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ASSOCIAÇÃO DE CICLOSPORINA E *Heteropterys aphrodisiaca* (NÓ-DE-CACHORRO) ADMINISTRADOS A RATOS WISTAR: ESTRUTURA, ULTRA-ESTRUTURA E MORFOMETRIA TESTICULAR

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Juliana Castro Monteiro

e aprovada pela Comissão Julgadora.

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Tu, que habitas sob a proteção do Altíssimo,
que moras à sombra do Onipotente,
dize ao Senhor: "Sois meu refúgio e minha cidadela,
meu Deus, em que eu confio."
É ele que te livrará do laço do caçador,
e da peste perniciosa.
Ele te cobrirá com suas plumas;
sob suas asas encontrarás refúgio.
Sua fidelidade te será um escudo de proteção.
Tu não temerás os terrores noturnos,
nem a flecha que voa à luz do dia,
nem a peste que se propaga nas trevas,
nem o mal que grassa ao meio-dia.
Caiam mil homens à tua esquerda e dez mil homens à sua direita;
tu não serás atingido.
Porém, verás com teus próprios olhos,
contemplarás o castigo dos pecadores,
porque o Senhor é teu refúgio.
Escolheste, por asilo, o Altíssimo.
Nenhum mal te atingirá,
nenhum flagelo chegará a tua tenda,
porque aos seus anjos ele mandou
que te guardem em todos os seus caminhos.
Eles te sustentarão em suas mãos
para que não tropeces em alguma pedra.
Sobre serpente e víbora andarás,
calcarás aos pés o leão e o dragão.
"Pois que se uniu a mim, eu o livrarei;
e o protegerei, pois conhece o meu nome.
Quando me invocar, eu o atenderei;
na tribulação estarei com ele.
Hei de livrá-lo e o cobrirei de glória.
Será favorecido de longos dias,
e mostrar-lhe-ei a minha salvação."

(Salmo 90)

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Aos meus pais,
Lucas e Marinete,
exemplos de perseverança e dignidade.

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SUMÁRIO

1. RESUMO

A ciclosporina A (CsA) possui potentes propriedades imunossupressivas e tem sido amplamente usada na terapia de transplantes de órgãos, aumentando as taxas de sobrevivência dos enxertos, e no tratamento de algumas doenças auto-imunes. Apesar de ser um importante medicamento, diversas reações colaterais são verificadas, entre elas a toxicidade testicular, levando à infertilidade masculina. *Heteropterys aphrodisiaca* é uma planta com indicações de estimulante e potente afrodisíaco, que aumenta o peso corporal e testicular e o volume das células de Leydig nos testículos de ratos adultos. Assim o efeito da associação dessas drogas foi estudado em ratos Wistar em idade reprodutiva, com avaliação da morfometria, estrutura e ultra-estrutura testicular. Foram utilizados 30 ratos divididos em 5 grupos: I- controle; II- tratamento com CsA; III- concomitante uso de CsA e *H. aphrodisiaca*; IV- pré-tratamento com *H. aphrodisiaca* por 30 dias, seguido de CsA por 26 dias; V- tratamento com *H. aphrodisiaca*. CsA foi administrada na dose de 15 mg/kg/dia e *H. aphrodisiaca* na dose de 0.5 ml de infusão preparada com 25g de raízes secas/100 ml água fervente. Os tratamentos foram administrados diariamente por gavagem, durante 56 dias. Aumento do peso corporal foi observado em todos os grupos, mas os grupos II e III tiveram menor ganho de peso. O peso dos testículos e dos órgãos sexuais acessórios, assim como a proporção e o volume dos compartimentos tubular e intersticial não alteraram nos grupos tratados. Com a administração de CsA houve aumento na proporção de tecido conjuntivo e redução na proporção de células de Leydig que, além disso, eram menores e com citoplasma mais denso que as células dos animais de outros grupos tratados. Por outro lado, a infusão de *H. aphrodisiaca* resultou em aumento do volume nuclear das células de Leydig. Os níveis de testosterona plasmáticos, o número de células de Leydig por testículo e por grama de testículo aumentou significativamente nos animais do grupo III. CsA causou degeneração das células germinativas, vacuolização nas células de Sertoli, espermátides arredondadas anormais e atrofia das organelas envolvidas na síntese de esteróides nas células de Leydig. *H. aphrodisiaca* causou vacuolização nas células de Sertoli, perda do contato e espaçamento entre as células germinativas e aumento de retículo endoplasmático liso e mitocôndrias nas células de Leydig. O tratamento concomitante e sequencial com

CsA e *H. aphrodisiaca* foi efetivo em manter a ultra-estrutura das células de Leydig. Nesses tratamentos, as alterações observadas no epitélio seminífero foram a combinação das modificações observadas em cada tratamento separadamente. Entretanto, *H. aphrodisiaca* exerceu um efeito protetor no epitélio germinativo, uma vez que as modificações encontradas nesses grupos foram menos severas em relação ao grupo tratado somente com CsA.

