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"EFEITOS DA INFUSÃO DE NÓ-DE-CACHORRO (*Heteropterys aphrodisiaca*, O. Mach.) SOBRE A MORFOLOGIA E ESTRUTURA TESTICULAR DE RATOS WISTAR ADULTOS, SUBMETIDOS A TREINAMENTO FÍSICO"

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Orientadora: Profa. Dra. Mary Anne Heidi Dolder

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Este trabalho e minha vida

são inteiramente dedicados à minha família...

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RESUMO

A espécie *Heteropterys aphrodisiaca* é amplamente utilizada como afrodisíaca. Estudos mostram o potencial efeito estimulante sobre o tamanho das vesículas seminais e de populações de células de Leydig em ratos, ambos andrógenos dependentes. Neste trabalho foi proposto o tratamento com a infusão de *H. aphrodisiaca* aliado ao exercício de resistência física com o intuito de observar seus efeitos nos testículos de ratos. Foram utilizados ratos Wistar (90 dias) divididos em 4 grupos (n=10, cada): controles - água, sedentário e com treinamento físico; tratados com *H. aphrodisiaca* (104mg/kg/dia) sedentário e com treinamento físico. O experimento teve duração total de 56 dias. Foi realizada dosagem de testosterona plasmática e avaliados parâmetros morfométricos e estereológicos do parênquima testicular. No epitélio seminífero, foram contados os números de células germinativas e somáticas, calculando-se o rendimento da espermatogênese, índices mitótico e meiótico e eficiência e capacidade de células de Sertoli. Foi realizado Western Blotting para quantificação da concentração de receptores de andrógeno, e TUNEL para determinação de porcentagem de células apoptóticas. A concentração de testosterona circulante foi significativamente maior nos animais tratados e sedentários. Os diâmetros médios tubulares dos animais treinados foram maiores quando comparados aos dos animais sedentários. Não ocorreram variações significativas quanto ao volume do tecido intersticial, na proporção de macrófagos, espaço linfático e vasos sanguíneos. Entretanto, ocorreram diminuições significativas na proporção de tecido conjuntivo em ambos os grupos que receberam a infusão vegetal. Houve aumentos significativos do diâmetro e volume nucleares das células de Leydig dos animais treinados. Houve diminuição significativa do número de células de Leydig por grama de parênquima testicular nos animais controle/treinados quando comparados aos outros grupos experimentais. O grupo tratado/treinado apresentou o maior índice de mitoses espermatogoniais, sendo significativamente maior que a média do grupo sedentário/tratado. As curvas de rendimento e índice mitótico possuem

mesmo padrão de distribuição, entretanto, para o índice meiótico, o grupo sedentário/tratado mostrou as maiores médias quando comparado aos dois grupos controle, sedentário e treinado. Não houve alteração de índices apoptóticos tampouco na concentração de receptores androgênicos entre os grupos experimentais. O protocolo de treinamento aplicado no presente experimento não acarretou em danos no parênquima testicular, como tem sido afirmado em outros experimentos envolvendo exercício aeróbico. Contudo, a infusão de nó-de-cachorro parece influenciar tanto no estímulo de células de Leydig, com aumento do volume celular, quanto no comportamento espermatogonial, induzindo mitoses e aumentando o rendimento geral do processo espermatogênico.

ABSTRACT

The species *Heteropterys aphrodisiaca* is traditionally used as an aphrodisiac. Some studies showed its stimulating potential of seminal vesicle and Leydig cell population growth, both androgen dependent. In this study, we proposed to observe the effects of *H. aphrodisiaca* infusion treatment combined with endurance exercise protocol. For this investigation, 40 adult Wistar rats (90 days) were used (4 groups, n = 10 each): two controls receiving water, sedentary and trained, and two treated with *H. aphrodisiaca* (104mg/kg/dia), sedentary and trained. The experiment lasted for 56 days. Plasma testosterone concentration was measured by radioimmunoassay. Morphometrical and stereological parameters were evaluated from the testicular parenchyma. The numbers of somatic and germ cells were estimated by counting the seminiferous epithelium cell population. Also, the spermatogenic yield, meiotic and mitotic index, efficiency and capacity of Sertoli cells were estimated. Western blotting was performed to quantify the concentration of androgen receptors, and TUNEL technique to determine the percentage of apoptotic cells within the seminiferous tubules. Control/trained animals showed a significant decrease in body mass gain compared to sedentary animals. The testosterone concentrations were significantly higher in treated/sedentary animals. The mean tubular diameters of trained animals were higher when compared to sedentary animals. There were no significant differences regarding the volume of interstitial tissue, the proportion and volume of macrophages, lymphatic space and blood vessels. However, the treated/sedentary group showed lower connective tissue content compared with the control/sedentary, as well as the diminished volume of Leydig cells, compared with the treated/trained group. There was a significant increase of both nuclear diameter and volume of Leydig cells in trained animals. On the other hand, there was a significant decrease of Leydig cell number per gram of testicular parenchyma in control/trained animals. Considering the spermatogenesis dynamics, both treated groups showed the highest spermatogenic yield, in relation to the control/trained group.

The treated/trained group showed the highest index of spermatogonial mitosis – significantly higher than the sedentary/treated one. Spermatogenic yield and mitotic index showed the same distribution pattern when plotted on graphs; however, the meiotic index was significantly higher only in the sedentary/treated group. The apoptotic indexes, as well as the androgen receptor concentrations were not affected by the protocols employed. Treatment with the plant infusion did not lead to an increase of the parameters analyzed. However, the infusion seems to stimulate either the Leydig cell (increase of cell volume) or the spermatogonial behavior, inducing more mitosis and increasing the spermatogenic yield.

SUMÁRIO

1. INTRODUÇÃO

Muitos preparados vegetais são empregados nas mais diversas culturas a fim de aumentar a performance social e sexual (Arletti et al., 1999). Tal fato chama atenção de diversos grupos de pesquisadores em relação à utilização de fitoterápicos para o beneficio de quem os utiliza. Tanto extratos quanto infusões vegetais contêm grande variedade de compostos fitoquímicos, como antioxidantes e citocinas, que auxiliam na atenuação de sintomas patofisiológicos relacionados principalmente a doenças causadas por radicais livres (Goel et al., 2006). Por possuírem vitaminas, flavonóides e compostos polifenóicos, os fitoterápicos atuam por diversos mecanismos essencialmente incumbidos de combater tanto os radicais livres endógenos, produzidos normalmente pelo corpo, quanto os de origem exógena (Ishige et al., 2001).

Muitos termos específicos como fitoterapia e adaptógenos tem sido utilizados nos últimos anos para descrever o uso de plantas na medicina. O termo fitoterapia relaciona-se ao estudo de extratos de origem vegetal com propriedades curativas, usados como promotores da saúde. Acredita-se que tais compostos aumentam a capacidade do corpo de responder a estímulos danosos, desencadeados por substâncias mediadoras de estresse, como os corticosteróides, catecolaminas e óxido nítrico (Panossian et al., 1999; Rege et al., 1999).

O termo adaptógeno foi primeiramente utilizado para classificar plantas e outras substâncias que aumentam a resposta não específica do corpo, protegendo-o de fatores de estresse (Brekhman & Dardymov, 1969). Adaptógenos têm sido recentemente definidos como reguladores metabólicos naturais que aumentam a habilidade do organismo de se adaptar a variações ambientais diversas e de impedir danos por parte desses fatores (Panossian et al., 1999).

Várias substâncias são utilizadas na medicina popular nas mais diferentes culturas (Sandroni, 2001). Algumas delas são identificadas farmacologicamente levando à compreensão de seu mecanismo de ação. Os árabes utilizam ambreína (*Ambra grisea*) para aumentar a libido. Esta droga aumenta a concentração de vários hormônios hipofisários e da testosterona sérica. Os chineses fazem uso do ginseng (*Panax ginseng*) para aumentar a potência sexual, segundo a medicina tradicional. O ginseng atua como antioxidante pelo aumento na síntese de óxido nítrico no endotélio de muitos órgãos, incluindo os corpos cavernosos. O aumento do desempenho físico, incluindo o sexual, atribuído ao ginseng tem sido frequentemente avaliado (Chen & Lee, 1995; Chen, 1996; Gillis, 1997; Kim et al., 1998; Nocerino et al., 2000). Entretanto, no Brasil, apesar da grande quantidade de plantas utilizadas como afrodisíacas, sabe-se muito pouco sobre sua ação e toxicologia.

A busca por novos afrodisíacos assim como do conhecimento do modo de ação daqueles já largamente utilizados na medicina tradicional é uma preocupação global e tem levado cientistas de vários países a pesquisarem sobre o assunto.

Alguns trabalhos relacionam o uso de plantas potencialmente afrodisíacas, como *Lepidium meyenii* ("maca"), ao aumento da produção espermática diária, ou mesmo aumento na população de espermatozóides viáveis no epidídimo (Gasco et al., 2007).

Os efeitos da *Eurycoma longifolia* ("tongkat ali, pasak bumi") têm sido avaliados na Malásia (Ang et al., 2000; Ang & Cheang, 2001; Ang & Ngai, 2001; Ang et al., 2001) com resultados positivos na estimulação sexual atuando, portanto como potencial afrodisíaco. Na Jordânia, El-Thaer et al. (2001) testaram em ratos, com efeito positivo, a função erétil do óleo da semente de *Ferula harmonis* ("zallouh"), enquanto no Sri Lanka Ratnasooriya & Dharmasiri (2000) analisaram o efeito das sementes de *Terminalia catappa* ("chapéu-de-sol, amendoeira-da-praia") no comportamento sexual e fertilidade de ratos adultos concluindo que, além do efeito esperado, o extrato pode ser usado também

para combater outras formas de disfunção sexual como a ejaculação precoce. Outros estudos que combinam os efeitos de várias espécies vegetais também se mostram eficientes, como no caso da *Ekebergia capensis* (“cape ash”), *Mondia whitei* (“gengibre branco”), *Aloe excelsa* (“aloe do Zimbábue”) e *Cucurbita pepo* (“abobrinha”) (Gundidza et al., 2009).

1.1. *Heteropterys aphrodisiaca* (Nó-de-cachorro)

A espécie *Heteropterys aphrodisiaca* é arbustiva, medindo de 0,6 a 2,0 metros de altura, sendo encontrada principalmente nos cerrados de Mato Grosso, Goiás e norte de Minas Gerais. Foi primeiramente descrita por Hoehne (1920) como tendo propriedades afrodisíacas e estimulantes, sendo atribuída à família Malpighiaceae por Othon X.B. Machado, em 1949 (Pio Corrêa, 1984). Historicamente a população usa as raízes desse arbusto como tônico ou estimulante e para o tratamento de debilidades do sistema nervoso (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).

Apesar de escassos, alguns estudos experimentais com esta espécie vegetal apontam melhora de memória em grupos de ratos idosos (Galvão et al., 2002), assim como promovem o aumento de massa corporal e testicular em ratos adultos jovens, tratados por 56 dias com o extrato aquoso da planta (Chieregatto, 2005). Sabe-se que o extrato de *H. aphrodisiaca* ministrado em ratos Wistar machos adultos, propiciou significativo aumento da libido, peso das vesículas seminais e volume das células de Leydig (Chieregatto, 2005). Além disso, o extrato da planta também se mostra eficaz contra efeitos deletérios da ciclosporina A (Monteiro et al., 2008). Aliado a isso, sabe-se que a administração da infusão continuada de *H. aphrodisiaca* a ratos Wistar machos adultos não causa efeitos colaterais

perceptíveis em órgãos alvo como fígado e rim quando expostos por longos períodos de tempo (Sbervelheri et al., 2009).

Embora a dosagem hormonal não tenha sido realizada nos experimentos de Chieregatto (2005), tais incrementos nos índices biométricos levam a crer que existe alguma influência hormonal sobre órgãos andrógeno-dependentes, que são sensíveis a alterações plasmáticas desses hormônios, principalmente da testosterona. Incrementos na produção e sensibilidade a androgênios favoreceriam o ganho de massa muscular, o aumento de peso corporal e de libido. Associando-se o tratamento com a infusão desta planta ao treinamento físico contínuo procura-se potencializar estes possíveis efeitos, uma vez que o exercício também aumenta a produção e secreção de testosterona.

1.2. O testículo

O testículo é um órgão com funções exócrina e endócrina, geralmente localizado no escroto e envolvido por uma espessa cápsula de tecido conjuntivo, a túnica albugínea. Funcionalmente, o testículo dos mamíferos pode ser dividido em dois compartimentos: um compartimento intertubular ou intersticial e o compartimento tubular, ou de túbulos seminíferos (Russell et al., 1990, Ross et al, 2003). Os principais componentes do compartimento intertubular são as células de Leydig, responsáveis pela produção da testosterona, os vasos sanguíneos e espaços linfáticos, nervos e uma população celular variável, constituída principalmente por fibroblastos e macrófagos, sendo os últimos associados diretamente às células de Leydig (Russell et al., 1990; Setchell, 1991, Hales, 2002).

O compartimento dos túbulos seminíferos constitui a maior parte do testículo, ocupando, na grande maioria dos mamíferos, de 70% a 90% do parênquima testicular (França & Russell, 1998; Godinho, 1999). Eles são constituídos por túnica própria, epitélio seminífero e lume tubular. A túnica própria reveste o túbulo externamente, sendo composta por células mióides ou peritubulares e matriz

extracelular. Juntamente com as células mióides, as células de Sertoli elaboram a membrana basal que serve de suporte estrutural para a própria célula de Sertoli e para as células germinativas.

