoforms of miosin during endurance training can be characterized as quantitative remodeling of the muscle, in that one isoform is substituted by another which is more adapted to the level of strength needed for long duration exercise (Baldwin & Haddad, 2002). In some studies, the endurance exercise promoted the transition of rapidly contracting fibers into a phenotype of slower fibers. This does not signify a transformation of type II into type I, but rather a change within the type II fiber subtypes, in the direction of types IIB→IID→IIA (Okumoto *et* al., 1996; Seene et al., 2005; Fontana, 2008), although the type of change will depend on the muscle analyzed. In our work, we did not find significant differences in the percentage of the muscle fiber types of the EDL of the different experimental groups. Our results differ from the results of Seene and collaborators (2005), where significant changes were found in the myosin isoforms after treadmill training. In spite of the fact that association of treatment with infusion and training did not cause significant alterations in the EDL muscle profiles, we did find a tendency of change in the direction of IIB→IID→IIA.

In this study, we found that the association of the plant infusion and endurance training increased volumetric density of mitochondria and capillaries, thus increasing the vascular system of the muscle tissue. On the other hand, exercise in control rats was not sufficient to alter these parameters, which is in disagreement with the results of Hoppeler et al. (1985), Howald et al. (1985), Poole and Mathieu-Costello (1996) and Galvin et al. (2007), that demonstrated the increase of volumetric density of mitochondria and/or capillaries in muscles of humans and rats after endurance training. Therefore, the association of the plant infusion with endurance exercise

could have induced capillary angiogenesis within and among skeletal muscles and, since the capillary and oxidative capacities are coupled, increased mitochondrial volume density was observed.

Therefore, the training protocol used in this study did not alter the muscle in control animals; however the association of *H. aphrodisiaca* and this exercise training protocol resulted in increased mean muscle fiber area, mitochondrial volume density and muscular vascularization. Thus, it can be concluded that the plant infusion and endurance training improved the endurance capacity of animals trained and treated for eight weeks.

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Table 1- Exercise protocol for treadmill running

Event	Week	Velocity (m/min)	Duration (min)
Treadmill adaptation	1	10.68	5
	2	12.42	7.5
	3	14.16	10
Training	1	14.16	20
	2	19.62	30
	3	19.62	40
	4-8	22.92	45

Table 02- Biometry and testosterone dosage of Wistar rats treated with *Heteropterys aphrodisiaca* infusion and submitted to treadmill training and controls.

	CS	**HS**	**CT**	**HT**
Body mass gain (g)	72.00 ± 12.41	68.5 ± 15.54	57.6 ± 12.02	61.00 ± 16.57
EDL mass (mg)	167 ± 19.00	183 ± 22.00	165 ± 25.00	180 ± 18.00
Relative weight EDL	0.040 ± 0.004	0.044 ± 0.005	0.041 ± 0.005	0.041 ± 0.005
Testosterone (ng/mL)	3.28 ± 0.894	5.13 ± 0.614*	3.08 ± 0.353	2.51 ± 0.55

CS- control sedentary; HS- *H. aphrodisiaca* sedentary; CT- control trained; HT- *H. aphrodisiaca* trained.
The values are the mean ± SD.
* The differences were significant for $p<0.05$ (ANOVA) compared with other groups by Duncan test.

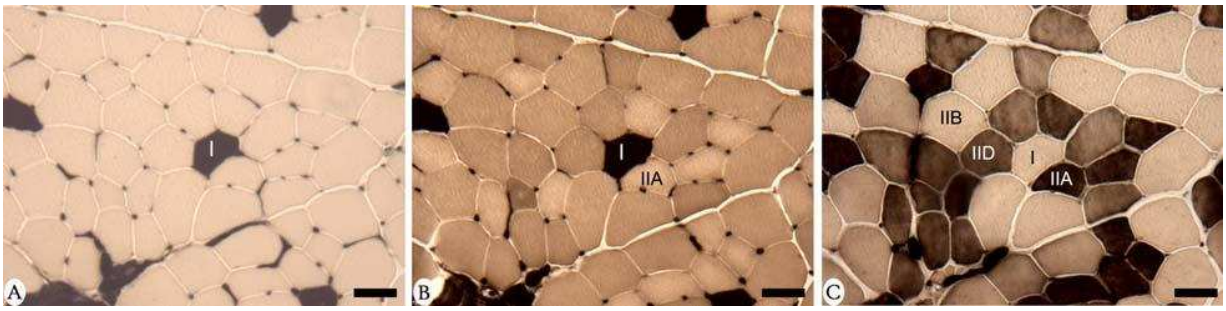

Figure 1- Histochemical mATPase reaction at pH 4.2 (A), pH 4.5 (B) and pH 10.6 (C) in serial sections of the EDL muscles of Wistar rats. I – type I fiber, IIA – type IIA fiber, IIB- type IIB fiber, and IID- type IID fiber. Scale bar: 20µm.