2. ABSTRACT

Cyclosporine A (CsA) has potent immunosuppressive properties and it has markedly improved the ability of transplant patients to survive grafts and is also used in some autoimmune diseases. However, the drug has several side effects, including testicular toxicity, leading to male infertility (and temporary impotence?). Stimulant and aphrodisiac properties has been attributed to the plant, *Heteropterys aphrodisiaca*. Data from other experiments suggests that the root extract can increase body and testicular weight and the volume of Leydig cells in rat testis. Thus, the present work was undertaken to study the association of the drug and the medicinal herb in Wistar rats, evaluating testicular morphometry, structure and ultrastructure. Thirty adult rats were used, divided into five groups: I- control; II– CsA; III– simultaneous use of CsA and *H. aphrodisi*aca; IV- *H. aphrodisiaca* for 30 days and CsA sequentially for another 26 days; V- *H. aphrodisiaca*. CsA was administered at a dose of 15 mg/kg/day and/or *H. aphrodisiaca* at a dose of 0.5 ml of the infusion prepared with 25g of roots/100ml boiling water, daily by gavage, during 56 days. Increased body weight was observed for all groups, but the animals that received CsA for 56 days showed the smallest body weight gain. The testis and accessory sex organs weights were unchanged. The volume and volumetric proportion of seminiferous tubules and interstitial compartments did not alter in all treated groups. Morphometry showed increased connective tissue volumetric proportion and decreased Leydig cell volumetric proportion in CsA-treated rats. The Leydig cells of CsA-treated animals were smaller and more dense than other groups. On the other hand, the *H. aphrodisiaca* infusion resulted in an increase in Leydig cell nuclear volume. The plasma testosterone levels and number of Leydig cells per testis increased significantly in group III. Using transmission electron microscopy, it was possible to ascertain that CsA caused germ cell degeneration, Sertoli cell vacuolization, abnormal round spermatids and atrophy of the organelles involved in steroid synthesis in Leydig cells. *H. aphrodisiaca* caused Sertoli cell vacuolization, loss of germ cell attachment and expanded intercellular spaces between germ cells and Leydig cells with more mitochondria and smooth endoplasmic reticulum. The simultaneous and sequential treatment with *H. aphrodisiaca* and CsA was effective in maintaining the Leydig

cell ultrastructure. In these treatments, the seminiferous epithelium alterations were a combination of the modifications observed for each isolated treatment. However it can be stated that *H. aphrodisiaca* had a protective effect on the testicular tissue, since less severe modifications were found for this group in relation to the rats that received only CsA.

3. INTRODUÇÃO

3.1. Estrutura testicular

Os testículos são envolvidos por uma cápsula fibrosa espessa, a túnica albugínea, a qual se invagina formando o mediastino testicular e dividindo o testículo em lóbulos. Esse órgão é constituído por dois compartimentos principais: o compartimento tubular e o compartimento intertubular ou intersticial.

No compartimento tubular encontram-se os túbulos seminíferos, que são alças contorcidas que têm suas extremidades conectadas na *rete testis* pelo túbulo reto (RUSSELL et al., 1990). O túbulo seminífero é circundado por uma camada externa de células mióides e tecido conjuntivo associado, a túnica própria, e por uma membrana basal bem definida adjacente ao epitélio seminífero, separando-o da túnica própria (ROSS et al., 2003). No epitélio seminífero são encontrados dois tipos celulares distintos: as células de Sertoli e as células germinativas (KARL & CAPEL, 1998).

As células de Sertoli têm mostrado importante papel na regulação da espermatogênese (RUSSELL et al., 1990). A estrutura da célula de Sertoli, revelada por microscopia eletrônica, é extremamente complexa. Esta célula estende-se a partir da base do túbulo e alcança o seu lúmen, tendo um comprimento de cerca de 90 μm no rato (FRANÇA & RUSSELL, 1998). São alongadas, apresentam citoplasma claro, mal delineado e de forma extremamente irregular. O núcleo, geralmente de forma ovóide ou triangular, apresenta cromatina finamente dispersa e nucléolo bem desenvolvido. Essas células não se dividem durante o período reprodutivo e são muito resistentes a condições adversas como infecções, desnutrição e raios X, que afetam mais facilmente as células espermatogênicas (RUSSELL & GRISWOLD, 1993). As células de Sertoli desempenham três funções principais: (1) fornecer suporte e controlar a nutrição das células germinativas, através da regulação da passagem dos nutrientes trazidos pelo sangue; (2) fagocitar e digerir, via lisossomos, os restos de citoplasma que se desprendem das espermátides; (3) secretar fluido para o lúmen (RUSSELL & GRISWOLD, 1993; SHARPE, 1994). Juntamente com as células mióides, as células de Sertoli produzem a membrana basal que

serve de suporte estrutural para a própria célula de Sertoli e para as células germinativas que se encontram na porção basal do epitélio seminífero.

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-leptóteno e leptóteno), e o compartimento adluminal, onde se encontram os espermatócitos primários a partir da fase de zigóteno, espermatócitos secundários e espermátides (RUSSELL et al., 1990; RUSSELL & GRISWOLD 1993; SHARPE, 1994). No lúmen tubular encontram-se o fluido secretado pelas células de Sertoli e os espermatozóides recém espermiados.

O compartimento intertubular, ou intersticial, é formado por tecido conjuntivo, nervos, vasos sanguíneos e linfáticos e células intersticiais ou de Leydig. Mastócitos e macrófagos são comumente vistos no intertúbulo sendo que, em algumas espécies, os macrófagos podem representar 25% das células do interstício (RUSSELL et al., 1990). A estrutura dos vasos linfáticos associados ao tecido intersticial demonstra enormes variações nas diferentes espécies (FAWCETT et al., 1973). Nos roedores, em geral, estão presentes espaços linfáticos peritubulares extraordinariamente grandes (SHARPE, 1994).

A célula de Leydig tem forma arredondada ou poligonal, núcleo central com nucléolo proeminente e citoplasma rico em mitocôndrias com cristas tubulares, abundante retículo endoplasmático liso, variável quantidade de lipídios e uma rede de peroxissomos, sendo a principal fonte de andrógenos (PAYNE et al., 1996; COOK et al., 1999; JUNQUEIRA & CARNEIRO, 2004). A produção de andrógenos ocorre por meio de estímulos do hormônio luteinizante (LH) em receptores na membrana plasmática das células de Leydig. Dentre os andrógenos sintetizados pelas células de Leydig, incluem-se a testosterona e a diidrotestosterona, responsáveis pela diferenciação do trato genital masculino e da genitália externa na fase fetal, pelo aparecimento dos caracteres sexuais secundários e pela manutenção quantitativa da espermatogênese a partir da puberdade (SHARPE, 1994; PELLENIEMI et al., 1996). Adicionalmente, a diidrotestosterona é responsável pela manutenção funcional das glândulas sexuais acessórias e do epidídimo (LUKE & COFFEY, 1994).