As células de Sertoli, através de junções de oclusão, dividem o epitélio seminífero em dois compartimentos: o compartimento basal, onde se localizam as espermatogônias e os espermatócitos primários na fase inicial da prófase meiótica (pré-leptótenos e leptótenos), e o compartimento adluminal, onde se encontram os espermatócitos primários a partir da fase de zigóteno, espermatócitos secundários e espermátides. No lume tubular encontram-se o fluido secretado pelas células de Sertoli e os espermatozóides recém espermiados (Ross et al., 2003).

1.3. A célula de Leydig

As células de Leydig foram observadas pela primeira vez pelo pesquisador Franz Leydig, em 1850, em um estudo comparativo da histologia reprodutiva de algumas espécies de mamíferos. Entretanto, somente com o avanço das técnicas de microscopia no final do século XIX tornou-se possível observar com mais precisão e detalhes tais células (Christensen, 1996). Elas apresentam formato poligonal, com diâmetro de cerca de 20μm; seu núcleo é esférico, contendo grande parte da cromatina em seu estado condensado e um ou dois nucléolos (Pelliniemi et al., 1996).

As células de Leydig possuem caráter eosinofílico, devido principalmente à grande quantidade de retículo endoplasmático liso e mitocôndrias em seu citoplasma (Ross et al., 2003). Em ratos, essas células estão intimamente associadas com os vasos sanguíneos presentes no espaço intertubular e cercadas por espaço linfático (Fawcett et al., 1973).

A célula de Leydig é bastante conhecida devido à sua principal função: a produção de andrógenos, principalmente a testosterona e a diidrotestosterona, que têm grande importância na diferenciação do trato genital masculino e da genitália externa na fase fetal (Pelliniemi et al., 1996).

Além disso, esses dois hormônios também estão relacionados como surgimento dos caracteres sexuais secundários e a manutenção do processo espermatogênico normal (Sharpe, 1994; Ross et al., 2003).

No início do desenvolvimento, a célula de Sertoli, responsável pela manutenção do processo espermatogênico no epitélio seminífero, regula a proliferação das células de Leydig, assim como a produção de hormônios esteróides por estas células. Já em animais adultos é o LH (Hormônio Luteinizante) o principal regulador e estimulador da síntese de testosterona (Cooke, 1996; Sylvester, 1996). Além da célula de Sertoli, outro tipo celular comumente encontrado em associação com as células de Leydig é o macrófago. Em testículos de animais adultos, os macrófagos intersticiais podem ser encontrados junto aos agrupamentos de células de Leydig. Esta interação pode estar relacionada à transformação, por parte do macrófago, de alguns esteróides produzidos pela célula de Leydig, com a consequente produção e liberação de testosterona e diidrotestosterona (Hales, 2002).

1.4. Esteróides anabólico-androgênicos

Os hormônios esteróides são produzidos pelas gônadas (ovário e testículo) e pelo córtex da supra-renal. Os esteróides anabolizantes, ou esteróides anabólico-androgênicos (EAA), são os hormônios sexuais masculinos relacionados aos caracteres sexuais. Após a puberdade, a quantidade crescente de secreção de testosterona faz o pênis, o escroto e os testículos aumentarem de tamanho. Além disso, este hormônio determina o desenvolvimento das características sexuais secundárias que distinguem o homem da mulher, como: distribuição dos pêlos pelo corpo, calvície, tom de voz, espessura da pele, desenvolvimento muscular, crescimento e retenção de cálcio nos ossos (Gebara et al., 2002; Guyton & Hall, 2002; Dohle et al., 2003).

As células responsáveis pela produção de testosterona no testículo são as células de Leydig. A síntese deste hormônio inicia-se com o colesterol que, após sucessivas oxidações é transformado em

pregnenolona. Durante a conversão da pregnenolona em testosterona, ocorre formação de dehidroepiandrosterona (DHEA) e de androstenediona. Estes hormônios são precursores da testosterona, sendo mais freqüentemente utilizados por atletas que desejam aumento de desempenho físico associado a desenvolvimento muscular acelerado. Tanto a DHEA quanto a androstenediona parecem exercer atividade androgênica fraca, sendo atribuída à sua transformação metabólica em testosterona e 5-alfa-dihidrosterona (DHT). Nos órgãos sexuais masculinos, cérebro e tecido adiposo, a testosterona é convertida em DHT pela enzima 5-alfa-redutase. A DHT é o principal metabólito ativo da testosterona e possui maior afinidade pelo receptor androgênico que a testosterona. Ele se transforma mais rapidamente no complexo hormônio-receptor e dissocia-se mais lentamente do que a testosterona (Kam & Yarrow, 2005).

A secreção de testosterona é regulada pelo hormônio luteinizante (LH), produzido pela hipófise anterior. A produção de LH é controlada por outro hormônio, o GnRH - hormônio liberador de gonadotrofinas - que é secretado pelo hipotálamo 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 esteróides anabolizantes ligam-se a receptores citoplasmáticos protéicos, formando o 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 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).

1.5. Exercícios de resistência física e seu efeito sobre o sistema reprodutor

O exercício de resistência física compreende várias respostas fisiológicas e adaptações crônicas que são críticas para o aumento de resistência muscular, força, hipertrofia e tolerância à atividade física. Vários experimentos vêm correlacionando os efeitos de tal modalidade de exercício sobre a produção e liberação de LH e testosterona (Häkkinen et al., 1988; Fry et al., 1998; Nindl et al, 2001; Tremblay et al., 2004). Sabe-se que a concentração plasmática de testosterona aumenta de 10% a 37% durante sessões de treinamento de "endurance" (resistência) ou de força. Este aumento se deve à redução do volume plasmático e a estimulações adrenérgicas ou a prováveis adaptações na síntese e/ou na capacidade secretória das células de Leydig. Estas alterações são baseadas no aumento da produção ou liberação de LH (Longcope et al., 1990, Kraemer & Ratamess, 2005) e no efeito estimulatório do lactato na secreção de testosterona (Lin et al., 2001). Na perspectiva de um atleta, tais aumentos podem atuar melhorando seu desempenho físico, uma vez que a testosterona age aumentando a quantidade de massa muscular e, consequentemente de força física, e diminuindo o acúmulo de gordura (Tremblay et al., 2004).

No entanto, outros autores concluíram que pode ocorrer diminuição da taxa de inativação ou de remoção da testosterona (Powers & Howley, 2000), assim como a diminuição da concentração deste hormônio após a prática de exercícios físicos (Fry et al., 1998; Hackney 2001; Nindl et al., 2001), levando à disfunção do sistema reprodutor masculino (Arce & De Souza 1993; Hackney 1996). Mudanças na dinâmica do parênquima testicular se devem à diminuição dos níveis de prolactina em repouso (De Leo et al., 2000).

Outro fator relevante observado durante estudos com animais ou humanos é que a prática de exercício extremo e intenso durante longos períodos pode levar a excessiva produção de espécies reativas de oxigênio (ERO's) (Temiz et al. 2000, Di Meo & Venditti 2001). Estas são sabidamente

causadoras de danos a macromoléculas incluindo ácidos graxos de cadeia poli-insaturada (PUFA), amplamente presentes em células da linhagem germinativa, causando danos às suas funções celulares (Lin et al. 1999, Radak et al., 2000; Ghosh et al., 2002), o que torna o testículo altamente susceptível ao estresse oxidativo.

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3. OBJETIVOS

3.1- Gerais

Observar os efeitos da administração conjunta da infusão de *Heteropterys aphrodisiaca* sobre o parênquima testicular de ratos Wistar machos adultos submetidos, ou não, ao exercício de resistência física (*endurance*) em esteira ergométrica, por um período de 56 dias.

3.2-Específicos

Avaliar os efeitos da infusão de *Heteropterys aphrodisiaca* sobre os compartimentos tubular e intersticial nos testículos de ratos sedentários ou treinados, através de análises morfométricas, estereológicas e imunohistoquímicas (técnica de TUNEL);

Investigar possíveis alterações das proporções dos componentes intersticiais, bem como alterações ultra-estruturais em células de Leydig devido ao tratamento com a infusão e/ou exercício de endurance;

Verificar possíveis alterações na concentração de receptores androgênicos nos testículos pela técnica de Western Blotting.

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 2 artigos e 1 revisão, sendo 1 submetido e 2 em fase final de elaboração, conforme descrito abaixo:

4.1- Revisão – Phytotherapy and spermatogenesis.

4.2 – Artigo 1 - Association of the infusion of *Heteropterys aphrodisiaca* and endurance training brings spermatogenetic advantages (Submetido à Biological Research).

4.3 – Artigo 2 - Association of *Heteropterys aphrodisiaca* with endurance exercise protected Leydig cell population in Wistar rats?

REVISÃO

Phytotherapy and spermatogenesis

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Summary

Plants possess a variety of phytochemicals such as antioxidants and cytokines that help to alleviate the pathophysiological symptoms of a number of free radical mediated diseases and overcoming environmental adversities of both physical and biotic origin. Phytotherapy means the study of the use of natural extracts as medicines or health-promoting agents. It is believed that some phytochemicals increase the capacity of the body to respond to stressful stimuli acting in response to stress mediators. Also, plants are commonly used as aphrodisiacs, increasing libido, potency and sexual desire.

Keywords*: adaptogen, phytotherapy, reproduction, testicle, antioxidant.*

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Introduction

Many plant extracts are traditionally employed among different cultures to improve social or sexual performance (Arletti et al., 1999). These reasons have turned the attention of researchers towards the plant kingdom. Plants possess a variety of phytochemicals such as antioxidants and cytokines that help to alleviate of the pathophysiological symptoms of a number of free radical mediated diseases and overcoming environmental adversities of both physical and biotic nature (Goel et al., 2006). Herbal products containing vitamins, flavonoids and polyphenolic compounds are beneficial through several mechanisms including scavenging of free radicals of both endogenous and exogenous origin (Ishige et al., 2001).

Phytotherapy is the study of natural extracts as medicines or health-promoting agents. They are believed to increase the body's capacity to respond to stressful stimuli acting in response to stress mediators such as corticosteroids, catecholamines, and nitric oxide (Panossian et al., 1999; Rege et al., 1999).

The term adaptogen was first used to classify plants and other substances that increase non-specific resistance of the body, protecting it from stressful factors (Brekhman & Dardymov, 1969). Thus adaptogens are natural metabolic regulators that increase the ability of the organism to adapt to environmental factors and to avoid damage from such factors (Panossian et al., 1999).

Several substances have been used in traditional medicine in different cultures (Sandroni, 2001). Some of them were pharmacologically identified, leading to an understanding their action mechanisms. The Arabs use ambrein (*Ambra grisea*) to increase libido; this species increases the concentration of several pituitary hormones and testosterone levels. The Chinese usually use ginseng (*Panax ginseng*) to increase sexual potency, according to traditional medicine. Improvement of sexual performance attributed to ginseng has been extensively evaluated. The ginseng extract acts as an antioxidant,

increasing the synthesis of nitric oxide in the endothelium of many organs, including the *corpus cavernosum* (Chen & Lee, 1995; Chen, 1996; Gillis, 1997; Kim et al., 1998; Nocerino et al., 2000). The search for new aphrodisiac products, as well as for the mode of action of those already used in traditional medicine, is a global concern and has led scientists from several countries to do research on the subject (Table 1).

This review proposed to focus on some recent achievements in the use of plant extracts in animal research, in regard to modifications within the spermatogenic process and sexual behavior or to protection against substances hazardous to spermatogenesis.

Fertility

The use of plant extracts as alternative treatments for infertile men has recently been emphasized by several authors who affirm that infertile men are more likely than fertile ones to have depressed total antioxidant capacity and lower levels of individual antioxidants (Comhaire & Mahmoud, 2003). Therefore, improved fertility observed in the studies discussed below might be due to the antioxidant effect of the most plant extracts studied so far.

The potency and fecundity of the male rats treated with *Satureja khuzestanica* essential oil (150 and 225 mg/kg) were significantly higher, as well as the fertility index and litter size, since the post implantation loss was significantly lower in mated females of the treated groups (Abdollahi et al., 2003; Haeri et al, 2006). These findings are partially explained by the higher germ cell number, also observed after *Butea superba* administration in adult Wistar rats (Manosroi et al., 2006). Other plants species as *Eurycoma longifolia* have been evaluated in Malaysia (Ang et al., 2000, Ang & Cheang, 2001; Ang & Ngai, 2001, Ang et al., 2001) with positive results in stimulating sexual act, classifying this herb as a potential aphrodisiac. In Sri Lanka, Ratnasooriya & Dharmasiri (2000) analyzed the

aphrodisiac potential of *Terminalia catappa* seeds on sexual behavior and fertility, concluding that the extract could also be used in some sexual disfunction treatments and in premature ejaculation.

Usually, the increased number of germ cells, and of Leydig cells are not related to any toxicological alterations (Abdollahi et al., 2003, Saadat et al., 2004; Manosroi et al., 2006), which indicates the use of the plant material as a stimulant. Several studies in lower mammals indicated that quite a few plant extracts could improve fertility in different mammal species after treatments (Obregon, 1998; Cicero et al., 2001; Muhammed et al., 2002), which could be the first step in the development of new medicines.

Relaxation of the corpus cavernosum

On sexual stimulation the axon terminals of parasympathetic nerves release nitric oxide (NO). The NO diffuses into smooth muscle cells that line the arteries of the *corpus cavernosum* (spongy erectile tissue) and activates the enzyme guanylate cyclase (GC). The latter converts the nucleotide guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). The cGMP in turn causes the smooth muscle cells around the penis to relax, leading to dilation and increased influx of blood into the penile tissue. This blood is essentially trapped in the penis and results in an erection (Palmer, 1999). The erection ceases after a while because cGMP is hydrolysed by phosphodiesterase type 5 enzyme, (PDE5) into inactive GMP (Drewes et al., 2003).