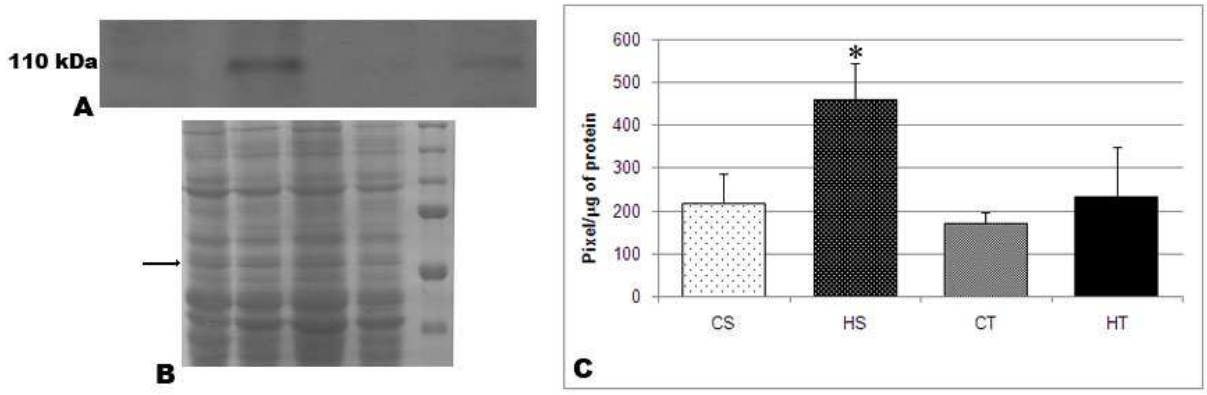

Figure 2- Western blot analysis of androgen receptor (AR) in rat EDL muscle. Fifty micrograms of crude muscle protein homogenate was fractionated by 10% of SDS-PAGE. AR protein was quantified by immunoblotting and antibody detection. A- Representative blot showing AR levels in EDL muscle. B- Coomassie blue-stained membrane demonstrates even loading. C- AR concentration in different groups. CS- Control sedentary, HS- *Heteropterys aphrodisiaca* sedentary, CT- Control trained, HT- *Heteropterys aphrodisiaca* trained. In C the values are mean ± SD. * The differences were significant for $p<0.05$ (ANOVA) compared with other groups by Duncan test. Arrow- Bands corresponding to tubuline (55kDa).

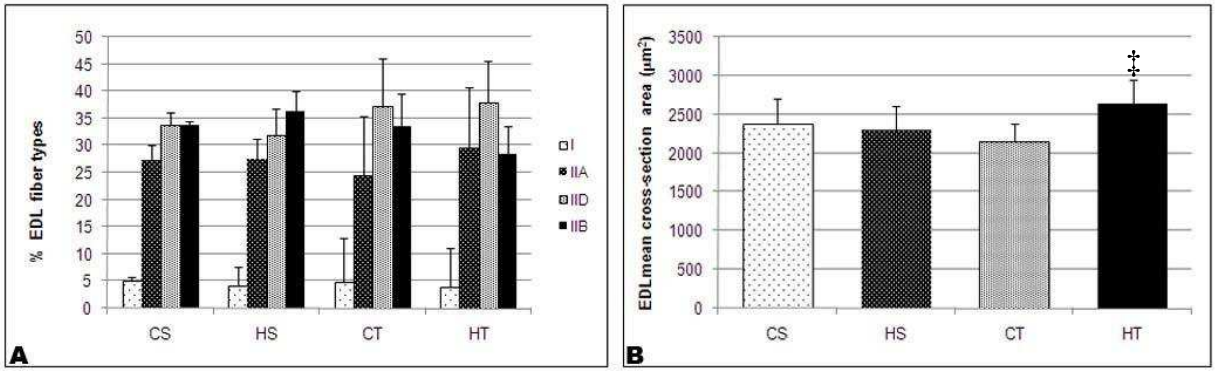

Figure 03- Percentage of different fiber types (A) and mean cross-section area (B) of EDL of Wistar rats treated with *Heteropterys aphrodisiaca* infusion and submitted to treadmill training. CS- control sedentary; HS- *H. aphrodisiaca* sedentary; CT- control trained; HT- *H. aphrodisiaca* trained. The columns are the mean ± SD. ‡ The differences were significant for $p<0.05$ (ANOVA) compared with CT group, by the Duncan test.

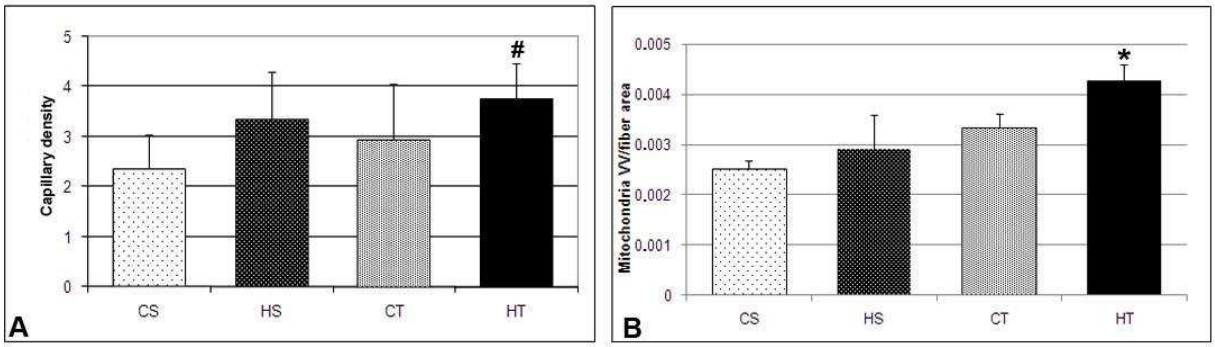

Figure 4- Effect of *Heteropterys aphrodisiaca* infusion and endurance training on muscular vascularization and mitochondrial content. A- Capillary density (ratio of capillary volume density and fiber volume density); B- Ratio of mitochondria volume density and mean fiber cross-section area. CS- Control sedentary, HS- *Heteropterys aphrodisiaca* sedentary, CT- Control trained, HT- *Heteropterys aphrodisiaca* trained. The values are mean ± SD.

# $p<0.05$ (ANOVA) compared with CS group by the Duncan test. * $p<0.05$ (ANOVA) compared with other groups by the Duncan test.

ARTIGO 3

Does *Heteropterys aphrodisiaca* administration and endurance training alter bones of mature rats?