3.2. Espermatogênese

A espermatogênese é um processo no qual as células germinativas são transformadas de células indiferenciadas diplóides (espermatogônias) em espermatozóides haplóides altamente especializados (SHARPE, 1994). Esse processo pode ser dividido em três fases de acordo com as considerações morfológicas e funcionais: (1) fase proliferativa (espermatogonial), na qual células sofrem rápidas e sucessivas divisões mitóticas, (2) fase meiótica (espermatocitogênica), onde o material genético é combinado e segregado, e (3) fase de diferenciação ou espermiogênica, na qual as espermátides transformam-se em células estruturalmente equipadas para alcançar e fertilizar o ovócito (FRANÇA & RUSSELL, 1998).

A espermatogênese depende do suporte coordenado e interações das células germinativas e somáticas (Sertoli, Leydig, macrófagos e peritubulares), além de vasos sanguíneos. Toda a regulação do processo é mediada pelo eixo hipotálamo-hipófise-célula de Leydig, mas igualmente importante é a regulação local das funções celulares através de fatores parácrinos e autócrinos (CREASY, 2001).

3.3. Ciclosporina A

Ciclosporina A (CsA) é um polipeptídeo cíclico com 11 aminoácidos, alguns N-metilados, isolada de micélios dos fungos *Tolypocladium inflatum* Gams e *Cylindrocarpon lucidum* Booth. É um peptídeo neutro, rico em aminoácidos hidrofóbicos e insolúvel em água (BOREL & KIS, 1991). Possui potentes propriedades imunossupressivas e tem sido amplamente usado na terapia de transplantes de órgãos, aumentando as taxas de sobrevivência dos enxertos, e no tratamento de algumas doenças auto-imunes.

A CsA liga-se a uma proteína específica, a ciclofilina, formando um complexo que inibe a atividade fosfatase da calcinerina, a qual regula a translocação nuclear e subseqüente ativação dos fatores de transcrição NFAT (Fator nuclear de ativação de células T), reduzindo assim a produção da interleucina 2 (IL-2), essencial na proliferação de linfócitos (MATSUDA & KOYASU, 2000; LANGONE & HELDERMAN, 2004). Recentes estudos indicam que CsA também bloqueia a ativação de JNK (c-Jun NH_2-

terminal kinase) e a rota de sinalização da proteína p58 desencadeada pelo reconhecimento do antígeno, sendo a CsA um inibidor altamente específico da ativação de células T (MATSUDA & KOYASU, 2000).

Apesar da poderosa propriedade imunossupressora, a CsA tem efeitos colaterais tanto em animais experimentais quanto em pacientes. Severa nefrotoxicidade associada com a droga é extensivamente documentada e é um fator dose-limitante para sua administração (MASON, 1990). Além disso, hepatoxicidade, tremor, hipertensão, hiperlipidemia, hipercalcemia, hipertricose, hiperplasia gengival (LEE & CANAFAX, 1996; READY, 2004), disfunção testicular (SEETHALAKSHMI et al., 1987, 1990 a e b) e ginecomastia (RAJFER et al., 1987) foram documentadas.

SEETHALAKSHMI et al. (1987), avaliando os efeitos da CsA na reprodução de ratos machos, observaram que a droga reduz o peso corporal e o dos órgãos reprodutivos e o número de espermatozóides testiculares e epididimais. Em adição, foram observados redução dose-dependente nos níveis de testosterona sérica e aumento nos níveis de LH e FSH. Essas modificações foram acompanhadas por degeneração de espermatócitos primários, espermátides arredondadas e espermatozóides. O desenvolvimento pós-testicular e a maturação de espermatozóides no epidídimo também foram afetados, uma vez que houve retenção das vesículas citoplasmáticas na peça intermediária dos espermatozóides e defeitos na cabeça e cauda dos mesmos. Essas alterações morfológicas podem explicar a diminuição da motilidade e da capacidade de fertilização dos espermatozóides de ratos tratados com CsA (SEETHALAKSHMI et al., 1987).

IWASAKI et al. (1995) observaram que o tratamento de ratos com CsA diminui o diâmetro dos túbulos seminíferos e a porcentagem de túbulos com espermatozóides, e aumenta os níveis séricos de FSH, o que indica injúria nas células de Sertoli. Redução no diâmetro dos túbulos seminíferos, no peso testicular, no número de espermatozóides no epidídimo e na fertilidade também foi observada em ratos que receberam CsA desde o nascimento até a idade adulta (SRINIVAS et al., 1998).

Um estudo para verificar se a CsA pode inibir a produção de andrógenos foi conduzido por RAJFER et al. (1987). Os resultados indicaram que a administração de CsA em doses iguais ou maiores que 15 mg/Kg/dia, por um mês, leva à diminuição nos níveis

séricos e intratesticulares de testosterona. Essas doses são administradas a pacientes transplantados e podem explicar o desenvolvimento de certos efeitos colaterais da droga, como por exemplo a ginecomastia, que ocorre como resultado do desequilíbrio na razão de andrógenos periféricos em relação ao estrógeno.

O mecanismo pelo qual a CsA pode afetar a reprodução em machos ainda não é claro. Inicialmente foi sugerido que alterações nos testículos poderiam ser resultado da toxicidade da droga no fígado e nos rins. É conhecido que o mau funcionamento do fígado e a falência renal podem influenciar as funções testiculares (LIM & FANG, 1975). Entretanto, essa possibilidade foi excluída, uma vez que animais que receberam baixas doses da droga (10 mg/Kg/dia) apresentaram alterações nos testículos e mantiveram as funções renais normais (SEETHALAKSHMI et al., 1987; IWASAKI et al., 1995).