Erectile disfunction is a serious clinical problem in adult men. The failure of penile erection could be due to impaired relaxation of the smooth muscle related to the increase in blood flow into the spaces of the *corpus cavernosum* and *corpus spongiosum* (Hnatyszyn et al., 2003). In 1998, the drug Viagra (Sildenafil citrate, Pfizer) was launched as the key to the end of impotence, increasing the

welfare mainly of elder men. Viagra is able to inhibit the hydrolyzing action of the PDE5 with the result that active cGMP can accumulate and prolong the erection by increased blood flow.

Recent studies have shown that different plant extracts could have a relaxant effect on the smooth muscle in rabbits (Antunes et al., 2001), Guinea pigs (Hnatyszyn et al., 2003) and rats penises (Hadidi et al., 2003), due to the possible involvement of flavonoids, coumarins, terpenes, and other related substances, which were reported to be relaxant (Chiou et al. 1998).

From a different point of view, while the synthetic drugs affect the sexual performance or potency by increasing copulatory acts, the extract of *Ferula harmonis* sustained sexual motivation with a higher mount rate (El Thaer et al., 2001; Hadidi et al., 2003). According to Antunes et al. (2001) a well known Brazilian commercial phytotherapic - Catuama® - brings about short lived, dose dependent relaxations on rabbit *corpus cavernosum.* Catuama is a mix of 4 plant extracts: catuaba (*Trichilia catigua*), muirapuama (*Ptychopetalum olacoides*), ginger (*Zingiber officinale*) and guaraná (*Paullinia cupana*), the latter being the most effective in causing relaxation of smooth muscle, when tested separately.

Protection against radiotherapy

The testis, an important organ of the male reproductive system, is appreciably radiosensitive because of the presence of rapidly proliferating stem cells, the spermatogonia (Oakberg, 1957). The adverse events of radiotherapy result in several abnormalities in spermatogenesis, which can cause infertility, low levels of sperm count, in frequency of abnormal spermatozoa and defective sperm functions (Shen & Ong, 2000).

Many of the plants used in the Indian and Chinese medicine have been reported to exhibit radioprotective ability (Shimoi et al., 1996; Goel et al., 1998, 2002; Uma Devi et al., 1999). The

commonly known Sea buck-thorn (*Hippophae rhamnoides*) is one of the most impressive herbal preparations that protect against radiation side effects. The fruits are reported to be an excellent vitamins source, especially of vitamin C (100–300 mg 100^{-1} g) (Goel et al., 2002). Also, the fruit oil is rich in vitamin E, K and carotenoids, flavonoids and tannins (Ianev et al., 1995; Spiridonov et al., 1997). In both lower (5 Gy ^{60}Co) and higher (10 Gy ^{60}Co) irradiation levels, the prior injection of the plant extract (30mg/kg of body weight) protected the testis and spermatogenesis (Goel et al, 2006). Despite the fact that the treatment alone did not alter the sperm count, the number of abnormal spermatozoa decreased with the intake of the plant extract, whereas the diameter of the seminiferous tubules increased. It is important to note that all animals exposed to 10 Gy whole body supralethal dose without prior-administration of radioprotector (RH-3), died within 12 days (Goel et al, 2006).

Although many fruits and vegetables represent a vast source of phytochemicals of varied chemical structure the data are still scarce and none of them have been studied extensively for their potential anticancer or chemopreventive efficacy (Ramos, 2008).

Protection against chemicals

The pesticides are one of the most potentially harmful chemicals liberated in the environment in an unplanned manner. Malathion is a pesticide widely used by farmers in many parts of the world and has been shown to produce some adverse effects, most of them to the male reproductive system, such as reduction of testes, epididymis, seminal vesicle and ventral prostate weight, as well as a decrease in fertility rate (Choudhary et al., 2008). However, the testicular damage caused by even a single dose of malathion can be reduced with intake of *Lepidium meyenii*, commonly known as “maca”, which also increase the lengths of spermatogenesis stages VII and VIII (Bustos-Obregón et al., 2005).

Cyclosporine A (CsA) has been used in medical treatment as an immunosuppressant after organ transplants and to treat several autoimmune disorders such as psoriasis and rheumatoid arthritis (Hagar, 2004). However, even in low doses CsA has certain adverse side effects, including degenerative changes of seminiferous tubules and impaired spermatogenesis leading to infertility (Iwasaki et al., 1995). The treatment combining CsA and a Brazilian herb, *Heteropterys aphrodisiaca*, has a potential protective effect on these damages (Monteiro et al., 2008). The testes of the animals treated daily with the aqueous infusion of this plant (56 days) have shown minor alterations under electron microscopic analysis, proving the efficiency of this species in protecting the testicular parenchyma.

Conclusion

Currently, there is a growing scientific interest in research on plants used in folk medicine, resulting in several studies of their purified active principle (Pitman, 1996, Srivastava et al., 2005). The use of these plants in therapy is driven by the promise of producing of more affordable medicines and the wide acceptance of natural products by the average person.

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Table 1. Species commonly known as aphrodisiac/tonic.

Species and Family	Native country	Part used	Traditional uses
Satureja parvifolia (Asteraceae)	Argentina	Leaves (D)	Relaxation of corpus cavernosum (1)
Haplopappus rigidus (Asteraceae)	Argentina	Aerial parts (A)	Relaxation of corpus cavernosum (1)
Senecio eriophyton (Asteraceae)	Argentina	Aerial parts (A)	Relaxation of corpus cavernosum (1)
Ferula harmonis (Umbelliferae)	Syria and Lebanon	Roots and seeds (M)	Increase the libido (2)
Heteropterys aphrodisiaca (Meliaceae)	Brazil	Roots (A)	Antioxidant, anti-rheumatic and aphrodisiac (3)
Satureja khuzestanica (Asteraceae)	Iran	Aerial parts (A)	Antioxidant, anti-diabetic, fertility increase (4, 5)
Butea superba (Leguminosae)	Thailand	Roots (A)	Rejuvenation and impotence, fertility increase (6, 7)
Eriosema kraussianum (Fabaceae)	South Africa	Roots	Erectile dysfunction, urinary disorders, expectorant (8, 16, 17)
Berberis aristata (Berberidaceae)	Himalaya and South India	Leaves, fruits and roots	Erectile dysfunction (9)
Berberis vulgaris (Berberidaceae)	Europe and Africa	Leaves, fruits and roots	Erectile dysfunction (9)
Coleus forskohlii (Lamiaceae)	India	Roots	Erectile dysfunction, treatment of congestive cardiomyopathy, glaucoma and asthma (10)
Triubulus terrestris (Zygophyllaceae)	Western Europe	Whole plant	Improve sexual desire and enhance erection (11)
Lepidium meyenii (Solanaceae)	Andean Mountains	Roots (A)	Enhancing fertility and sexual behavior (12,13, 15)
Catuama*	Brazil	-	analgesic, antibacterial, cardiotonic,purgative and vaso-dilatory and aphrodisiac (14)

A = aqueous extract; M = methanol extract; D = Dichloromethane extract.

(1): Hnatyszyn et al., 2003; (2): Hadidi et al., 2003, (3): Monteiro et al., 2008; (4) Haeri et al, 2006; (5): Abdollahi et al., 2003, (6): Soonthorn, 1931; (7): Manosroi et al, 2006. (8): Drewes et al., 2002; (9): Chiou et al., 1998; (10): Mulhal et al, 1997; (11): Adaiakan et al., 2000; (12): Cicero et al., 2001; (13): Muhammad et al., 2002; (14): Antunes et al., 2001; (15): Gonzales et al., 2001; (16): Hutchings et al., 1996; (17): Watt and Breyer-Brandwijk,1962.

* Brazilian natural medicine: *Paullina cupana, Trichilia catigua, Zingiber officinalis* and *Ptychopetalum olacoides* combined extracts.

ARTIGO 1

Association of the infusion of *Heteropterys aphrodisiaca* and endurance training brings spermatogenic advantages

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Abstract

The species *Heteropterys aphrodisiaca* is commonly used as a stimulant by popular medicine in the Cerrado, a savanna-like biome, Brazil. Recent studies have proved its protective effects on testes of animals submitted to treatment using Cyclosporin A, as well as its stimulus to increase testosterone secretion. Therefore, the present study was designed to analyze whether the association of the plant infusion and endurance exercise could potentiate the stimulating effect. The animals were separated into 4 groups: two control (sedentary and trained) receiving water and two treated (sedentary and trained) receiving the plant infusion daily (104mg/day). The proportion of the seminiferous tubule compartment and interstitium was analyzed. Within the seminiferous epithelium, the number of Sertoli and germ cells were counted in order to evaluate whether the treatment would alter the spermatogenic dynamics, analyzing: the spermatogenic yield, the mitotic and meiotic indexes, the total number of germ cells and the Sertoli cell support capacity. Trained and treated animals showed increased spermatogenic yield and spermatogonia mitosis, and no significant differences in apoptotic indexes. Despite the results showing the same pattern regarding yield and mitotic index, the meiotic index was higher in the sedentary/treated group. Therefore, the *H. aphrodisiaca* infusion increased both the testosterone production and the spermatogonia mitosis, thus increasing the spermatogenic yield.

Key Words: germ cells, phytotherapy, seminiferous epithelium, spermatogenesis, testosterone.

Introduction

Nowadays, there is growing scientific interest in research on plants used in folk medicine, resulting in several studies of their active components (Pitman, 1996, Srivastava et al., 2005). The use of these plants in therapy is driven by the potential production of more affordable drugs and because of the wide popular acceptance of natural products, especially in countries with lower economic resources, such as Brazil (Corrêa et al., 2000). Despite the potential richness of medicinal flora, scientific studies and journals that address this theme are still scarce. In general, Brazil has not made satisfactory use of its biodiversity and the popular knowledge for the development of herbal agents and compounds (Calixto, 2005).

The species, *Heteropterys aphrodisiaca,* is a shrub that belongs to the Malpighiaceae Family, found mainly in the Cerrado region, a savanna-like biome, in the states of Mato Grosso, Goiás and northern Minas Gerais (Pio Corrêa, 1984). Popular use of the roots of this plant has been a tonic or stimulant and to treat nervous system weaknesses (Pio Corrêa, 1984; Pott and Pott, 1994; Guarim Neto, 1996). On the other hand, *H. aphrodisiaca* is one of the most famous aphrodisiacs in the Middle-West Brazil, popularly known as nó-de-cachorro, raiz de Santo Antônio and cordão de São Francisco (Pott and Pott, 1994; Guarim Neto, 1996).

Endurance exercise elicits several physiological responses and chronic adaptations that are critical for increasing muscular strength, hypertrophy and tolerance to physical activity. Several studies correlate the effects of exercise to the production and release of LH and testosterone (Häkkinen et al., 1988; Fry et al., 1998; Nindl et al, 2001; Tremblay et al., 2004). From the perspective of an athlete, the increased hormonal levels may act by improving performance and results, since testosterone stimulates

the development of muscle and strength, as well as decreasing fat tissue accumulation (Tremblay et al., 2004).

Based on previous studies, which inferred that *H. aphrodisiaca* could increase male libido and act as an aphrodisiac species (Chieregatto, 2005; Monteiro et al., 2008), the present study was designed to assess the possible stimulating effects of *H. aphrodisiaca* infusion on the seminiferous epithelium mainly by inducing germ cell division, in the testes of adult Wistar rats, maintained sedentary or trained on a treadmill.

Material and Methods

Herb harvesting and experimental groups

The *H. aphrodisiaca* samples were harvested in Nova Xavantina (Mato Grosso) and identified by comparison with samples kept in the Herbarium of the Federal University of Mato Grosso, under the registration number 23,928.

The roots were dried at room temperature, protected from direct incidence of sunlight, and then fragmented. Infusions were made of fragments (25g) put into 100mL of distilled water at boiling point. The infusion remained for four hours to cool down, then was filtered and stored at 4^{o}C for up to four days, as proposed by Chieregatto (2005). The rats were weighed every week and the variation was considered in order to calculate the concentration of the infusion to be prepared.

The Central Animal House (Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório - CEMIB) in the State University of Campinas (Unicamp) provided the male Wistar rats (*Rattus norvegicus albinus*) (90 days old) used in this study. The animals were divided into 4 groups (n = 10): a control sedentary (*CtlSed*) and a control trained group (distilled

water) (*CtlTra*), a sedentary treated with *H. aphrodisiaca* (104mg/day) (*HaSed*) and a group that was trained and treated (*HaTra*). Food (commercial diet) and water were provided *ad libitum*. The plant infusion was given daily by gavage (0.5mL/animal). The same procedure was repeated with the control animals, which only received distilled water. The treatment lasted eight weeks.

The animals were kept in the Animal Facility of the Department of Cell Biology and handled in accordance with the rules of the Ethics Committee of the Institute of Biology, UNICAMP. The project had been previously submitted to the same Committee (protocol number: 734-1) and it acceptance is registered under the number 1234-1.

Training protocol

The trained animals were submitted to a training protocol that consisted of running on a treadmill built specially for small animals, with 7 individual lanes and manual control of speed, five days a week for 8 weeks, based on previously set protocols (Moraska et al., 2000; Smolka et al., 2000; Demirel et al., 2001). First, all animals were subjected to an adaptation period (pre-training), until they reached the optimal degree of effort for the initial training phase (Table I).

Biological samples

Forty-eight hours after the end of the training protocol (56 days), animals were weighed and anesthesia was injected in the left hind leg with a mixture of Xylazine and Ketamine, 5 and 80 mg/kg, respectively. The blood was collected by cardiac puncture of the left ventricle and centrifuged at 10000 rpm (4^{o}C) for 5 minutes. The testosterone assay was performed by chemiluminescence.