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Abstract

Heteropterys aphrodisiaca infusion, alone or associated with endurance training, was investigated in rat bones in relation to their mechanical properties, collagen content and morphology. Male rats were divided into four groups (n = 8): CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT– control trained, HT- *H. aphrodisiaca* trained. The training protocol consisted in running on a motorized treadmill, 5 times a week, for 8 weeks, with weekly increase in treadmill velocity and duration. Control groups received water while HS and HT groups received *H. aphrodisiaca* infusion (104 mg/animal) by gavage during the 8 weeks. Tibiae were frozen for collagen dosage and biomechanical analysis or preserved in Karnovsky's fixative, then processed for histomorphological analysis by scanning electron microscopy. The HT group showed significantly higher yield load and yield stress in the tibiae three-point bending test. The maximum load, stiffness, maximum stress and elastic modulus were statistically similar for the experimental groups. The hidroxyproline content, morphometrical and stereological data were not significantly different for the four groups. Scanning electron microscopy showed more lacunae and Havers canals in the bone of trained animals, moreover the osteons were more disorganized, when compared with sedentary groups. These alterations may indicate that the bone of trained animals was being remodeled. However, after 8 weeks of training, it was not possible verify alterations in morphometrical measurements, collagen content, stiffness and modulus of elasticity of the trained and treated animals.

Keywords: Biomechanics, Hidroxyproline, morphometry, scanning electron microscopy, tibiae.

Running Title: *H. aphrodisiaca* and endurance training alter bone?

Introduction:

The primary function of bone is to provide protection and mechanical support for the body. In addition, bones participate in the regulation of calcium homeostasis (BUCKWALTER et al., 1996). Bone consists of a dry weight of 65% mineral and 35% organic matrix (BUCKWALTER et al., 1996; JEE, 2000). The collagen network accounts for most of the organic phase of this tissue and provides bone with tensile strength as well as a matrix for mineral deposition, which, in turn, confers rigidity (BAILEY et al., 1999). Bone collagen fibers are predominantly type I, but a small amount of type III and V collagen has been reported to be associated with vasculature and osteocytes, respectively (BAILEY et al., 1999). Type I fibers are composed of parallel aligned, end-over-lapped and quarter staggered molecules. This precise organization of molecules in the fibrils allows head to tail cross-linking of the molecules for strength, and at the same time provides a nucleation site for the deposition of calcium apatite in the gap regions generated in the fiber (LANDIS et al., 1996).

It is well accepted that physical exercise improves health (KIVINIEMI et al., 2007). It strengthens bones, improves cardiorespiratory fitness, speed-strength and lipid profiles (VAINIONPAA et al., 2007). Exercise can potentially increase resistance to skeletal fragility, and it is commonly considered that mechanical stimulation exerts its influence through structural adaptation and the accrual of bone mass (CARTER, VAN DER MEULEN and BEAUPRE, 1996). Rodent models of exercise have shown a link between mechanical loading of bone with increased bone mass, cross-sectional geometric properties, and maintenance or increase in mechanical properties (KODAMA et al., 2000; NOTOMI et al., 2001). However, the improvement in mechanical properties cannot be fully explained by changes in the size and shape

of bones, and mechanical loading can also affect the quality of extracellular matrix (WALLACE et al., 2007; KOHN et al., 2009). Physical exercise may change the pace of collagen network remodeling and therefore significantly affect the generation of collagen cross-links (BRAMA et al., 2002; KOHN et al., 2009), and consequently, bone mechanical properties.

Heteropterys aphrodisiaca O. Mach. (Malpighiaceae) is a Brazilian plant found mainly in the "Cerrado" regions of Mato Grosso and Goiás states (PIO CORRÊA, 1984; POTT and POTT, 1994). It was described by Hoehne (1920) as a plant with stimulant and aphrodisiac properties, and it is known as "nó-de-cachorro", "nó-de-porco" and "cordão-de-São-Francisco". *H. aphrodisiaca* root infusion is used in popular medicine as a tonic or stimulant, for the treatment of nervous debility, nervous breakdown and for muscle and bone weakness. In previous studies, the association of endurance training with *H. aphrodisiaca* resulted in more organized collagen bundles and more resistant tendons to support high loads from intense muscle contraction (MONTEIRO et al., 2009).

In this study, we investigated the effect of the association of endurance training with *H. aphrodisiaca* administration on the size, collagen content, mechanical properties and morphology of tibia of Wistar male adult rats.

2 Material and Methods:

2.1 Animals

Adult Wistar rats, 90 days old, were obtained from the Multidisciplinary Center for Biological Investigation - CEMIB (State University of Campinas, Campinas, SP, Brazil). The rats were housed, three per cage, under standard conditions with 12hrL:12hrD. Animals were

provided with commercial rat food and water *ad libitum*. The Institutional Committee for Ethics in Animal Care and Use of this University approved the experimental protocol (number 1233-1).

2.2 Medicinal Plant

H. aphrodisiaca roots were collected in February 2007, in Mato Grosso State, Brazil. The species was identified by comparison with the voucher herbarium specimen of the plant at the Herbarium of the Federal University of Mato Grosso, Brazil (number 23928). The dried roots were crushed and powdered using a grinding mill. The infusion was routinely prepared by pouring 100mL of boiling water over 25 g of powdered roots, which were allowed to steep for 4 hours, then filtered using filter paper. The infusion was prepared every four days and stored in the refrigerator (4ºC). The yield was an infusion of 68.66 mg of dry extract (6.866% w/v) and a yield of 6.832% (w/w) in terms of initial crude dry weight of plant material. The doses of *H. aphrodisiaca* were selected according to previous studies (MONTEIRO et al., 2008).