Um mecanismo de ação da CsA nos órgãos reprodutores masculinos poderia ser devido à diminuição da produção de testosterona (SEETHALAKSHMI et al., 1987; IWASAKI et al., 1995). Está bem documentado que a espermatogênese e maturação dos espermatozóides são dependentes de altas concentrações de andrógenos intratesticulares e circulantes, respectivamente (SHARPE, 1994). A redução nos níveis plasmáticos de testosterona pode ser devido à degeneração das células de Leydig ou inibição da esteroidogênese testicular (SEETHALAKSHMI et al., 1987). CAVALLINI et al. (1990) relataram atrofia das células de Leydig em ratos tratados com CsA. Essa atrofia foi principalmente devido à diminuição de mitocôndrias e retículo endoplasmático liso, ou seja, de organelas que contém enzimas para a biossíntese de testosterona. Além disso, foi mostrado que há aumento de vesículas citoplasmáticas contendo colesterol nas células de Leydig dos animais tratados. KRUEGER et al. (1991), estudando como a biossíntese de testosterona em ratos pode ser afetada pela CsA, encontraram redução nos receptores de LH das células de Leydig, o que significativamente reduz a capacidade dos testículos em responder a concentrações normais ou elevadas de LH circulante. Também foi observada redução na enzima citocromo P450-dependente que cliva as cadeias laterais de colesterol mitocondrial, sendo esta atividade um passo limitante na esteroidogênese, além da redução da atividade do complexo enzimático citocromo microssomal 17α-hidroxilase que é P-450-dependente.

Recentes estudos ultra-estruturais (MASUDA et al., 2003) avaliaram as mudanças nas células espermatogênicas e na espermiogênese de ratos tratados com CsA. Os túbulos seminíferos estavam atrofiados e com descamação de espermátides arredondadas. Muitos corpos residuais contendo um ou mais flagelos foram encontrados no lúmen dos túbulos seminíferos. Esses corpos residuais não foram fagocitados pelas células de Sertoli e acumularam-se nos ductos epididimais. Além disso, observaram-se espermátides arredondadas degeneradas e espermatozóides anormais no epidídimo, com desconexão da cabeça e cauda e flagelos anormais. Esses resultados sugerem que a CsA pode reduzir a capacidade fagocitária das células de Sertoli, causar má formação de espermátides e fragilidade dos espermatozóides (MASUDA et al., 2003).

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

Esta espécie foi descrita por HOEHNE (1920) como tendo propriedades afrodisíacas e estimulantes, sendo chamada de *Heteropterys afrodisiaca*, e atribuída à família Malpighiaceae por Othon X.B. Machado, em 1949 (PIO CORRÊA, 1984). É uma planta arbustiva, de 0,6 a 2,0 metros de altura, encontrada no cerrado de Mato Grosso, Goiás e norte de Minas Gerais. Normalmente, a população usa as raízes como tônico ou estimulante e para o tratamento de debilidades do sistema nervoso (PIO CORRÊA, 1984; POTT & POTT, 1994; GUARIM NETO, 1996).

H. afrodisiaca, do ponto de vista etnobotânico, 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).

RIZZINI (1983) descreve as raízes de *H. afrodisiaca* como sendo estimulantes e fazendo parte de um elenco de 65 espécies de plantas com princípios psicoativos conhecidas no Brasil.

GUARIM NETO (1996), em seus estudos etnobotânicos realizados em 25 cidades do estado do Mato Grosso, relata que o chá preparado com as raízes é empregado como depurativo do sangue, dentre outras indicações. Entretanto, o emprego mais difundido é sob a forma de garrafadas, tidas como afrodisíacas.

Estudos com esta espécie encontraram resultados interessantes envolvendo propriedades que mostraram melhora da memória em experimento com grupos de ratos idosos (GALVÃO et al., 2002).

CHIEREGATTO (2005) relata que ratos tratados com diferentes concentrações do extrato de *H. afrodisiaca,* por 56 dias, apresentaram aumento na massa corporal e testicular, bem como aumento significativo na proporção de interstício no parênquima testicular. Essa diferença foi devido, principalmente, ao aumento na proporção de células de Leydig em relação aos animais controle e aos tratados com *Anemopaegma arvense* (vergateza), outra espécie considerada afrodisíaca no centro-oeste brasileiro. Foi encontrado também, aumento na altura do epitélio seminífero e no diâmetro tubular e redução no comprimento dos túbulos seminíferos. As células de Leydig apresentaram maior volume individual e volume total no testículo, em relação aos demais ratos controle e tratados com vergateza. Como não houve variação no número total de células por testículo, o aumento volumétrico descrito é justificado pela hipertrofia individual destas células. De acordo com CASTRO et al. (2002), o volume nuclear da célula de Leydig está altamente correlacionado com o nível de testosterona testicular e plasmático. Então, CHIEREGATTO (2005) propõe que os animais tratados com *H. aphrodisiaca* apresentavam níveis mais elevados de testosterona que o grupo controle, apesar de não verificar aumento significativo na massa das glândulas vesiculares, as quais são andrógeno-dependentes. Entretanto, a massa corporal desses animais apresentou-se significativamente maior do que aquela dos animais controle, provavelmente porque a testosterona promoveu efeito anabolizante na massa muscular dos animais tratados com *H. aphrodisiaca*.

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5. OBJETIVOS

5.1. Gerais

Avaliar se os efeitos da administração de doses terapêuticas de Ciclosporina nos testículos de ratos Wistar podem ser aliviados pela administração, concomitante e seqüencial, da infusão de *Heteropterys aphrodisiaca.*

5.2. Específicos

- Analisar as alterações morfológicas, através de microscopia de luz e eletrônica de transmissão, nos testículos de ratos Wistar;
- Avaliar quantitativamente, através de análises histométricas, possíveis alterações nos componentes do testículo;
- Quantificar os níveis plasmáticos de testosterona nos animais controle e tratados.

6. RESULTADOS

A partir dos resultados obtidos no presente trabalho foram elaborados dois artigos científicos:

Artigo 01 - Can *Heteropterys aphrodisiaca* Infusion Counterbalance the Colateral Effects of Cyclosporine A on the Male Reproductive System?