The animals were perfused with saline solution (0.9%) and fixed with Karnovsky's fixative (4% paraformaldehyde and 4% glutaraldehyde in 0.1 mol/L phosphate buffer at pH 7.2) for 20 minutes

each. After perfusion, the testes were removed, weighed and put into new Karnovsky's fixative, at the same concentration, where they remained for 24h.

After fixation, the fragments were dehydrated in ethyl alcohol for embedding in glycol methacrylate. The blocks were cut with a manual microtome (4µm) and the sections stained with toluidine blue/sodium borate, 1%.

Testicular morphometry

The gonadosomatic index (GSI) was calculated dividing the gonadal weight (GW) by the body weight (BW): GW/BWx100. The volume of the testicular parenchyma was obtained by subtracting the tunica albuginea volume from the testis volume. According to Paula et al. (2002), as the density of the testicle is approximately 1 (1.03 - 1.04), the testicular mass was considered equal to its volume.

The volumes (mL) of the testicular parenchyma components (seminiferous tubules and interstitium) were estimated from the proportion (%) occupied by them within the testis. A total of 15 digital images (400x), per animal, were used in order to calculate the proportion between both compartments. The images were analyzed using the software Image Pro Plus (v. 6.0). A grid containing 200 intersections was placed on the images and the points on the tubular and interstitial compartments were counted.

The mean seminiferous tubule diameter was obtained by randomly measuring 30 tubular cross sections, as circular as possible. Since the tubular diameter remains constant in adult male rats throughout the seminiferous cycle, it was unnecessary to consider the stage of the epithelium within the cycle (França and Russell, 1998). These sections were also used to measure the seminiferous epithelium height, which was taken from the basal membrane to the tubular lumen. The epithelium height for each tubule was the average of four diametrically opposed measurements. The total length

(TL) of the seminiferous tubules, per testicle, was estimated from previous knowledge of the volume occupied by these structures within the parenchyma, as well as from the mean tubular diameter: STV/πr2 (STV = seminiferous tubule volume; πr2 = tubule cross section area; r = diameter/2).

Germ cell line counting

The estimated population of different cell types that make up the seminiferous epithelium in Stage 1 was based on counts of the nuclei of germ cells and nucleoli of Sertoli cells (Swierstra, 1968, Curtis and Amann, 1981, Amann and Schanbacher, 1984). The following populations were quantified in 10 seminiferous tubule cross sections: A-type spermatogonia (SPTGA), primary spermatocytes at pre-leptotene/leptotene (SPT Pl/L) and pachytene (SPT P), round spermatids (RSPD) and Sertoli cells (S). Cell populations were corrected numerically considering section thickness and nuclear or nucleolar diameter, the latter in the case of Sertoli cells, as done by Amann and Almquist (1962). The average nuclear diameter is the average of 30 nuclei diameters of each cell type studied, for each animal.

The following were determined from these populations: efficiency coefficients of spermatogonial mitosis (SPT Pl/L/SPTGA), spermatogenesis yield (RSPD/SPTGA), meiotic index (RSPD/SPT P) and Sertoli cell supporting index by the total of spermatogenic cells ((SPTGA + SPTC Pl/L + SPTC P + RSPD)/S).

TUNEL assay

In order to detect apoptosis, the TUNEL technique (Terminal deoxynucleotidyl transferase dUTP nick end labeling) was performed on paraformaldehyde fixed sections (5µm). TUNEL assay was made according to the Calbiochem kit protocol (#QIA33). In brief, tissue sections

were deparaffined and hydrated in ethyl alcohol, incubated with proteinase K 1% (20 minutes at room temperature), washed in distilled water and incubated with 3% hydrogen peroxide in methanol for 5 minutes, to quench endogenous peroxidase activity. Slides were then incubated with Tdt Equilibrium Buffer in distilled water in a humid chamber at room temperature for 20 minutes and subsequently with Tdt enzyme in Tdt mix for an hour (37^{o}C). Immunoreactive cells were detected by incubating the sections with a mixture of 3,3-diaminobenzidine tetrachloride (DAB), for 13 minutes in a humid dark chamber. Sections were counterstained with Harris' hematoxylin, dehydrated in ethanol, cleared in xylene and mounted. Apoptotic nuclei were stained in brown. Extra slides of the same material were used as negative and positive controls. No color reaction was observed when TdT enzyme was omitted from the procedure. Positive control slides were incubated with DNAse (1,500U/µl) in tris-buffered saline (TBS) containing $MgCl_2$ (10mM) and bovine serum albumin (1mg/mL) for 10 minutes, prior to the endogenous peroxidase blocking process (Figure 1A-C). The same TUNEL staining steps described above were taken. Four slides, one per group, were stained at each time, avoiding discrepancies when comparing results.

Apoptotic index

Two hundred and fifty round seminiferous tubules cross sections from five animals per experimental group were evaluated for the appearance of apoptotic nuclei, at 400x magnification. Counting was based on previous studies previous studies of Turner et al. (1997), Kimura et al. (2003), Li et al.(2009). The mean number of apoptotic cells per tubule cross section was recorded, as well as the maximum number of positive cells per cross section. The proportion of apoptotic cells was also calculated considering only positive (+) tubules. In order to determine apoptotic rates, the number of

each type of TUNEL-positive germ cell was divided by the total number of the corresponding type of germ cell within the seminiferous sections.

Statistic analysis

Analysis of variance (one-way ANOVA) plus Duncan's test was used to compare differences between groups. Values of $p<0.05$ indicate significant differences. Two-way ANOVA was used to determine whether either the *H. aphrodisiaca* infusion or training protocol influenced the patterns analyzed, and whether there was interaction between the infusion and exercise. The software Statistica (v. 8.0, Tulsa, OK, USA) was used for all statistical analysis. All values were expressed as mean ± SEM.

Results

Hormonal assay

The sedentary animals that received the plant infusion showed significant increase in total testosterone concentration (Fig. 2). The trained animals did not show any alterations in this parameter compared with the control sedentary animals. There was no interaction between training on a treadmill and the infusion of the plant in the final outcome of total testosterone (two-way ANOVA).

Testicular morphometry

There were no significant alterations in the testicular and body weight throughout the study, as well as the GSI. The proportions between seminiferous tubules and interstitium remained constant among the experimental groups (Table II).

On average, both groups submitted to the exercise protocol showed significantly higher tubular diameters compared to the sedentary ones. On the other hand, the sedentary animals showed longer seminiferous tubules, so that, despite the differences, the final volume remained constant among all treatments (Table III). The epithelium height was not significantly altered.

Germ and Sertoli cells

The number of spermatogonia decreased in animals treated and trained compared with the control trained ones (Table IV). There was also a decrease in the number of pre-leptotene and pachytene cells in animals treated with *H. aphrodisiaca,* both sedentary and trained. However, the number of round spermatids remained constant (Table IV).

The spermatogenic yield, the meiotic index and the spermatogonial mitosis were calculated using the average values of the germ cell populations. Animals that received the infusion and were submitted to the exercise protocol had the highest spermatogenesis yield average and spermatogonial mitosis rate, compared with the control trained group and with both sedentary groups. The mitotic index and spermatogenesis yield showed the same tendency among the groups, however, for the meiotic index, the sedentary group receiving the infusion showed the highest average when compared to the two control groups, sedentary and trained (Fig. 3).

Apoptotic assay

All apoptotic results are listed on Table V. Sedentary rats that received plant treatment showed the lowest number of apoptotic cells per tubule and per positive tubule cross sections ($p<0.05$). Also, the proportion of tubules showing at least one apoptotic germ cell was significantly lower in this group.

None of the germ cells populations showed any significant alterations. Besides, apoptosis was noted mainly in spermatogonia and primary spermatocytes, and occasionally in round spermatids.

Discussion

The presented data showed that both biometric data and spermatogenesis were not affected by the proposed endurance protocol. However, *H. aphrodisiaca* infusion seems to play an important role improving testosterone secretion as well as cell division, increasing spermatogenesis yield and the meiotic index.

Morphometry techniques have frequently been used to help in comparisons between experimental groups, thus adding more reliability to the final diagnosis. The present study was based on the principles of morphometry and stereology, in order to describe possible alterations within the testicular functions and structure of Wistar rats under exercise and/or treatment with *Heteropterys aphrodisiaca* infusion.

According to the protocol suggested for the present study there were no differences in the body and testicular weights after treatments, thus the gonadosomatic index did not show significant variations among groups. However, Chieregatto (2005) and Monteiro et al. (2008) working with the same animal strain and infusion dose found a greater weight gain in those rats treated with *H. aphrodisiaca* infusion, which was correlated with the increased testosterone secretion in those animals. Although the sedentary, treated animals showed an increase in testosterone concentrations, there were no alterations in the reproductive organs' weights.

The seminiferous tubule compartment occupies most of the testicular parenchyma (86%), containing somatic (Sertoli cells) and germ cell lineages (Russell and França, 1995), and thus is

important during the entire spermatogenic process. Several studies have related the tubular diameter and length, as well as the seminiferous epithelium height and the tubular proportion within the parenchyma to the daily sperm production. The intraperitoneal injection of alcoholic extracts of *Momordica charantia* (Naseem et al., 1998), and piperine (Malini et al., 1999) in rats led to a reduction of the tubular diameter, thus reducing the sperm production by severe modifications of the epithelium. On the other hand, Chieregatto (2005) reported that after treatment of rats with *Anemopaegma arvense* infusion, it would be possible to increase those parameters, while not increasing the total tubular volume within the parenchyma. The present study showed significant decrease of tubular diameter in sedentary rats. Seminiferous tubules are made of three basic components: *tunica propria*, seminiferous epithelium and lumen, where sperm is released after spermiogenesis (Ross et al., 2003). Once the epithelium height and total length did not change after treatment and/or exercise, and the *tunica propria* is really thin and was apparently normal in all animals, the decrease of diameter could be due to a smaller lumen.

Morphological analysis did not show any damage or major alterations due to the administration of *H. aphrodisiaca* infusion and/or the endurance exercise. The total volume occupied by the seminiferous tubules remained statistically the same among the experimental groups, despite the numerical variations of such parameter as diameter and length. Since the volume formula for the tubular compartment calculation includes both tubular diameter and length, the association of the variations observed for these parameters was related to the maintenance of the total volume of the seminiferous tubules. The longer the tubules, the narrower their diameters, while the shorter the tubules, the wider their diameters.

The Sertoli cell population established during the initial development of the male reproductive system determines the daily sperm production in normal and sexually mature animals (Orth et al.,

1988; Hess et al., 1993). This hypothesis is based on the fact that each Sertoli cell is able to support a limited number of germ cells (Russell and Peterson, 1984; França and Russell, 1998). The daily spermatogenic efficiency, which is the number of spermatozoa produced daily per gram of testis, is positively related to the number of germ cells supported by the Sertoli cell (Russell and Peterson, 1984; Sharpe, 1994; França and Russell, 1998), since the interactions between Sertoli and germ cells are crucial to maintain normal sperm production (Griswold, 1995). In the present study, the number of Sertoli cells was constant for all treatments, which was also observed for the total number of germ cells.

The number of spermatogonia and primary spermatocytes in leptotene and pachytene was significantly lower in the treated, trained animals, leading to a higher mitotic index. Besides, the lower number of cells is not due to apoptosis, as was shown by the TUNEL technique. On the contrary, the number of apoptotic cells was significantly lower in the sedentary and treated animals, suggesting that the administration of the drug could act as a substance protecting the tubules by preventing apoptosis and/or seminiferous damage, as shown by Monteiro et al. (2008), who used *Heteropterys aphrodisiaca* infusion to protect the seminiferous epithelium against Cyclosporin A administration.

Even with a lower number of primary spermatocytes in both HA treated groups, the meiotic indexes of these groups were higher, compared to control ones. The process of meiosis seemed to occur in a more efficient way, producing as many round spermatocytes as the control groups, as well as the same total number of germ cells.

Several drugs, such as Bisphenol A (Li et al., 2009), lead to increasing of apoptotic cell types within either the seminiferous epithelium (spermatogonia, primary spermatocytes and spermatids) or the interstitium (Leydig cells), causing loss of germ cell lineages and decreasing fertility rates, as well as decrease in plasma testosterone levels and testis weight, associated with morphological changes,

sperm count and motility (Takao et al., 1999; Aikawa et al., 2004). Germ cell apoptosis can also be related to a combination of factors, such as alterations in hormonal parameters and testicular oxidative stress (Chaki et al., 2006).

The results observed after long term treatment with the aqueous infusion of *Heteropterys aphrodisiaca* showed that there were no negative changes within the seminiferous epithelium, even after a prolonged treadmill endurance protocol. Indeed, the results showed a significantly lower number/proportion of apoptosis in the testis of the treated, sedentary animals. The protective potential of this species was shown previously by Mattei et al. (2001) and Monteiro et al. (2008), who demonstrated the increasing of antioxidant species in the brain of old rats or protection of the seminiferous epithelium after exposition to Cyclosporin A, respectively. Corroborating the above cited results, the testosterone concentration in the plasma of the same experimental group was significantly higher, also showing that the Leydig cells were preserved and not affected by the treatment.

Therefore, according to the presented data, *Heteropterys aphrodisiaca* infusion seems to play an important role in increasing the testosterone secretion and on the spermatogonial behavior, inducing mitosis and increasing the spermatogenic yield.

Acknowledgments

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Figure 1. Histological view of the testicular parenchyma showing several seminiferous tubule cross sections in negative (A) and positive (B) TUNEL controls. Brown color shows apoptotic nuclei. C: apoptotic germ cells within the seminiferous epithelium (arrows). In the inset, note the dense chromatin on the edges of a characteristically apoptotic nucleus (arrow head). ST: seminiferous tubule; Int: interstitium; star: lymphatic space. Bars: A and B = 100µm; C = 25µm; detail = 5µm.