2.3 Study Groups and Experimental Protocol

Thirty-two male rats were divided into four groups (n=8/group): control sedentary (CS), *H. aphrodisiaca* sedentary (HS), control trained (CT), *H. aphrodisiaca* trained (HT). The HS and HT received *H. aphrodisiaca* infusion by gavage (104 mg/animal) daily, during the 8 weeks of training or sedentary period, whereas the control groups (CS and CT) received 0.5mL of distilled water. Trained rats (CT and HT groups) were allowed to adapt to treadmill running for a 3 week period, prior to the beginning of the experimental protocol, which consisted of low to moderate level exercise carried out daily for 5 days a week (Table 1). After adaptation, trained rats were subjected to 8 weeks of intensive aerobic exercise (treadmill running), also on a weekly cycle of

5 consecutive exercising days followed by a two day rest, as shown in Table 1 (adapted from MORASKA et al., 2000; SMOLKA et al., 2000; DEMIREL et al., 2001;, FONTANA et al., 2008). This program is a form of endurance training and should not be compared with power training (FONTANA et al., 2008).

2.4 Surgical Procedures

Forty-eight hours after the last training, the rats were anesthetized with xylazine chloride (Anasedan, Vetbrands, São Paulo, Brazil) and ketamine chloride (Cetamin, Syntec, Cotia, Brazil) (5 and 80 mg/Kg body weight, respectively). The left and right tibiae were harvested. All soft tissues were removed from the bones. Right tibiae lengths were measured according Lammers and collaborators (1998), they were wrapped in aluminum foil and kept at -20°C for future biomechanical testing and hydroxyproline analysis. The left tibiae were preserved in Karnovsky's fixative for morphological analysis

2.5 Scanning Electron Microscopy

Tibia samples (n=4/group) were immersed in Karnovsky's fixative for 24 h and decalcified with formic acid, formaldehyde and sodium citrate 0.5M. The bones were then rinsed three times with 0.1 M sodium phosphate buffer, pH 7.2, and then dehydrated in an ascending ethanol series prior to critical point drying. The specimens were mounted on stubs, sputter-coated with gold and examined with the scanning electron microscope (Jeol-JMS 560).

2.6 Biomechanical Test

A three-point bending model test was adopted for measuring the mechanical properties of bone tissues (n=8/group) using a material testing system (MTS, model Teststar II). The span of the two support points was 21 mm, and the deformation rate was 3 mm/min. Load-displacement data were transported to a computer and acquired by software. Original data were used to calculate the structural properties: yield load, maximum load and stiffness. Stiffness was computed as the slope of the linear portion of the load-displacement curve. After testing the specimens in three-point bending test, the failure sites of all bone specimens were photographed together with a standard measurement, using a high-resolution digital camera at a standard distance, according to HUANG et al (2003). Cross-sectional parameters, including cortical bone thickness and cross-sectional area (CSA) of cortical bone, were measured from the photographs using the software Image Pro Plus (v6.1, Media Cybernetics). The cross-sectional moment of inertia (I) was calculated under the assumption that the cross sections were elliptical: $I=\pi/64[ab^3-(a-2t)x(b-2t)^3]$, where ***I*** is the cross-sectional moment of inertia, *a* is the width of the cross section in the mediolateral direction, *b* is the width of the cross section in the anteroposterior direction, and *t* is the average of the cortical thickness (TURNER and BURR, 1993). Data of load-displacement were transferred to a stress–strain curve using the following equations: $\sigma = F.L.c/4I$ and $E = Stiffness.L^3/48I$, **w**here σ is stress, ***c*** is the maximal distance from pixels to the line crossing the center of mass, ***F*** is the applied load (N), ***E*** is elastic modulus, and ***L*** is the span between the two supporting points of the bending fixture (mm). Then, the material properties (yield stress, maximum stress and elastic modulus) were measured. Yield load and yield stress were determined following the 0.2%-offset method (TURNER and BURR, 1993). A line 0.002-strain offset and parallel to the linear part of the stress–strain curve was constructed. The intersection point of this 0.002-strain offset line and the stress–strain curve was called the yield

stress. The original loading value of this point was called the yield load. The yield point (yield load and yield stress) is the imaginary point that divides the elastic region of the plastic region. It is defined as the highest force that the material can withstand, without leaving any permanent deformation when unloaded.

2.7 Hidroxyproline Analysis

To quantify hydroxyproline, the same tibia samples used for biomechanical analysis (n=4) were dehydrated in acetone for 48 h and, subsequently, for another 24 hours in a mixture of chloroform and ethanol, at a ratio of 2:1. The tibia fragments were then hydrolyzed in 6N HCl (10 mg of tissue/mL), for 18 h at 120^{o}C, and the hydrolysate was neutralized with 6N NaOH. After, the samples were then treated with chloramine T solution and perchloric acid/aldehyde, as described by STEGEMANN and STALDER (1967). After incubation for 15 min at 60^{o}C, the material was cooled and the absorbance was measured at 550nm in a spectrophotometer, Ultrospec 2100 (Pro Amersham Biosciences, England). The amount of hydroxyproline in the sample was calculated by comparison with a standard curve of hydroxyproline, and expressed as mg/g of wet tissue.

2.8 Statistical Analyses

The Statistica software (v 8.0) (Tulsa, OK, USA) was used for the statistical analysis. All data were presented as mean ± SD and a value of $p<0.05$ was considered significant. The statistical comparison was determined using one-way ANOVA followed by the post hoc Duncan test.

3 Results:

There was an increase of body mass in all groups during treatment, but the trained groups of animals gained less mass than those of sedentary animals (Figure 1).