Artigo 02 - Could *Heteropterys arphrodisiaca* Infusion Help Maintain Testicular Ultrastructure Altered by Cyclosporine Use?

Can *Heteropterys aphrodisiaca* Infusion Counterbalance the Colateral Effects of Cyclosporine A on the Male Reproductive System?

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Abstract

Cyclosporine A (CsA) is known to have testicular toxicity, leading to male infertility. Stimulant and aphrodisiac properties have been attributed to the plant, *Heteropterys aphrodisiaca*. Data from previous experiments suggest that the root extract can increase the volume of Leydig cells in rat testis. Thus, the present work was undertaken to evaluate the association of the drug and the medicinal herb in Wistar rats, applying testicular morphometry. Thirty rats were used, divided into five groups: I-control; II–CsA; III–simultaneous use of CsA and *H. aphrodisiaca*; IV-*H. aphrodisiaca* for 30 days and CsA sequentially for another 26 days; V-*H. aphrodisiaca*. Daily administration by gavage was carried out, during 56 days, of water (sham), CsA at a dose of 15 mg/kg/day and *H. aphrodisiaca* at a dose of 0.5 ml of the infusion prepared with 25g of roots/100ml boiling water. Increased body weight was observed in all groups, but the animals that received CsA for 56 days showed the smallest body weight gain. The testis and accessory sex organs weights were unchanged. Morphometry showed a increase in connective tissue volumetric proportion and decreased Leydig cell volumetric proportion, in CsA-treated rats. The Leydig cells of CsA-treated animals were smaller, with very dense cytoplasm in relation to other groups. On the other hand, the *H. aphrodisiaca* infusion resulted in an increase in Leydig cell nucleus volume. The plasma testosterone levels and number of Leydig cells per testis increased significantly in group III. The association of CsA and *H. aphrodisiaca* is effective in maintaining rat testis structure.

Key Words: Cyclosporine, *Heteropterys aphrodisiaca*, Wistar rats, testes, morphometry, testosterone.

Introduction

Cyclosporin A is a highly lipophylic cyclic undecapeptide, produced by the fungi *Cylindrocarpon lucidum* Booth and *Tolypocladium inflatum* Gams (Hess & Colombani, 1986). It is known to possess a variety of biological and physiological actions such as antiparasitic, fungicidal, anti-inflammatory effects and immunosuppressive properties. Up to now, it has been used in medical treatment as an immunosuppressant after organ transplantation and as a cure for several autoimmune disorders such as psoriasis, atopic dermatitis, idiopathic nephritic syndrome, inflammatory bowel disease and rheumatoid arthritis (Hagar, 2004). However, CsA has certain adverse side effects, including renal and hepatic cytotoxicities and testicular dysfunction. CsA administration caused degenerative changes of seminiferous tubules and impaired spermatogenesis. Anomalous sperm are found in the epididymal ducts, as well as and decreased sperm count and motility, resulting in male sterility (Seethalakshmi et al, 1987; Iwasaki et al, 1995). Seethalakshmi et al (1987) and Rajfer et al (1987) suggested that the spermatogenesis inhibition manifested resulted from suppression of testosterone production by the drug.

Heteropterys aphrodisiaca O. Mach. (Malpighiaceae) was described by Hoehne in 1920 as a plant with stimulant and aphrodisiac properties and its botanical name was given by Othon Machado in 1949 (Machado, 1949). The plant is found mainly in the "cerrado" regions of the states of Mato Grosso and Goiás in central Brazil, and is also known as 'nó-de-cachorro', 'nó-de-porco' and 'cordão-de-São-Francisco' (Pio Corrêa, 1984; Pott and Pott, 1994). The local population uses *H. aphrodisiaca* roots as an aphrodisiac, a tonic or stimulant and for the treatment of nervous debility and nervous breakdown (Pio Corrêa, 1984; Pott and Pott, 1994). Qualitative analysis of the plant extract showed glycosides, polyphenols, tannins, alkaloids, saponins and anthracene derivatives as major components (Galvão, 1997). Data from the literature suggest that the root extract can increase body and testicular weight, the diameter of seminiferous tubules and individual and total Leydig cell volume in rat testis (Chieregatto, 2005).

Because of the frequency with which long-term CsA therapy is being used in the organ transplant population and in autoimmune disease patients, and the early age of many

of these patients, the side effects can be serious. Therefore, we administered therapeutic doses of CsA, such as those used in clinical treatment. Our primary aim was to determine whether or not the effects of therapeutic doses of CsA could be alleviated by simultaneous and sequential administration of *H. aphrodisiaca* infusion, evaluating the testicular morphometry.

Materials and Methods

Experimental animals

Adult Wistar rats, 90 days old and weighting 314-380g, were obtained from the Multidisciplinary Center for Biological Investigation (State University of Campinas, Campinas, SP, Brazil). The rats were housed, three per cage, under standard conditions with 12hrL:12hrD. Animals were provided with rat feed and water *ad libitum*. The Institutional Animal Care and Use Committee of this University approved the experimental protocol.

Medicinal plant

Roots of *H. aphrodisiaca* were collected in February 2005, in Mato Grosso State, Brazil. *H. aphrodisiac* was identified by comparison with the voucher herbarium specimen of the plant of the Herbarium Federal University of Mato Grosso, Brazil (number 23928). The roots were crushed and powdered using a grinding mill. The infusion of *H. aphrodisiaca* was routinely prepared by pouring boiling water over the powdered roots, that were allowed to steep for 4 hours and filtered using filter paper. This produced an infusion of 68.66 mg of extract dry weight per ml of infusion (6.866% w/v) and a yield of 6.832% (w/w) in terms of initial crude dry weight of plant material.