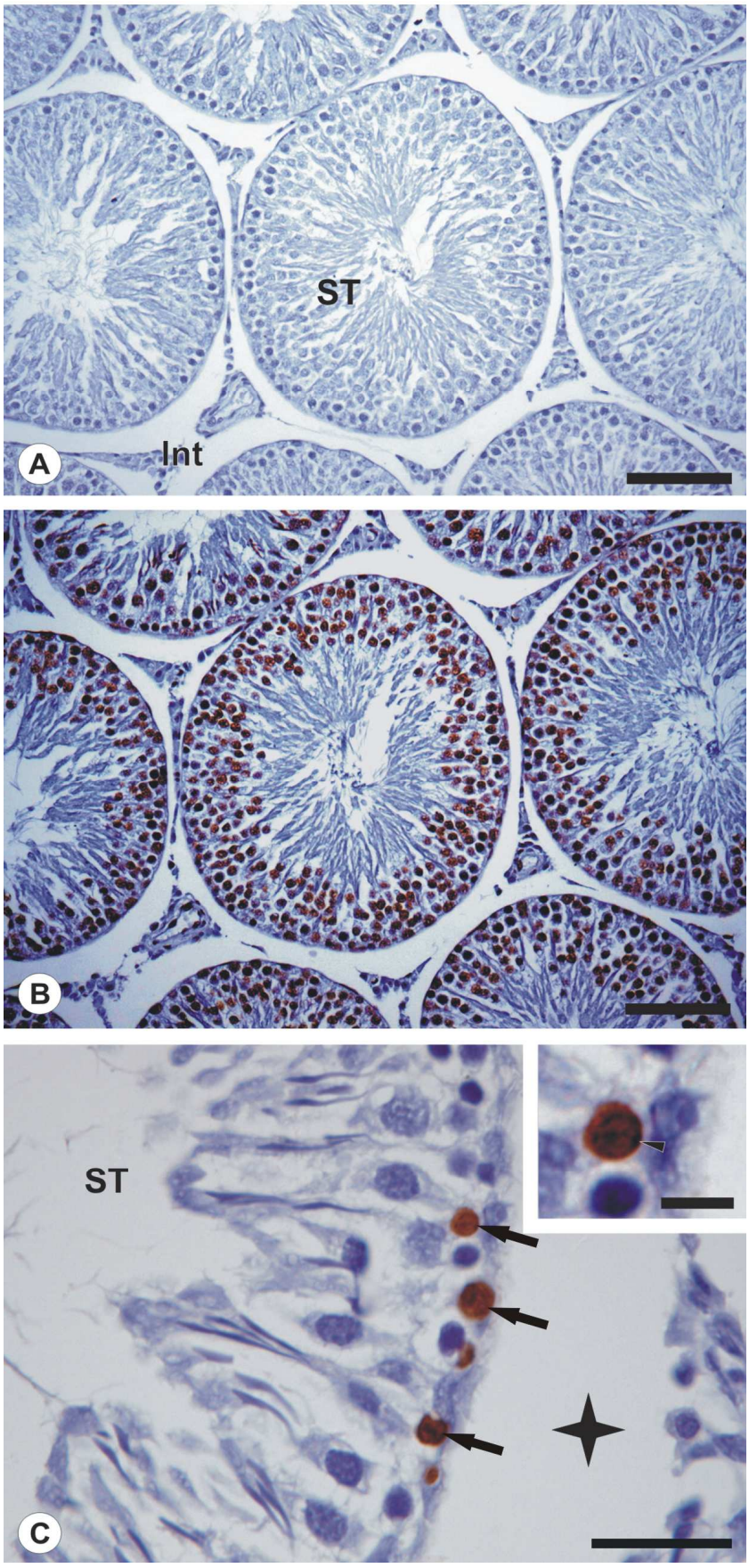

ST
Int
A
B
ST
C

Figure 2. Testosterone concentration (ng/mL) (mean ± SEM; n=10). Same letters do not differ by Duncan's test ($p > 0.05$). CtlSed: Control sedentary, HASed: Treated and sedentary, CtlTra: Control trained and HATra: Treated and trained.

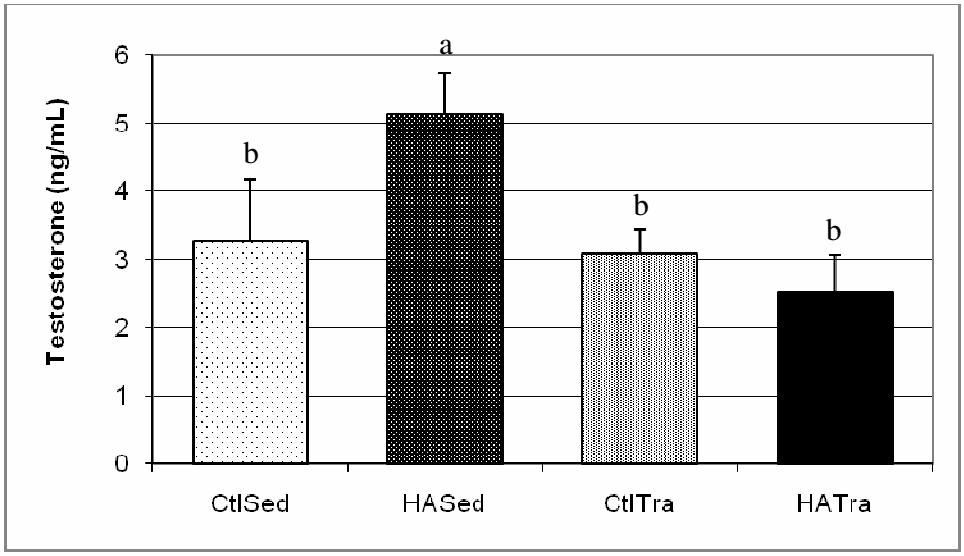
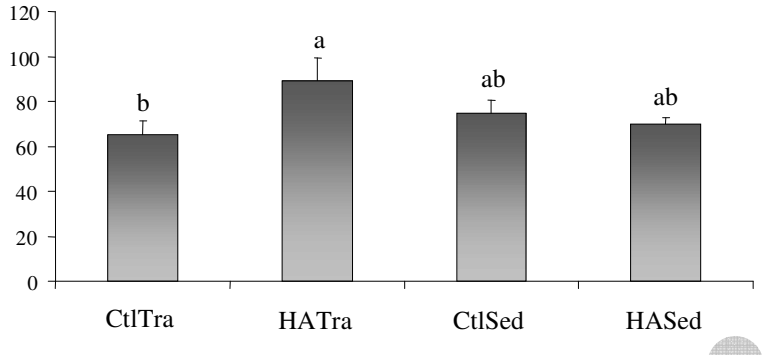


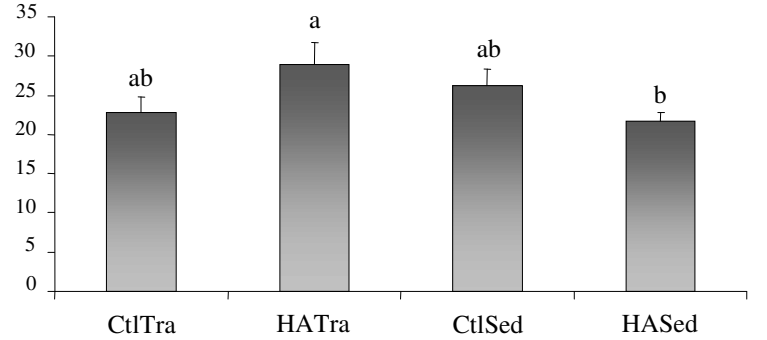
Figure 3. Spermatogenesis dynamics. **A.** Spermatogenic yield; **B.** Spermatogonial mitosis and; **C.** Meiotic index. Values are mean ± SEM (Means with the same letters do not differ significantly; Duncan test; $p>0.05$, $n=10$). CtlTra: Control trained, HATra: Treated and trained, CtlSed: Control sedentary and HASed: Treated and sedentary.

Spermatogenic
Yield



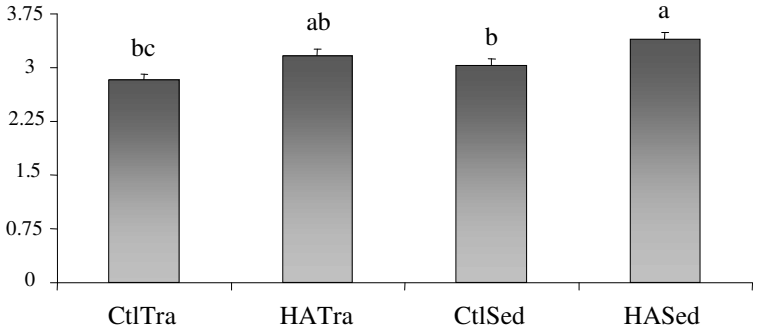
A

Spermatogonial
Mitosis



B

Meiotic
Index



C

Tables

Table I. Treadmill protocol.

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 II. Body and testicular weight, gonadosomatic index and tubules/interstitium ratio of Wistar rats treated with *Heteropterys aphrodisiaca* infusion and/or submitted to treadmill endurance training (mean ± SD, for body and testicular weight and GSI, whereas mean ± SEM for seminiferous tubules and interstitium).

Parameters	**CtlTra**	**HATra**	**CtlSed**	**HASed**
Body weight (g)	403.60 ± 33.17[a]	416.30 ± 40.92[a]	417.10 ± 25.47[a]	410.40 ± 44.48[a]
Testicular weight (g)	3.37 ± 0.14[a]	3.58 ± 0.10[a]	3.43 ± 0.08[a]	3.54 ± 0.01[a]
GSI (%)	0.83 ± 0.03[a]	0.86 ± 0.04[a]	0.82 ± 0.02[a]	0.86 ± 0.02[a]
Seminiferous tubules (%)	86.36 ± 0.82[a]	86.97 ± 0.63[a]	85.56 ± 0.81[a]	85.88 ± 1.22[a]
Interstitium (%)	13.64 ± 0.82[a]	13.03 ± 0.63[a]	14.44 ± 0.81[a]	14.12 ± 1.22[a]

*Same superscripts indicate lack of statistical difference as analyzed by Duncan's test ($p>0.05$; $n=10$). *CtlSed* and *CtlTra*: Control Sedentary and Trained, respectively; *HASed* and *HATra*: Sedentary/Treated and Trained/Treated, respectively.

Table III. Testicular morphometry of adult Wistar rats treated with *Heteropterys aphrodisiaca* infusion and/or submitted to treadmill endurance training (mean ± SEM).

Parameter	CtlTra	HATra	CtlSed	HASed
Tubular diameter (μm)	316.28 ± 4.84[a]	310.56 ± 7.46[a]	295.84 ± 3.75[b]	297.28 ± 4.74[b]
Epithelium height (μm)	107.26 ± 1.68[a]	104.31 ± 3.01[a]	100.03 ± 1.73[a]	103.38 ± 2.60[a]
Tubular length/testis (m)	17.48 ± 0.76[b]	19.09 ± 0.89[ab]	20.27 ± 0.57[a]	21.25 ± 1.08[a]
Tubular volume/testis (mL)	1.37 ± 0.06[a]	1.43 ± 0.05[a]	1.39 ± 0.04[a]	1.46 ± 0.05[a]

*Same superscripts indicate lack of statistical difference as analyzed by Duncan's test ($p>0.05$; $n=10$). *CtlSed* and *CtlTra*: Control Sedentary and Trained, respectively; *HASed* and *HATra*: Sedentary/Treated and Trained/Treated, respectively.

Table IV. Germ cells populations within seminiferous epithelium of Wistar rats treated with *Heteropterys aphrodisiaca* infusion and/or submitted to treadmill endurance training (mean ± SEM).

Number of cells (x10^6)	**CtlTra**	**HATra**	**CtlSed**	**HASed**
Sertoli/gram of testis	11.57 ± 0.74^{a}	11.15 ± 0.71^{a}	13.43 ± 0.72^{a}	12.66 ± 0.76^{a}
Spermatogonia	1.35 ± 0.08^{a}	1.04 ± 0.14^{b}	1.18 ± 0.08^{ab}	1.25 ± 0.07^{ab}
Spermatocytes (Pre-leptotene/Leptotene)	29.33 ± 0.77^{a}	26.68 ± 0.97^{b}	29.57 ± 1.04^{a}	26.51 ± 0.88^{b}
Spermatocytes (Pachytene)	29.48 ± 0.58^{a}	25.62 ± 0.76^{b}	27.94 ± 0.87^{a}	25.58 ± 0.96^{b}
Round spermatids	83.30 ± 2.69^{a}	80.64 ± 1.70^{a}	84.81 ± 3.51^{a}	86.39 ± 3.40^{a}
Germ cells (Total)	143.46 ± 3.46^{a}	133.98 ± 2.87^{a}	143.51 ± 5.02^{a}	139.73 ± 4.97^{a}

*Same superscripts indicate lack of statistical difference as analyzed by Duncan's test ($p>0.05$; n=10). *CtlSed* and *CtlTra*: Control Sedentary and Trained, respectively; *HASed* and *HATra*: Sedentary/Treated and Trained/Treated, respectively.

Table V. Apoptotic cells detected by TUNEL technique (mean ± SEM).