3.1 Morphometry analyses

There were no statistically significant differences in tibia length, proximal width, cortical bone thickness and cross-sectional moment of inertia between the sedentary and trained groups (Table 2). The distal width of the tibiae of animals of the HT group was significantly greater than in the CS group.

3.2 Scanning Electron Microscopy analysis

Scanning electron microscopy analysis of the bones of trained animals showed apparent increase in lacunae, which indicated the increase in osteocyte number in the cortical bone. The number of Havers canals also increased, suggesting an increase in bone vascularization of trained animals. The bones of trained animals showed more disorganized osteons, which may indicate that these bones were in the process remodeling. However, no morphological difference in the compact bone of control and treated trained rats could be identified (Figure 2).

3.3 Biomechanical Parameters

The structural and material mechanical properties of bone tissue were measured in this study. The animals trained and treated with *H. aphrodisiaca* (HT) showed significantly higher yield load and yield stress (Figure 3A and 4A, respectively) in the tibiae three-point bending test.

The maximum load (Figure 3B), stiffness (Figure 3C), maximum stress (Figure 4B) and elastic modulus (Figure 4C) were not significantly different among the four groups.

3.4 Hydroxyproline dosage

Hydroxyproline is an indicator of collagen concentration in tissues. The hydroxyproline content was similar in all groups (Figure 5).

4 Discussion

Many previous studies have used either young, old or surgically osteopenic rodents to determine effectiveness of exercise-induced loading on the skeleton. This may be justified by the importance of achieving peak bone mass and minimizing bone loss, but these models increase confounding factors due to accelerated bone growth or loss due to age alone. The study of skeletally mature, intact young adult rodents more directly address the normal adult skeletal adaption to mechanical loading (WARNER et al., 2006). As such, we studied 90-day-old male rats, and this discussion mostly focuses on previous studies which have included the effects of endurance exercises in the skeletally mature rats. We used the *Heteropterys aphrodisiaca* infusion to evaluate whether this plant, alone or associated with endurance training, exerts some effect on the mechanical properties and collagen content of rat bone, as was observed in tendons (MONTEIRO et al., 2009).

Changes in body mass can influence the mechanical properties of bone. The present study, however, showed no significant difference in body mass between control and trained groups. The present results, therefore, were not dependent on body mass, and the differences shown in bone

properties were likely a consequence of the exercise and/or of the treatment with *H. aphrodisiaca*.

Exercise has been shown to change bone morphometry in experimental animals. Cortical bone area increased following an exercise program in swine (RAAB et al., 1991) and rats (NEWHALL et al., 1991; WHEELER et al., 1995). However, other studies with rats and mice showed that the endurance exercise did not alter the morphometric measurements of bone of exercised animals (FORWOOD and PARKER, 1991; WARNER et al., 2006; ISAKSSON et al., 2009). In our study, the length, proximal width and cortical bone thickness displayed no statistical differences between the sedentary and trained groups. The statistically greater distal width of tibiae of the HT group was not considered important because this point does not support any major muscle.

The discrepancy in results described in the literature for morphometrical data could be due to differences in the exercise protocol, animal age and species, and the overall length or intensity of the exercise study. Moreover, the difference of these results could be explained by the proximo-distal location of the functional adaptations of limb bone to mechanical loading. Femoral rates and amounts of bone formation were significantly greater than those of the tibiae in exercised mice (PLOCHOCKI et al., 2008). Ontogenetically constrained bone formation in distal limb elements may be an evolutionary adaptation to conserve bone mass and maintain energetic efficiency during high stride frequency locomotion (PLOCHOCKI et al., 2008). However, there may also be a biomechanical explanation for proximodistal differences in bone growth in mammals with tapered limbs. Muscular contractions exert larger loads on bone than body mass during running activities because muscles work as poor lever arms and require great force (Frost, 1999). In rodents, mammals with tapered limbs, the muscle mass is concentrated proximally on

the limb. Because bone growth is, in part, regulated by mechanical stress, heavier muscle proximal skeletal elements can be expected to experience greater loading and consequently greater modeling than distal skeletal elements under conditions of intense running (PLOCHOCKI et al., 2008).

The effects of exercise on the biomechanical properties of bone using experimental animals have yielded controversial results. WHEELER and collaborators (1995) showed that tibial stiffness, the energy absorbed and the angle of twist at failure were affected by exercise. Conversely, WARNER (2006) and ISAKSSON (2009) and collaborators showed that treadmill exercise did not alter the mechanical properties of bone of the adult mice and rats, respectively. Our results were in agreement with the last results cited, since we showed that treadmill running alone did not alter the structural and material properties of tibiae bone. However, the difference of results might be due to the duration and intensity of exercise used in each study. WHEELER and collaborators (1995) explained that the duration of exercise may affect the bone mineralization more strongly than the intensity of exercise in rats.

In this study, the association of endurance exercise and the plant treatment determined tibiae with higher yield load and yield stress, when compared with control trained and sedentary animals. Having the significantly higher yield load and yield stress implies that the HT group bones sustained more elastic deformation or strain. These alterations in biomechanical properties could be related to bone mineral density and the collagen organization in the bone. According to ISAKSSON et al. (2009), exercise speeded up the rate of reorientation of the collagen structure, e.g, arrangement of fibrils especially in the longitudinal direction, rather than increased collagen formation. This could be the case in our study, since the quantity of collagen did not increase, as did the yield load and the yield stress of the tibiae.

Contrary to the findings for tibiae, a previous study associating the treatment with this plant and endurance exercise showed that the animal's tendons had a significant increase in mechanical properties and of collagen content**,** resulting in stronger tendons, able to support intense muscular contraction. Moreover, the tendons analyzed by polarized microscopy showed brighter collagen fibers, possibly indicating highly compacted bundles (MONTEIRO et al., 2009).