Treatment protocol

Thirty animals were used, divided into five groups:

I - control (sham);

II – CsA treatment for 56 days;

III - simultaneous use of CsA and *H. aphrodisiaca* infusion for 56 days;

IV- *H. aphrodisiaca* infusion for 30 days and CsA sequentially for another 26 days;

V- only *H. aphrodisiaca* infusion for 56 days.

Cyclosporine A (Sandimmun Neoral – Oral Solution, 100mg/ml - Novartis Pharma AG, Switzerland) was administered at a dose of 15 mg/kg/day dissolved in 0.5 mL of distilled water and *H. aphrodisiaca* at a dose of 0.5 ml of the infusion prepared with 25 g of roots/100 ml in distilled boiling water. All animals were weighed weekly and the CsA dose was calculated accordingly. The treatments and water (sham) were administered daily by gavage, during 56 days, since this interval represents the duration of spermatogenesis according to Russell (1990).

Preparation of tissue for light microscopy

Rats were anesthetized with Xylazine and Ketamine (5 and 80 mg/Kg body weight, respectively) and the abdomen and thoracic cavity was opened. The animals were fixed by whole body perfusion. After a brief saline wash to clear the blood vessels, the animals were perfused with glutaraldehyde 2.5% and paraformaldehyde 4% in 0.1 M phosphate buffer, at pH 7.2 for 25-30 min. The testis, epididymis, seminal vesicle and coagulation gland were dissected out. Testis tissue was post fixed in the same solution overnight and prepared for embedding in hydroxyethyl methacrylate (Historesin®, Leica), using routine technique. Subsequently, 3 µm thick sections were obtained, stained with toluidine blue and observed in light microscope.

Hormone assay

Before perfusion, blood samples were drawn from the caudal vena cava to measure testosterone levels in peripheral blood. Testosterone in plasma of rats was measured by radioimmunoassay method by using the commercial Kit Testosterone Total RIA, Catalogue # DSL 4000. The reading was realized in accountant Gamma Wizard-Perkin Elmer.

Biometric parameters

The reproductive organs were carefully freed from surrounding fat and connective tissue and weighed. One testis was dissected to remove the tunica albuginea. The gonadosomatic index (GSI), that is, the percent of body mass allocated to the testis, was obtained from testes mass divided by body weight of each animal x 100.

Volume and volumetric proportion (%) of the testis compartments

All morphometrical measurements were performed using Image Pro Plus software on Olympus BX-40 microscope. The volumetric proportions of various testicular tissue components were determined on methacrylate-embedded testes. A point-counting system was used to measure volumetric proportion of seminiferous tubules and interstitium. A 432-point grid was superimposed over each section viewed using a 40x objective lens, where ten randomly sampled areas were examined per tissue section (4320 points). Points were classified as one of the following: seminiferous tubule, Leydig cell, blood vessels, lymphatic space and connective tissue. The number of point intersections superimposed on the above components was counted and the volumetric proportion of testicular components was obtained by dividing the number of intersection points on each structure by the total number of points, multiplying by 100. The volume of each testis component was determined as the product of the volumetric proportion and testicular volume. For subsequent morphometric calculations, the specific gravity of testis tissue was considered to be 1.0 (Mori and Christensen, 1980). To obtain a more precise measurement of testis volume, the tunica albuginea was excluded from testis weight.

Leydig cell morphometry

Individual volume of a Leydig cell was obtained from the nucleus volume and the proportion between nucleus and cytoplasm. The average diameter of the Leydig cell nucleus was obtained assessing 10 cells/animal with the Image Pro Plus software associated to an Olympus BX-40 microscope. For this value, the $4/3\pi r^3$ formula was used, where "r" was the mean nucleus radius that was determined to estimate the nuclear volume of each Leydig cell. To calculate the proportion between nucleus and cytoplasm, a 432-point grid

was superimposed over each section viewed using a 1000X magnification. Over one thousand points on Leydig cells were counted for each animal. The number of Leydig cells per testis was estimated from the Leydig cell's individual volume and the volume occupied by Leydig cells in the testis parenchyma.

Statistical analysis

All data was presented as the mean ± SEM (standard error mean) and analyzed via ANOVA (Tukey test) by using the System for Statistical Analysis (SAEG 9.0). The significance level was $p<0.05$.

Results

Organ weights and plasma testosterone levels

A statistically significant difference ($p<0.05$) in gain of body weight was observed between groups that received CsA for 56 days (II and III), which gained less weight than the control group (Table 1). Group V had higher values compared to the control and the other treated groups; however, statistical significance was not observed between control and group V rats, due to the higher SEM in the latter group. For this group, the average body weight was reduced, due to the unusually small weight gain of one of the rats of this group. The gonadosomatic index and the testis, epididymis, seminal vesicle, coagulation gland, dorso-lateral and ventral prostate weights were not significantly different in any of the groups studied (Table 1). Plasma testosterone levels increased significantly in group III (Table 1).

Volume and volumetric proportion measurements

There was no difference in the volumetric proportion of testicular compartments between control and treated animals (Figure 1). The volume of the testis components and volumetric proportions of the intertubular compartment components are given in Table 2 and 3, respectively. The morphometric measurements for the intertubular compartment volume components were similar except for the connective tissue, where there was a

significant increase in all groups that received CsA compared with the control group. The volumetric proportion of connective tissue was significantly increased in group IV compared with the control group. The volumetric proportion of Leydig cells and blood vessels was significantly reduced in group II and group III, respectively, compared with the control group.

Leydig cell morphometry

The Leydig cell morphometry data can be found in Table 4. No statistically significant differences were observed between the control and the treated groups for the nuclei diameter of Leydig cells. The volume of a Leydig cell in the treated groups was not significantly different from that of the control rats. On the other hand, the nuclear volume of Leydig cells was significantly increased in group V, when compared with the control and other treated groups. The cytoplasmic volume of a Leydig cell was not significantly different among all experimental groups. The total number of Leydig cells per testis and per gram of testis increased significantly in group III.