Parameters/Groups	**CtlTra**	**HATra**	**CtlSed**	**HASed**
Maximum/tubule	2.50 ± 0.45^{a}	3.00 ± 1.47^{a}	3.00 ± 1.05^{a}	0.40 ± 0.24^{a}
Mean/tubule	0.16 ± 0.07^{ab}	0.19 ± 0.09^{a}	0.15 ± 0.06^{ab}	0.01 ± 0.005^{b}
Mean/+tubule[¥]	1.72 ± 0.35^{a}	1.60 ± 0.67^{a}	1.59 ± 0.26^{a}	0.40 ± 0.24^{b}
% Tubules apoptosis	8.50 ± 2.87^{a}	8.00 ± 3.16^{a}	8.40 ± 2.32^{a}	0.80 ± 0.49^{b}
Cell type (%)				
Spermatogonia	11.08 ± 1.18^{a}	12.12 ± 7.18^{a}	7.27 ± 4.54^{a}	2.50 ± 2.50^{a}
Primary spermatocyte	3.22 ± 1.18^{a}	1.84 ± 0.75^{a}	3.31 ± 1.91^{a}	$0.00 \pm 0.00^{*}$
Round spermatid	0.31 ± 0.18^{a}	0.14 ± 0.14^{a}	0.43 ± 0.18^{a}	$0.00 \pm 0.00^{*}$

Same superscripts indicate lack of statistical difference as analyzed by Duncan's test ($p>0.05$; n=5). *CtlSed* and *CtlTra*: Control Sedentary and Trained, respectively; *HASed* and *HATra*: Sedentary/Treated and Trained/Treated, respectively.

[¥]Analysis carried on only on cross sections containing apoptotic cells.

*Absence of apoptotic primary spermatocyte and spermatid in the analyzed slides.

ARTIGO 2

Association of *Heteropterys aphrodisiaca* with endurance exercise protected Leydig cell population in Wistar rats?

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Key Words: Phytotherapy, testis, spermatogenesis, interstitium, Leydig cells.

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Abstract

Heteropterys aphrodisiaca is one of the most famous aphrodisiacs in the Middle-West Brazil. Long term administration of the plant infusion enhanced the production of antioxidant species, such as superoxide dismutase in old rats and protected against the deleterious effects on the testicular parenchyma caused by constant intake of cyclosporin A, with no reported side effects. Thus, the present study aimed to evaluate whether *H. aphrodisiaca* infusion would be able to protect the testicular interstitium against the oxidative stress due to long term endurance training. The infusion was administered daily (104mg/animal) by gavage to sedentary and trained animals, divided as follows: control/sedentary (*CtlSed*), control/trained (*CtlTra*), treated/sedentary (*HASed*) and treated/trained (*HATra*). Treated/sedentary animals showed significantly higher serum testosterone concentration. Lower number of Leydig cells was noticed in control/trained animals, although treated/trained did not show any alteration in cell population parameters. Despite these alterations, Leydig cells ultra-structural analysis, as well as androgen receptor concentrations remained unaltered after treatment. Therefore, it seems that the plant infusion could act enhancing testosterone production in sedentary animals, as well as preventing Leydig cell loss due to strenuous physical activity.

Introduction

There is growing scientific interest for research on plants used in traditional medicine, leading to several studies of their active components (Pitman, 1996, Srivastava et al., 2005). The use of these plants to treat diseases is driven by the potential production of more affordable drugs and because of the wide popular acceptance of natural products, especially in countries where the greater part of the population still lives in the country side or does not have enough resources to buy regular medication, such as occurs in Brazil (Corrêa et al., 2000). Despite the potential richness of medicinal flora,

scientific studies and journals that address this theme are still scarce (Ferreira, 1998). Brazil has not made satisfactory use of its biodiversity and the popular knowledge for the development of herbal compounds and agents (Calixto, 2005).

The species *Heteropterys aphrodisiaca* is a Brazilian shrub that belongs to the Family Malpighiaceae, found mainly in the Cerrado biome of Mato Grosso, Goiás and northern Minas Gerais States (Pio Corrêa, 1984). It is one of the most famous aphrodisiacs in the Middle-West Brazil, popularly known as nó-de-cachorro, raiz de Santo Antônio and cordão de São Francisco (Pott & Pott, 1994; Guarim Neto, 1996). Previous studies have shown that long term administration of the plant extract containing this herb could enhance the production of antioxidant species, such as superoxide dismutase in old rats (Mattei et al., 2001) or protect against the deleterious effects on the testicular parenchyma caused by constant intake of cyclosporin A (Monteiro et al., 2008), with no major side effects on kidneys or hepatic tissues (Sbervelheri et al., 2009).

Endurance exercise has several physiological responses and chronic adaptations that are critical for increasing muscular strength, hypertrophy and tolerance to physical activity. Several studies correlate endurance activity to the production and release of LH and testosterone (Häkkinen et al., 1988; Fry et al., 1998; Nindl et al, 2001; Tremblay et al., 2004). From the perspective of an athlete, this increase might improve performance, since testosterone stimulate the increase of muscle and strength, as well as decreasing the accumulation of fatty tissue (Tremblay et al., 2004). Despite the great variety of experiments regarding hormonal (androgenic) content after physical exercise, studies relating endurance and reproductive organ composition, such as the testicle and its interstitium, are still scarce. Some relate that endurance exercise could enhance the hypogonadal male condition (Hackney, 2008), reducing testosterone production (Bennell et al., 1996) and leading to deterioration of semen characteristics and testis reduction (Di Luigi et al., 2001).

The present study was designed to evaluate whether the treatment with *H. aphrodisiaca* infusion combined, or not, with treadmill exercise could interfere on the testis interstitial composition and arrangement, as well as its effects on Leydig cell population.

Material and Methods

Herb harvesting

H. aphrodisiaca samples were harvested in Nova Xavantina (Mato Grosso) and identified by comparison with the voucher specimen from the Federal University of Mato Grosso Herbarium (registration number 23,928).

The roots were dried at room temperature, protected from sunlight, crushed and then powdered using a grinding mill. Infusion was made pouring 100mL of boiling water over 25g of the crushed roots. A study performed by Marques and colleagues (2007) showed that water is the main extractor of polar solid extractable substances. The mixture was allowed to steep for four hours to cool down, then filtered and stored at 4^{o}C for up to four days, as proposed by Chieregatto (2005) and Monteiro et al. (2008, 2010). This procedure produced an infusion of 68.66 mg of extract dry weight per mL of infusion (6.866% wt/vol) and a yield of 6.832% (wt/wt) in terms of initial crude dry weight of plant material. The animals were weighed once a week and the weight variation considered when calculating the concentration of the infusion to be administered.

Experimental groups

The Multidisciplinary Center for Biological Research (CEMIB) in Unicamp provided the male Wistar rats (*Rattus norvegicus albinus*) (90 days old) used in this study. The animals were divided into 4 groups (n = 10): a control, sedentary group (distilled water), a control, trained one, a sedentary group

treated with *H. aphrodisiaca* (104mg/animal/day) and a treated and trained group. Food (commercial diet) and water were provided *ad libitum*. The amount of food intake was recorded weekly in order to check possible variations among groups. Plant infusion was given daily by gavage (0.5mL/animal). The same procedure was repeated with the control animals, which only received distilled water. The treatment lasted eight weeks.

The animals were kept in the Department of Cell Biology Animal Care Laboratory and handled in accordance to the Ethics Committee of the Institute of Biology, UNICAMP. The project had been previously submitted to the same Committee (protocol number: 734-1) and is registered under the number 1234-1.

Training protocol

The trained animals underwent a training protocol that consisted of running on a treadmill built specially for small animals, with 7 individual lanes and manual control of intensity, five days a week for 8 weeks, based on pre set protocols developed by Moraska et al. (2000), Smolka et al. (2000) and Demirel et al. (2001) (Table 1). All animals went through an adaptation and conditioning period (pre-training), until they reached the optimal degree of effort for the initial training phase (Table 1).

Biological samples

Forty-eight hours after the end of the training protocol, animals were weighed and anesthesia was injected in the left quadriceps with a mixture of Xylazine and Ketamine (5 and 80 mg/kg, respectively). Blood samples were taken from the left ventricle by heart puncture and kept refrigerated (4^{o}C). The samples were centrifuged at 5000 rpm for 5 minutes. Plasma was collected and the total testosterone was measured by chemi-luminescence in the Alvet Laboratory (Sorocaba, SP, Brazil).

The animals were perfused with saline (0.9% NaCl solution) and fixed with Karnovsky's fixative (paraformaldehyde 4% and 4% glutaraldehyde in phosphate buffer 0.1 mol/L, pH 7.2) for 20 minutes each. After perfusion, the left testes was removed, weighed and put into new Karnovsky fixative solution for 24h. The right one was dissected in order to obtain the weight of the albuginea and testicular parenchyma.

Light microscopy

After fixation, testicular fragments were dehydrated in ethyl alcohol for further embedding in glycol methacrylate. The blocks were cut with a manual microtome (4μm) and the sections stained with toluidine blue/sodium borate, 1%, for further stereology and morphometry procedures.

Stereology

In order to get the proportion of interstitial components (macrophage, lymphatic space, blood vessels, connective tissue and Leydig cells), 15 digital images from this compartment were randomly taken (400x magnification). In both cases, a 200 point grid was placed over each image (Figure 1A-B). All images were analyzed using the Image Pro Plus (v 6.0) software.

The volume of both interstitium and seminiferous tubules was estimated from the proportion occupied by each compartment within the testes and from the parenchyma net volume. The latter was obtained by subtracting the tunica albuginea volume from the total testes volume. According to Paula et al. (2002), since the density of the testicle is around 1 (1.03 1.04), the testicular mass was considered equal to its volume. A total of 15 digital images (400x), per animal, were used in order to calculate the proportion between both compartments.

Scanning electron microscopy (SEM)

After perfusion, the tissue was fixed in Karnovsky's fixative and rinsed three times with 0.1 M sodium phosphate buffer, pH 7.2. Thus, 6 baths in increasing sucrose solutions (0.5, 1.0, 1.5, 2.0, 2.5 and 3%) were made to prepare the tissue to be frozen in liquid nitrogen, then fractured, post fixed in 1% osmium tetroxide, rinsed and dehydrated in an ascending ethanol series prior to critical point drying. The specimens were mounted on aluminum stubs, sputter-coated with gold and examined under scanning electron microscope (Jeol–JMS 560). SEM was made in order to describe interstitium morphology (Figure 2).

Leydig cell volume

The calculation of the Leydig cell individual volume was performed using a grid with 121 intersections placed on 1000x magnification micrographs. A total of 2000 points were counted on the cytoplasm and the nucleus of Leydig cells, per animal, thus determining the proportion (%) between nucleus and cytoplasm (Figure 1C). In addition, the nuclear diameters of 30 cells, per animal, were measured. Thereafter, the nuclear volume: $4/3\pi R^3$ (R = nuclear radius), the cytoplasmic volume, (% cytoplasm x nuclear volume) / (% nucleus), and the volume of each Leydig cell (nuclear volume + cytoplasmic volume) were calculated.

In order to calculate Leydig cells number per testicle the total volume occupied by these cells was divided by the average volume of one Leydig cell.

Leydig cells ultra-structure

After perfusion, testicular fragments were put into new Karnovsky fixative solution for 24h. Afterwards, they were post fixed with osmium tetroxide 1% for 2 hours and routinely dehydrated in

ethyl alcohol and acetone for further Epon resin embedding. Semi and ultra thin sections were made with an automatic ultra microtome (Leica); the latter were counterstained with uranyl acetate 3% and lead citrate 2% (30 and 5 minutes, respectively). The specimens were observed under Zeiss Leo 906 electron microscope to see if there were any ultra-structural alterations in Leydig cells.

Western Blotting

Testes were weighed and homogenized for 1 min with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in 500µl of RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), left for 2 hours on ice and centrifuged at 15000xg for 5 min. Protein content in the supernatant was determined by Bradford's micromethod (Bio-Rad Laboratories, Hercules, CA). 25µg of protein were resolved by SDS-PAGE using 10% gels under reducing conditions. Primary and secondary antibody incubations were made using Millipore SNAP i.d..

Proteins were electro-transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences), which were assembled into cassettes, blocked with Chemiluminescent Blocker (Millipore, WBAVDCH01) for 15 seconds and put into blot holders (Millipore, WBAVDBH03). The membranes were probed with the antibody anti-androgen receptor (Sc-816, Santa Cruz Biotechnology), diluted at 1:200 in TBS-T for 10 minutes and passed through the filter membrane for 15-20 seconds. The excess of antibody was rinsed three times with TBS-T. The procedure was repeated using the HRP-conjugated goat anti-rabbit IgG (DAKO Envision System, #K1491). The bands were developed with DAB (3,3'- diaminobenzidine). Quantification of the AR bands was made by measuring the optical densities using Scion Image software Alpha 4.0.3.2 (Scion Corporation).

Statistic analysis

Analysis of variance (one-way ANOVA) plus Duncan's test was used to compare differences between groups. Values of $p<0.05$ indicate significant differences. Two-way ANOVA was used to determine whether either the *H. aphrodisiaca* infusion or training protocol influenced the patterns analyzed, and whether there was interaction between the infusion and exercise. The software Statistica (v. 8.0, Tulsa, OK, USA) was used for all statistical analysis.

Results

Hormonal assay

Sedentary animals that received the plant infusion showed significant increase in total plasma testosterone concentration (Fig. 3). Trained animals did not show any alterations in this parameter compared to the control/sedentary animals. There was no interaction between training on a treadmill and the infusion of the plant in the final outcome of total testosterone (two-way ANOVA).

Interstitial tissue

Interstitial tissue data are shown on Table 2. There were no significant differences between seminiferous tubules and interstitium proportions and interstitial volume after the treatments. Also, no alterations were observed either on the organizational structure of the interstitium or for macrophage, lymphatic space and blood vessels proportions. However, both treated groups showed significant decrease of connective tissue content, compared to their respective control groups.