Scanning electron microscopy analysis of the bone showed an increase in lacunae and Havers canals for trained animals. Moreover, the osteons of trained animals were more disorganized than in the sedentary groups, which suggest that the bone of trained animals were being remodeled.

Therefore, 8 weeks of training did not show alterations in morphometrical measurements, hidroxyproline content and other mechanical properties (stiffness and modulus of elasticity) of the bone of trained and treated animals, as was found in the tendons of animals that received *H. aphrodisiaca* and endurance training. Studies with longer training periods should be undertaken to determine whether alterations will ensue with further exercise.

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Table 1- Exercise protocol for treadmill running

Event	Week	Velocity (m/min)	Duration (min)
Treadmill adaptation	1	10.68	5
	2	12.42	7.5
	3	14.16	10
Training	1	14.16	20
	2	19.62	30
	3	19.62	40
	4-8	22.92	45

Table 2- Morphometric data for the tibiae

	CS	HS	CT	HT
Length (mm)	42.80 ± 1.28	42.02 ± 1.01	41.39 ± 1.07	41.77 ± 0.63
Proximal width (mm)	7.80 ± 0.25	7.03 ± 0.44	7.24 ± 0,35	7.25 ± 0.20
Distal width (mm)	4.61 ± 0.4	4.79 ± 0.38	4.88 ± 0.28	5.18 ± 0.24#
Cortical bone thickness (mm)	0.63 ± 0.03	0.60 ± 0.05	0.60 ± 0.04	0.62 ± 0.08
Cross-sectional moment of inertia (mm^4)	1.18 ± 0.21	1.24 ± 0.26	1.14 ± 0,3	1.39 ± 0.53

CS- control sedentary; HS- *H. aphrodisiaca* sedentary; CT- control trained; HT- *H. aphrodisiaca* trained.
The values are the mean ± SD.
# $p<0.05$ compared with CS group by Duncan test.

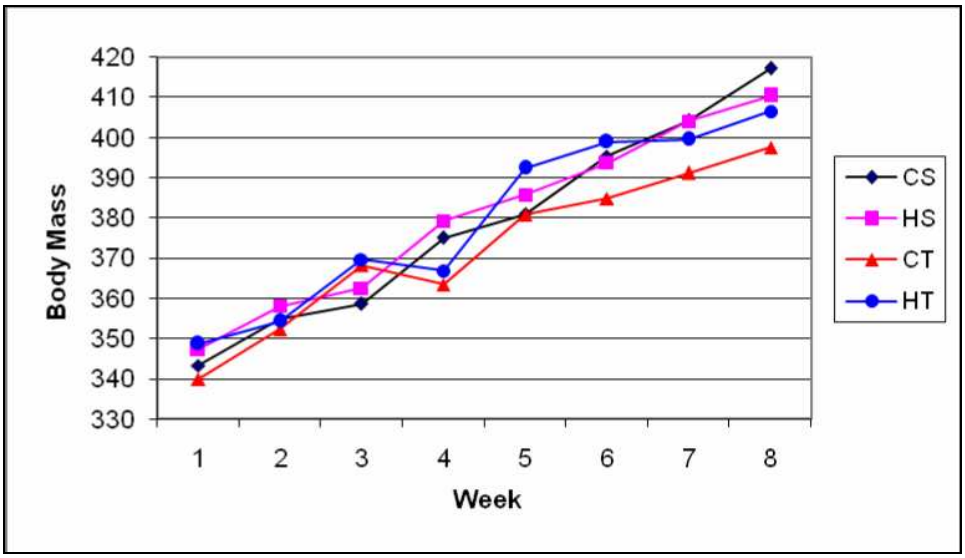

Figure 1- Body mass (g) of sedentary and trained Wistar male rats and/or treated with *H. aphrodisiaca* infusion.