Histology

Light microscopy of treated groups and controls revealed few histological changes in the testis structure. The seminiferous epithelium of all animals studied showed the cell and tissue architecture expected for full spermatogenic activity. Germ cells of all types were arranged in a complex stratified epithelium. The Sertoli cells showed typical mature morphology, notably large basally located nuclei with a prominent nucleolus (Figure 2). In treated rat testes, interstitial tissue was, for the most part, similar to that of the control animals, containing vascular elements, connective tissue, macrophages, lymphatic space and Leydig cells (Figure 3). However, the testicular interstitial tissue of rats that received only *H. aphrodisiaca* infusion contained clusters of Leydig cells with large nuclei, suggesting intensified metabolism, when compared to the control group and the animals that received only CsA. The majority of the Leydig cells of animals that received only CsA were smaller in size and this was confirmed by the present morphometric results.

Moreover, the shape of Leydig cells in these animals was elongated and sometimes irregular

Discussion

We administered therapeutic doses of CsA, such as those used in clinical treatment. Our primary aim was to determine whether or not the effects of therapeutic doses of CsA on testes and other reproductive organs could be alleviated by simultaneous or sequential administration of *H. aphrodisiaca* infusion. Since therapeutic doses were used, the damages found in literature with toxic doses were not observed in this experiment, such as decreasing weight of reproductive organs and testosterone levels.

The body weight in experimental animals has been assessed to determine the effect of the treatment on the general health of the animals (Seethallakshmi et al, 1987). The treatment with CsA caused decreased body weight (Seethallakshmi et al, 1987; Iwasaki et al, 1995) when high doses were administered (above of 20 mg/Kg of BW) but body weight was not affected with physiological doses, of 5 to 20 mg/Kg of BW (Sikka et al, 1988; Villanúa et al, 1996). In the present study, we observed an increase in body weight in the control and in all treated animals, but in the CsA-treated animals and CsA and *H. aphrodisiaca* simultaneous treatment, the body weight gain was statistically lower than other groups. Considering that we used physiological doses of CsA, these lower body weight gain can be related to the length of treatment with the drug (56 days).

Chieregatto (2005) observed a significant gain in body weight in *H. aphrodisiaca* treated animals and suggested that the weight gain was due to the anabolic property of testosterone on the muscle mass of these rats. The same effect could have occurred in this study for the animals that received only *H. aphrodisiaca*, since the same dose and treatment period was used.

Cyclosporine A treatment did not produce changes in testicular weight or in the weights of the other reproductive organs, in agreement with some authors (Rajfer et al, 1987; Seethallakshmi et al, 1990a; Villanúa et al, 1996) but differing from others (Sikka et al, 1988; Seethallakshmi et al, 1990b). These differences can be related to different doses of

CsA, as well as length of period and route of drug administration in the various studies. It is likely that species sensitivity, dose, time and drug administration route in the different treatments are the primary reasons for obtaining contrasting results (Sprando et al, 1998).

Chieregatto (2005) found significantly increased testicular weights for rats that received *H. aphrodisiaca* infusion, but the seminal vesicle weight did not increase. Greater testicular weight was associated with significantly higher interstitial tissue volume, since the seminiferous tubule volume did not alter between control and treated groups. In spite of following the same doses and treatment period as those used by Chieregatto (2005), the testicular and sexual accessory organs weights did not alter with the *H. aphrodisiaca* infusion administration in the present study.

Functionally, the mammal testis is divided in two basic compartments: the tubular (or spermatogenic) and the intertubular (or androgenic) portions. In rats, 90% (volumetric proportion) of testicular parenchyma is occupied by seminiferous tubules and 10% by interstitial tissue (Russell and França, 1995; Kim and Yang, 1999). In the present study, different values were found for these portions, but volume in the treated animals was similar to the control, suggesting that the treatments did not alter the volume or the volumetric proportion of testicular compartments. In the present study, a reduction in the volumetric proportion of blood vessels was observed in the simultaneous treatment with CsA and *H. aphrodisiaca.* The significance of this finding is not clear since the testes of these animals were less affected than in the CsA-treated animals.

The animals that received only *H. aphrodisiaca* showed a mean volumetric proportion of Leydig cells that was numerically higher than controls, but the mean values recorded were not significantly different. Although the bulk of the data suggests no significant difference in these parameters, we suggest that volumetric proportion of Leydig cells is slightly higher in animals treated with *H. aphrodisiaca*. This is based on a rather consistent trend in numerical values and observation of histological slides and ultrastructural analysis (data not included), made in this research and the earlier study (Chieregatto, 2005).

On the other hand, the CsA-treated animals showed a decrease in volumetric proportion and volume of the Leydig cells. The administration of 20 mg/Kg of CsA daily,

for 14 days, resulted in Leydig cells undergoing pronounced degeneration and the treatment of rats with 40 mg/Kg left only blood vessels and fibroblasts in the interstitium (Seethallakshmi et al, 1987). This result corroborates the lower volumetric proportion of the Leydig cell and the rise in the connective tissue volume in the rats that received only CsA, in the present study.

The treatments with CsA or *H. aphrodisiaca* did not alter the plasma testosterone levels of the treated animals, with the exception of the group that received simultaneous treateatment with CsA and *H. aphrodisiaca*. Reduction in testosterone levels was previously described in both sexually mature and immature CsA-treated rats (Seethallakshmi et al, 1987; Sikka et al, 1988; Seethallakshmi et al, 1990a). However, these authors also found decreased intratesticular levels of testosterone using a different CsA dose from that employed in the present study (20-40 mg/Kg body weight in sexually mature rats versus 15 mg/Kg body weight in this research). Using a lower dose of CsA in adult male rats (7.5 mg/Kg body weight administered, orally, for 28 days), Rajfer et al (1987) did not find the previously described inhibitory effect on testicular testosterone, although plasma levels of testosterone tended to be suppressed. Vexian et al (1990) found an increase in the activity of the 5 alfa-reductase in peripheral tissues, after CsA treatment (7.5 or 10 mg/Kg/day) in humans. Moreover, Krieger et al (1991) found a decrease in plasma T during CsA therapy (25 or 40 mg/Kg body weight, subcutaneous, for 6 days) with reduction in testicular testosterone with the higher dose. Collectively, these data suggest differential effects of the drug on the endocrine system. The alterations are dependent on the dose and the length of period of treatment.