Leydig cells

There was a significant increase of Leydig cell parameters in trained animals (Table 3). Nuclear diameter and volume, as well as cytoplasmic and cell volumes were also significantly higher than

sedentary ones. On the other hand, the cell number per gram of testicular parenchyma showed the same results in comparison to the treated/trained group; only the control/trained animals had significantly lower cell numbers per gram of testis (Table 3). The electron microscopy micrographs did not show any significant effects on morphology and organization of Leydig cell nuclei and cytoplasm in both treatments (infusion and exercise) (Figure 4).

Western Blotting

No significant differences were observed in the androgen receptor content among groups (Figure 5).

Discussion

Morphometry and stereology have been increasingly employed in various fields of biological sciences, in order to facilitate comparison of results and confer more confidence in a diagnosis. The present study was based on the principles of stereology and morphometry to evaluate possible changes in testicular structure of rats subjected to endurance exercise and/or treated with *Heteropterys aphrodisiaca* infusion, a Brazilian plant with related stimulating effects.

Changes in testosterone concentrations may be related to the ability of animals to adapt to the pattern of exercise (Viru & Viru, 2005). Thus, new incentives are needed to maintain the increased production and secretion of this hormone (Tremblay et al., 2004). In the present study, time and exercise load was increased periodically, which would eliminate such accommodation before the end of the training protocol. Nevertheless, the amount of plasma testosterone was significantly higher in sedentary animals that received the plant infusion, leading us to believe that the infusion stimulated testosterone production/secretion in that specific group.

Some researchers observed that endurance exercise reduces blood flow to the testicles and causes a low level of testosterone secretion, thus affecting some degree of spermatogenesis (Hackney, 2008). Based on the specific training protocol presented so far, the results showed that endurance exercise did not strongly influence plasma testosterone concentration, in agreement with Lucia et al. (1996) and Duclos (1996).

The interstitial tissue contains several components related to the maintenance and functioning of the organ as a whole. Modified proportions of these components can lead to irreversible changes, such as sterility, since all components are closely associated with each other, such as the Leydig cells, macrophages and myoid cells (Ichihara et al., 1993; 2001; Hales, 2002). From the present study the volume occupied by the connective tissue has diminished with treatment, even with no further stress such as the exercise routine. Souza et al. (2005) have shown that the Leydig cell population was directly affected after a long term running protocol, which was also observed in the present study in control/trained animals. Intensive exercise-induced oxidative stress may be responsible for some cases of low reproductive activities. Oxidative stress is imposed on male reproductive system by intensive exercise, which may interfere with testicular steroidogenesis and spermatogenesis (Manna et al., 2003). Changes in reproductive progress may be related to the administration of compounds that directly affect Leydig cells (Mantovani and Maranghi, 2005). It is important to indicate that even after long term endurance exercise, it seemed that the infusion intake along with the training protocol helped to prevent further Leydig cell loss by oxidative stress. Protection of this cell type was widely discussed by Monteiro and colleagues (2008) who administered the same *H. aphrodisiaca* infusion concentration to male Wistar rats that underwent treatment with the immunosuppressive drug Cyclosporin A. The plant infusion was shown to be helpful to prevent degeneration of this cell type, probably because of its role of enhancing antioxidant species production (e.g. superoxide dismutase) (Galvão et al., 2002). Despite

the lower number of cells per gram of parenchyma, control/trained animals showed the highest individual Leydig cell volume, which may be related to the maintenance of testosterone secretion at the same levels as the other experimental groups, not considering the treated/sedentary group. Besides, no ultra-structural changes were confirmed by qualitative analysis of Leydig cells in transmission electron microscopy micrographs.

Endogenous androgens, mainly testosterone and its active metabolite dihydrotestosterone (DHT), exert most of their effects by binding to the androgen receptor (AR) (Patrão et al., 2009). The AR belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors. In the adult male mouse and rat testes, AR is detected predominantly in the nuclear region of Sertoli, peritubular myoid, Leydig and perivascular smooth muscle cells (Sar et al., 1990; Zhu et al., 2000; Zhou et al., 2002).

The impact of lacking AR in organs such as the testicles has to do with optimal spermatogenesis operation. Important constituents would lose the ability to perform their functions, such as steroidogenesis in Sertoli and Leydig cells and muscular tone in smooth muscle and myoid cells. Poorly developed Sertoli cells would lead to spermatogenesis arrest at the diplotene primary spermatocyte stage prior to completion of the first meiotic division; the impact of lacking AR in Leydig cells would mainly affect steroidogenic functions leading to spermatogenic arrest at the round spermatid stage. Lack of AR in the smooth muscle cells and peritubular myoid cells in mice results in similar fertility despite decreased sperm output (Wang et al. 2009). Despite some previous results that showed Leydig cell numbers to be affected by endurance exercise, no significant difference was detected in AR concentration among the experimental groups, neither in Sertoli and germ cell numbers (Gomes et al., 2009), nor in Leydig cells and interstitial constituents.

Therefore, despite some results showing a clear increase of plasma testosterone concentration due to *Heteropterys aphrodisiaca* intake, the present data indicates that the plant infusion was able protect the Leydig cells against possible deleterious effects of endurance exercise, since the final population of this cell remained unaltered in treated/trained animals.

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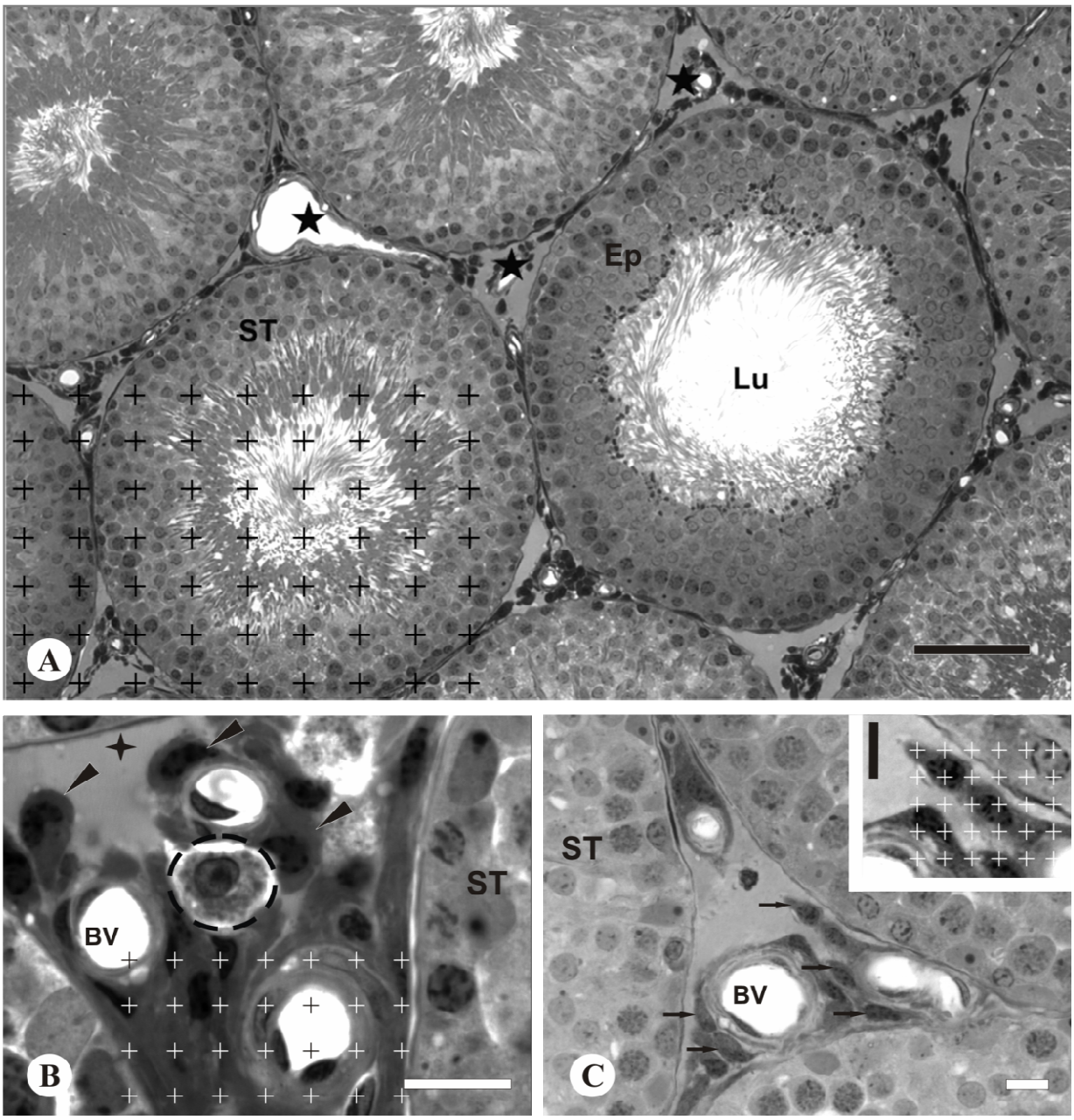
FIGURES

Figure 1. A. Panoramic view of the testicular parenchyma, showing seminiferous tubules (ST) and interstitial space (stars). Seminiferous tubules are essentially composed of the seminiferous epithelium (Ep) and the lumen (Lu). **B.** On the left corner an example of grid used to determine the proportion between both compartments. On the other hand, the interstitial space shows a variety of different components, such as: Leydig cells (arrowheads), macrophages (dashed circle), blood vessels (BV) and lymphatic space (star). Proportions among them were calculated using a grid like the one shown in B**. C.** Note the large number of Leydig cells (stars) within the interstitial space, and a model of grid used to evaluate the proportion of nucleus/cytoplasm (inlet). Bars: A - 75µm; B-C and inlet: 15µm.


Ep
ST
Lu
A
B
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ST
ST
BV
C

Figure 2. Testicular parenchyma scanning electron micrographs. The parenchyma is composed of seminiferous tubules (ST) and interstitium (white arrows), which contain a large amount of cells and connective tissue (star) (**A** and **B**). **C**: Note the intricate organization of the interstitial tissue, which is well irrigated by blood vessels (BV). There is a substantial amount of connective tissue surrounding the tubules, presenting large spaces (arrowheads) that allow the flow of lymph throughout. **D**: In a larger magnification it is possible to see a great number of cells among collagen fibers (black arrows) as well as large spaces, called fenestrae, in between them (asterisks). Bars: A-C: 150 µm; D: 50 µm.

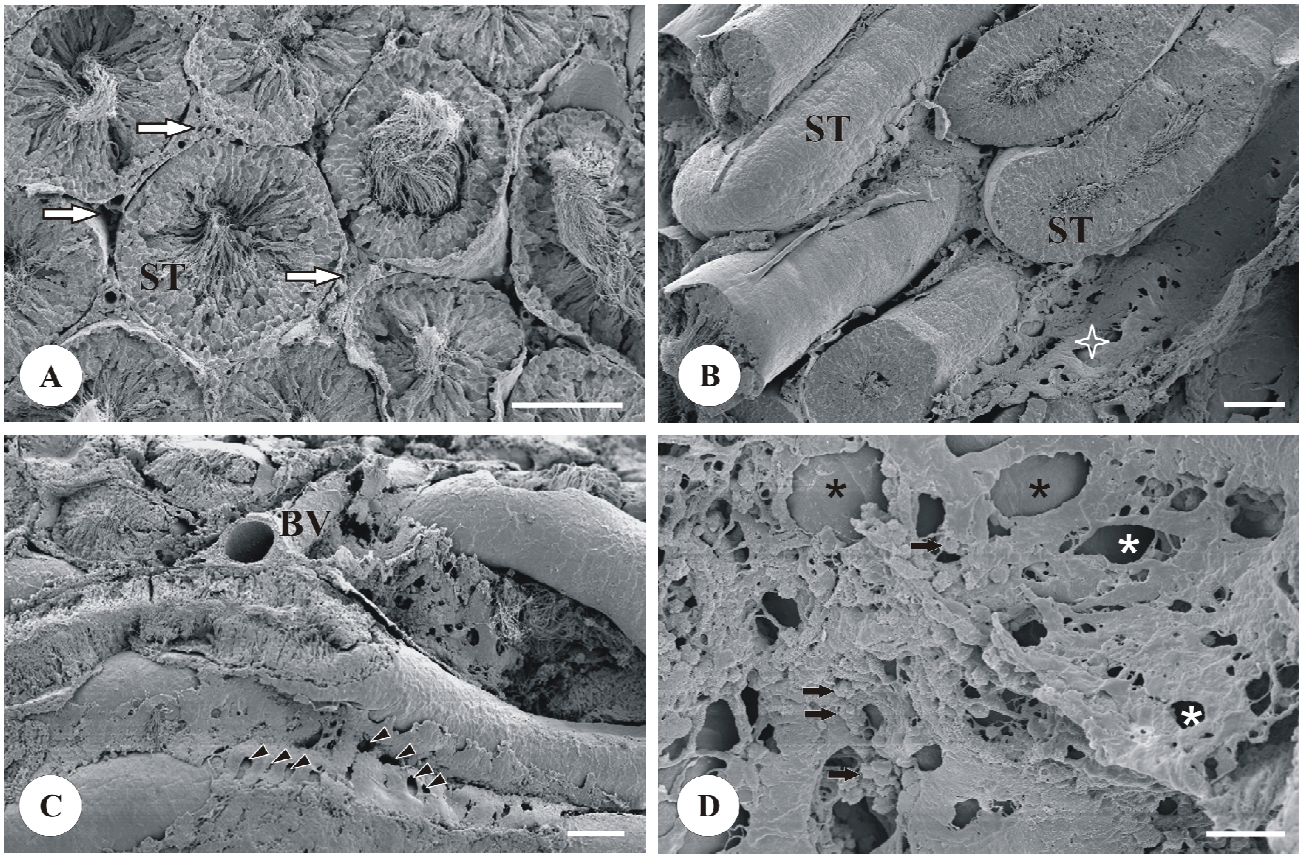

Figure 3. Plasma **t**estosterone concentration (ng/mL) (mean ± standard error). *CtlSed* and *CtlTra*: Control Sedentary and Trained, respectively; *HASed* and *HATra*: Sedentary/Treated and Trained/Treated, respectively.