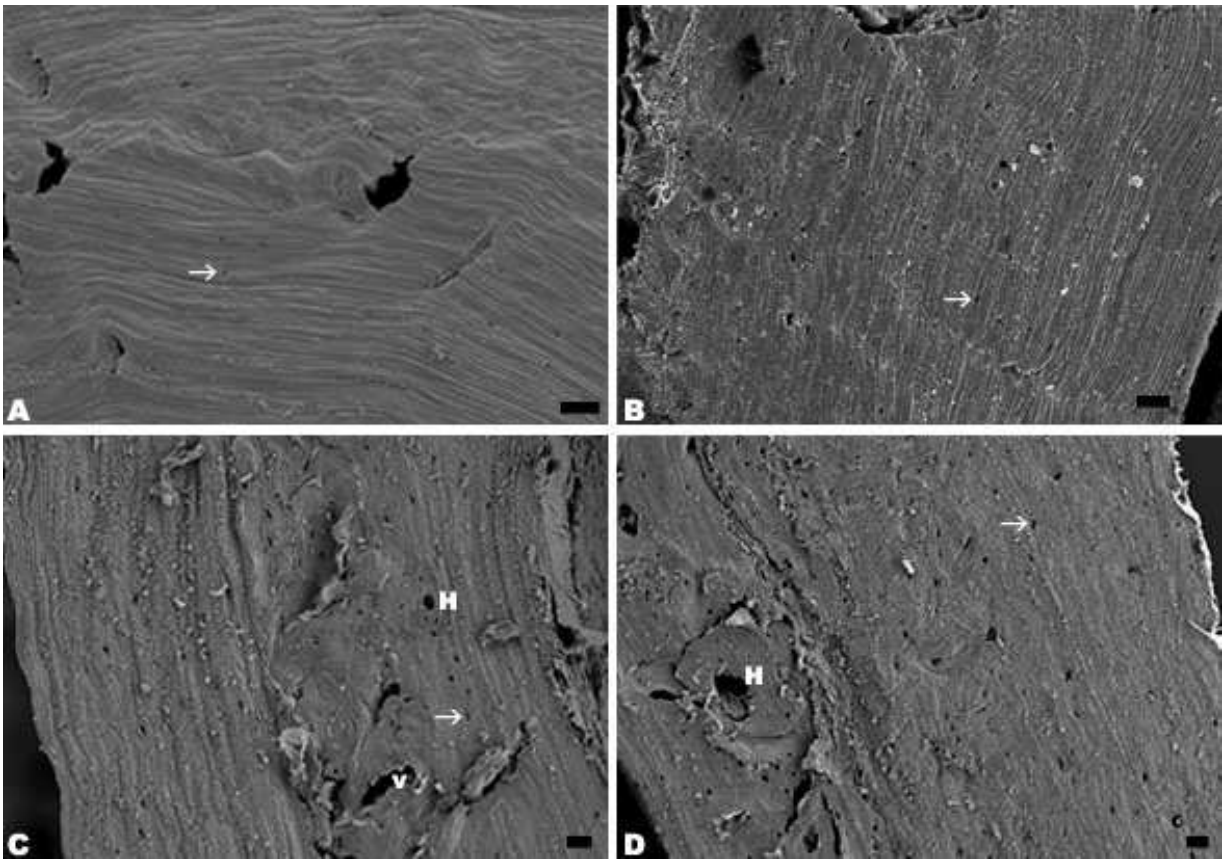

Figure 2- Scanning electron microscopy of cross-section of the tibia. A- Control sedentary, B- *H. aphrodisiaca* sedentary, C- Control trained, D- *H. aphrodisiaca* trained. Arrow- lacuna, **H** - Havers canal, **v** – Volkmann canal. Bar= 20 µm.

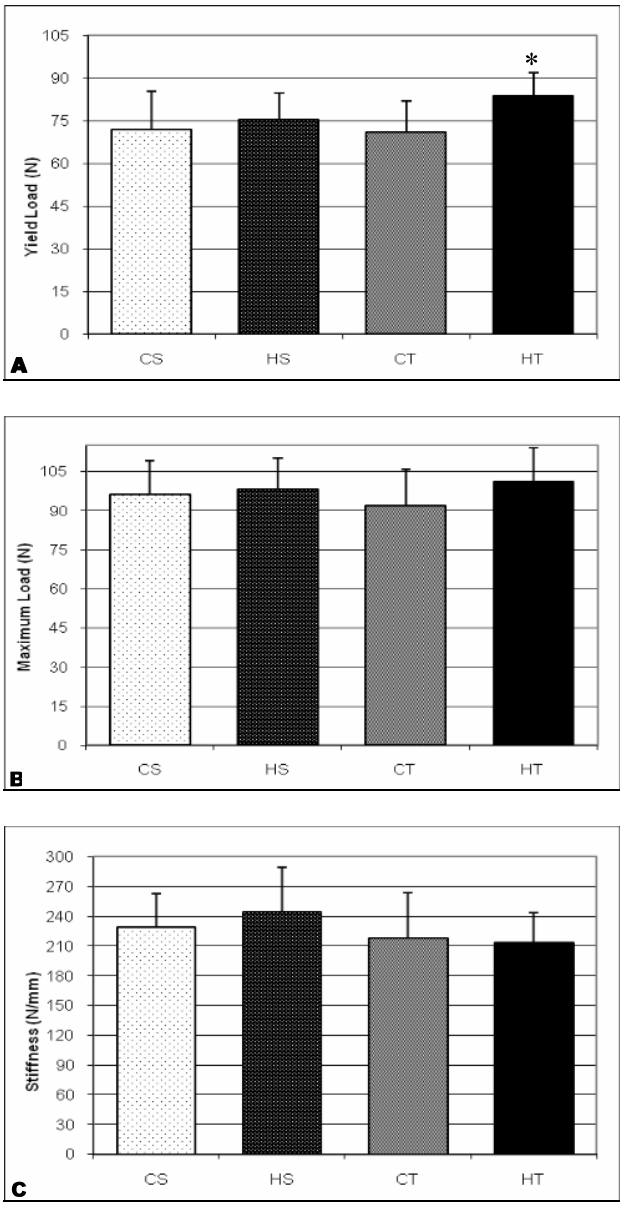

Figure 3- Structural biomechanical properties estimated by three-point bending test of tibiae from control and *H. aphrodisiaca* sedentary and trained rats. A- Yield load, B- Maximum load, C- Stiffness. CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT- control trained, HT- *H. aphrodisiaca* trained. The columns are the mean $\pm$ SD. * Differences were significant for $p<0.05$ (ANOVA) compared with CS and CT groups, by the Duncan test.

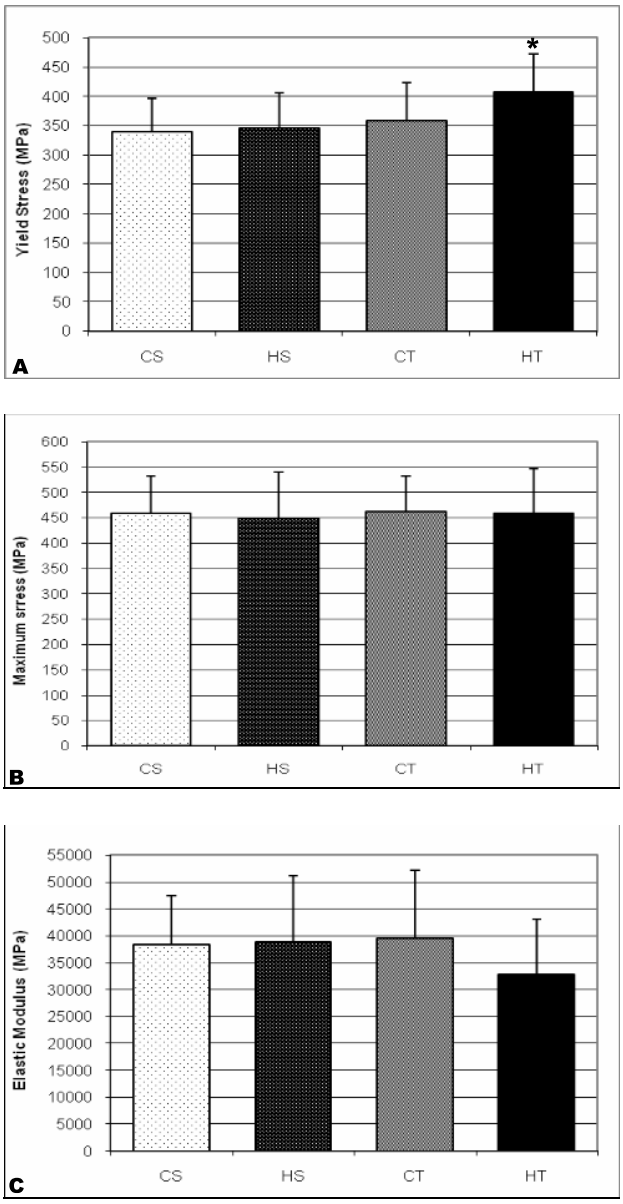

Figure 4- Material biomechanical properties estimated by three-point bending test of tibiae from control and *H. aphrodisiaca* sedentary and trained rats. A- Yield stress, B- Maximum stress, C- Elastic modulus. CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT- control trained, HT- *H. aphrodisiaca* trained. The columns are the mean $\pm$ SD. * Differences were significant for $p \leq 0.05$ (ANOVA) compared with CS group, by the Duncan test.

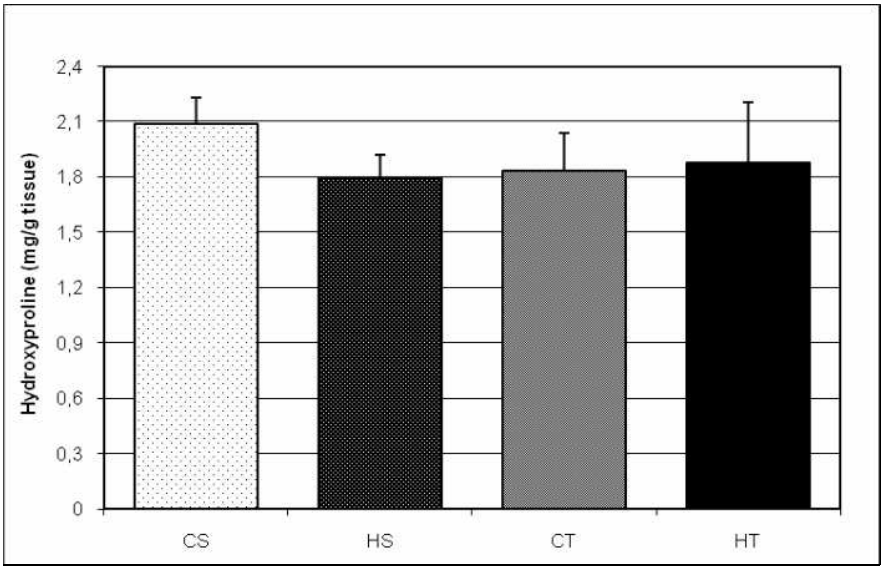

Figure 5- Hydroxyproline dosage of tibiae from control and *H. aphrodisiaca* sedentary and trained rats. CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT- control trained, HT- *H. aphrodisiaca* trained. The columns are the mean $\pm$ SD.

CONSIDERAÇÕES FINAIS

A infusão de *H. aphrodisiaca* em animais sedentários não causou alterações no sistema musculoesquelético de ratos Wistar, porém aumentou os níveis plasmáticos de testosterona e a concentração de receptores de andrógeno no músculo.

O protocolo de treinamento utilizado neste estudo não modificou os parâmetros analisados no músculo e osso dos animais controles treinados, porém reduziu a quantidade de proteínas não-colagênicas no tendão desses animais.

A associação da infusão da planta com o treinamento resultou em tendões mais resistentes para suportar as cargas geradas pelas contrações musculares repetidas. Também aumentou a densidade de capilares e a densidade volumétrica de mitocôndrias do músculo, sugerindo um aumento da capacidade de *endurance* dos animais. A observação da morfologia do osso sugere que o mesmo estava em processo de remodelamento. Possivelmente, o tempo de treinamento não foi suficiente para causar alterações significativas na biomecânica do osso analisado.

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1233-1**, sobre **"Efeito anabolizante da infusão de nó-de-cachorro (*Heteropterys aphrodisiaca*) associada ao exercício físico em ratos wistar machos: análise do sistema músculo-esquelético e coração"**, sob a responsabilidade de **Profa. Dra. Mary Anne Heidi Dolder / Juliana Castro Monteiro**, 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 28 de março de 2007.

CERTIFICATE

We certify that the protocol nº **1233-1**, entitled **"Anabolic effect of *Heteropterys aphrodisiaca* infusion associated to the physical excercise in male Wistar rats: analysis of the skeletal muscle system and heart"**, 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 March 28, 2007.

Campinas, 28 de março de 2007.

Profa. Dra. Ana Maria A. Guaraldo
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