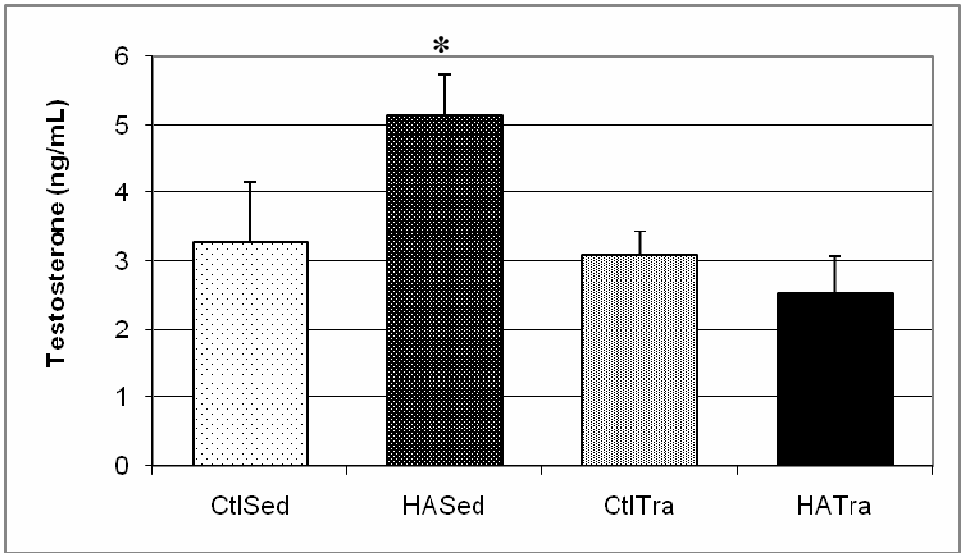

Figure 4. Leydig cell ultrastructure. **A.** Control/sedentary group. **B.** Control/trained. **C.** Treated/sedentary group. **D.** Treated/trained group. Star: Nucleus, Gc: Golgi complex, mit: mitochondria, arrowheads: rough endoplasmic reticulum. Bars: A-C = 15 μm; D = 20 μm.

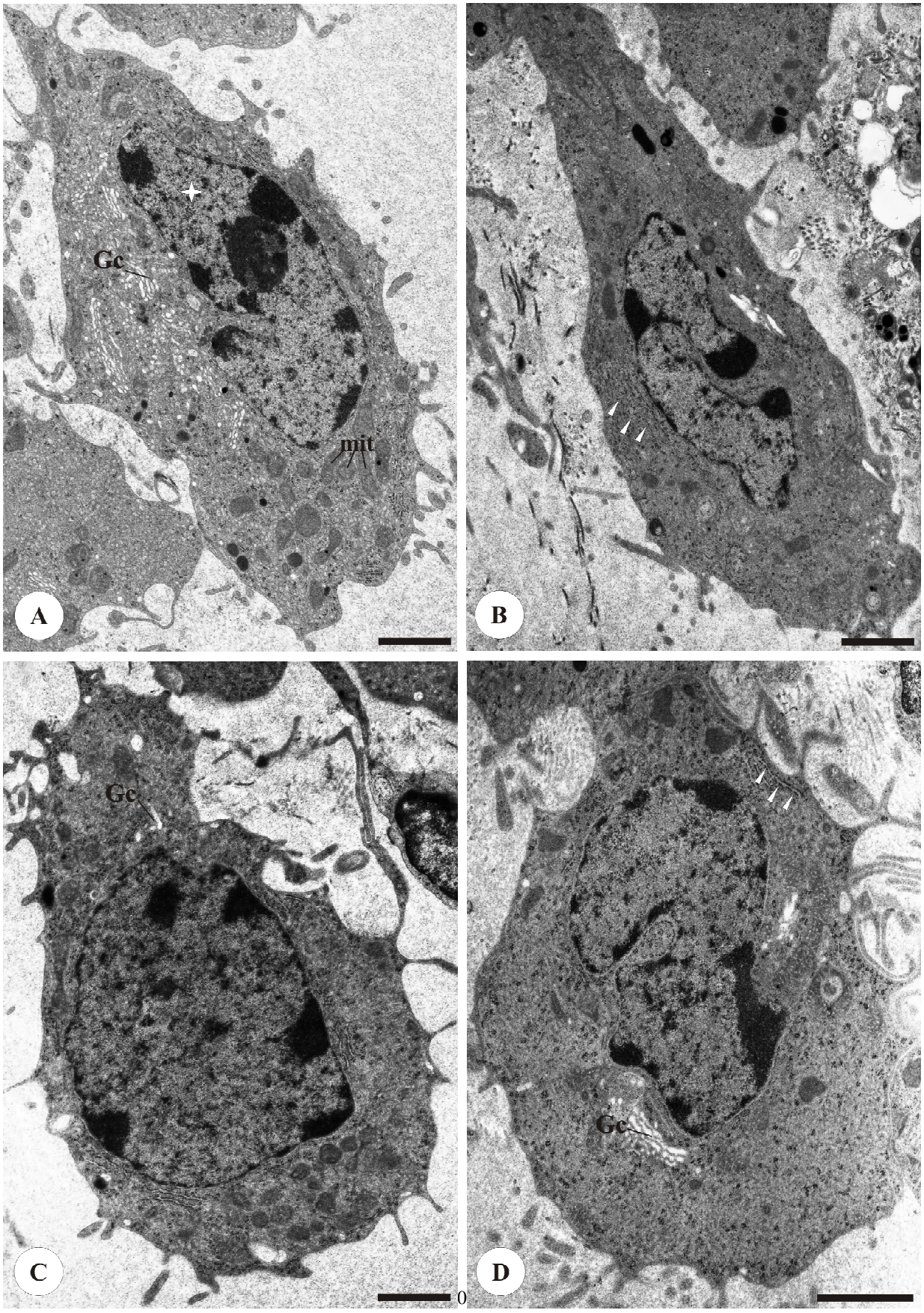
Gc
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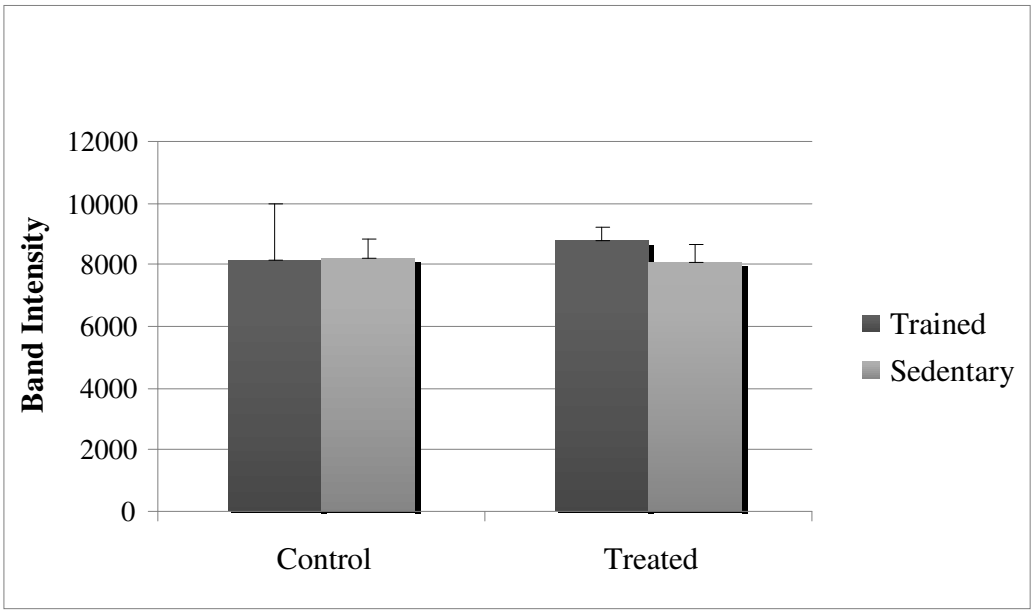
Figure 5. Western blotting (androgen receptor concentration).



TABLES

Table 1. Treadmill protocol.

Activity	Week	Speed (m/min)	Time (min)
Pre-training	1	10	5
	2	12.5	7.5
	3	15	10
Training	1	15	20
	2	20	30
	3	20	40
	4-8	22.5	45

Table 2. Interstitium volume and composition. Values are mean ± SEM.

Interstitium	**CtlSed**	**HASed**	**CtlTra**	**HATra**
Volume ($x10^{-3}$ mL)	257.46 ± 17.66[a]	237.77 ± 19.95[a]	245.16 ± 20.87[a]	289.19 ± 25.20[a]
Components (%)				
Macrophage	1.62 ± 0.20[a]	1.64 ± 0.13[a]	1.95 ± 0.18[a]	1.71 ± 0.10[a]
Lymphatic space	58.44 ± 1.65[a]	61.85 ± 2.26[a]	55.48 ± 3.35[a]	57.58 ± 3.57[a]
Blood vessels	9.01 ± 1.19[a]	8.46 ± 1.11[a]	9.78 ± 1.52[a]	10.88 ± 1.62[a]
Connective tissue	17.01 ± 1.38[a]	13.45 ± 0.91[b]	17.33 ± 1.09[a]	13.32 ± 1.45[b]
Leydig cells	13.92 ± 0.53[a]	14.60 ± 0.99[a]	15.46 ± 1.81[a]	16.51 ± 1.44[a]

Same superscripts indicate lack of statistical difference as analyzed by Duncan's test ($p>0.05$; $n=5$). *CtlSed* and *CtlTra*: Control Sedentary and Trained, respectively; *HASed* and *HATra*: Sedentary/Treated and Trained/Treated, respectively.

Table 3. Leydig cell morphometry and stereology. Values are mean ± SEM.

Leydig Cell	CtlSed	HASed	CtlTra	HATra
Number per gram of parenchyma ($x10^6$)	24.30 ± 2.05^a	23.51 ± 2.87^a	18.92 ± 1.60^b	25.15 ± 2.27^a
Nuclear diameter (µm)	6.20 ± 0.06^b	6.13 ± 0.09^b	6.50 ± 0.07^a	6.49 ± 0.11^a
Nuclear volume (μm^3)	125.43 ± 3.96^b	121.50 ± 5.02^b	144.10 ± 4.25^a	144.05 ± 7.15^a
Cytoplasm volume (μm^3)	425.28 ± 29.22^b	420.93 ± 18.27^b	553.98 ± 29.17^a	499.90 ± 31.22^a
Cell volume (μm^3)	550.71 ± 30.21^a	542.43 ± 21.72^b	698.08 ± 31.92^a	643.95 ± 37.24^a

Same superscripts indicate lack of statistical difference as analyzed by Duncan's test ($p>0.05$; $n=5$). *CtlSed* and *CtlTra*: Control Sedentary and Trained, respectively; *HASed* and *HATra*: Sedentary/Treated and Trained/Treated, respectively.

5. Considerações Finais

A administração da infusão de *Heteropterys aphrodisiaca,* assim como o treinamento em esteira seguindo o protocolo proposto pelo presente estudo não causou alterações nas proporções entre compartimentos tubular e intersticial nos testículos dos animais experimentais.

Houve aumento significativo da concentração de testosterona plasmática em animais tratados/sedentários, assim como menor número e proporção de figuras apoptóticas de células da linhagem germinativa neste grupo, indicando o potencial antioxidante desta espécie.

Os animais tratados com a infusão da planta e submetidos ao treinamento físico apresentaram maior índice de mitose espermatogonial, quando comparados aos animais tratados/sedentários. O rendimento da espermatogênese foi significativamente maior nos animais tratados/treinados comparando-se com seu respectivo grupo controle. Além disso, o grupo tratado/sedentário apresentou índice meiótico significativamente maior quando comparado a ambos os grupos controle, tratado e sedentário.

Os componentes do tecido intersticial não sofreram alterações importantes durante o período experimental, salvo o tecido conjuntivo que, em ambos os grupos tratados apresentaram reduções significativas quando comparados a seus respectivos grupos controle. As células de Leydig dos animais treinados apresentaram os maiores valores para diâmetro e volume nuclear, maiores volumes citoplasmáticos e celulares. Entretanto, o número total deste tipo celular por grama de parênquima testicular apresentou-se significativamente menor em animais controle/treinados. Dessa forma, propõe-se que a administração concomitante da infusão de *Heteropterys aphrodisiaca* pode atuar protegendo as células de Leydig contra os efeitos deletérios do exercício físico crônico prolongado.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha Tese de Doutorado intitulada "EFEITOS DA INFUSÃO DE NÓ-DE-CACHORRO (*Heteropterys aphrodisiaca*, O. Mach.) SOBRE A MORFOLOGIA E ESTRUTURA TESTICULAR DE RATOS WISTAR ADULTOS, SUBMETIDOS A TREINAMENTO FÍSICO":

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

(x) tem autorização da(s) seguinte(s) Comissão(ões) de Bioética ou Biossegurança*:
Comissão de Ética na Experimentação Animal CEEA/UNICAMP, sob Protocolo(s) nº 1234-1.

** Caso a Comissão seja externa à UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Aluno: Marcos de Lucca Moreira Gomes

Orientador: Mary Anne Heidi Dolder

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

Nome: Ana Maria Aparecida Guaraldo
Função:

Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da Comissão de Ética no Uso de Animais
CEUA/UNICAMP