H. aphrodisiaca infusion, as a pre-treatment or simultaneous to CsA treatment was effective in maintaining the Leydig cell structure. This cell is the main producer of androgens, since the animals of these groups did not present alterations in morphology nor volumetric proportion of Leydig cells as was found in animals treated only with CsA. The animals of group that received simultaneous treatment with CsA and *H. aphrodisiaca* presented a greater number of Leydig cells in the testis, which would explain the higher levels of plasma testosterone in this group. Castro et al (2002) observed a significant correlation between the number of Leydig cells and both plasma and testicular levels of

testosterone in rabbit. It suggested that the increase in testosterone might occur as a consequence of the increase in the population of androgen-secreting cells.

Although the plasma concentration of testosterone was not affected in rats that received only the plant infusion, the morphometric data (diameter and nuclear volume of the Leydig cells), as well as structural and ultrastructural aspects (not shown) suggest that this group presented a superior androgen production in relation to the other animals. Future studies will be untaken to confirm this hypothesis. The discrepancy of the plasma testosterone levels in the present study might be due to differences in the hour of collection of blood samples in all the animals. The plasma levels of testosterone can vary in pulses throughout the day, according to the peaks of LH liberation by the pituitary gland (Genuth, 2004). Also, the plasma testosterone levels follow a regular daily tendency; the levels are about 25% lower at 8:00 pm in relation to the level at 8:00 am (Genuth, 2004).

The results of the present study suggest that the association of CsA and *H. aphrodisiaca* is effective in maintaining rat testis structure based on our histological and morphometrical data, since the morphometric alterations typical of the CsA group were not found in other groups. The simultaneous treatment with CsA and *H. aphrodisica* infusion is more effective, since there was a significant increase in the Leydig cell numbers found in these rats. Although the plasma testosterone levels in *H. aphrodisiaca*-treated animals did not increase, the morphometrical and morphological data indicated that testicular androgen production was probably greater than in the control and CsA-treated animals. The discrepancy of plasma testosterone levels in the present study might be due to differences in the time of day at which blood samples were collected for all the animals. Therefore, we suggest that the higher testosterone level exerted an anabolic effect on the muscle mass of these rats, rather than a sexual stimulant effect, since the accessory sex organs weights were unchanged. Clearly, additional research must be untaken to evaluate this possibility.

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Tables

Table 1- Biometric data and testosterone levels in adult Wistar treated with CsA and/or *Heteropterys aphrodisiaca*

Parameters	Group I	Group II	Group III	Group IV	Group V
Body Weight gain (g)	94.00 ± 16.80^{b}	40.17 ± 1.45^{a}	41.83 ± 5.09^{a}	95.83 ± 15.03^{b}	106.17 ± 25.73^{b}
Testis weight (g)	1.58 ± 0.06	1.47 ± 0.07	1.56 ± 0.06	1.64 ± 0.02	1.57 ± 0.08
Gonadosomatic index (%)	0.82 ± 0.05	0.73 ± 0.04	0.79 ± 0.03	0.73 ± 0.01	0.71 ± 0.05
Epididymis weight (g)	0.54 ± 0.02	0.52 ± 0.2	0.56 ± 0.02	0.53 ± 0.01	0.59 ± 0.03
Seminal Vesicle weight (g)	1.11 ± 0.13	0.92 ± 0.04	0.94 ± 0.04	1.07 ± 0.04	1.11 ± 0.07
Coagulating Gland weight (g)	0.21 ± 0.02	0.21 ± 0.01	0.22 ± 0.01	0.22 ± 0.02	0.22 ± 0.01
Dorso-lateral Prostate weight (g)	0.35 ± 0.03	0.39 ± 0.03	0.36 ± 0.04	0.34 ± 0.02	0.39 ± 0.03
Ventral Prostate weight (g)	0.44 ± 0.04	0.40 ± 0.02	0.39 ± 0.03	0.51 ± 0.02	0.45 ± 0.02
Testosterone (ng/mL)	1.30 ± 0.44^{a}	1.84 ± 0.43^{ab}	3.59 ± 0.75^{b}	1.40 ± 0.35^{a}	1.07 ± 0.18^{a}

Group I - control; Group II – CsA; Group III - CsA + *H. aphrodisiaca;* Group IV - *H. aphrodisiaca* followed by CsA; Group V- *H. aphrodisiaca.*

The values are the means ± SEM; n = 6 for each group;

[a-b] In each row, values with different superscripts are significantly different ($p < 0.05$).

Table 2- Volume (mL) of the testis components in adult Wistar treated with CsA and/or *Heteropterys aphrodisiaca*

Parameters	Group I	Group II	Group III	Group IV	Group V
Seminiferous tubule	1.21 ± 0.05^{ab}	1.02 ± 0.05^{a}	1.05 ± 0.06^{ab}	1.19 ± 0.03^{ab}	1.24 ± 0.05^{b}
Interstitial Tissue	0.37 ± 0.10	0.45 ± 0.04	0.50 ± 0.05	0.45 ± 0.03	0.30 ± 0.03
Lymphatic space	0.22 ± 0.08	0.30 ± 0.04	0.32 ± 0.05	0.26 ± 0.05	0.16 ± 0.02
Connective tissue	0.05 ± 0.01^{a}	0.06 ± 0.00^{b}	0.06 ± 0.01^{b}	$0.07 \pm 0.0b^{b}$	0.03 ± 0.01^{a}
Blood vessels	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
Leydig Cell	0.08 ± 0.02	0.06 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.01

Group I - control; Group II – CsA; Group III - CsA + *H. aphrodisiaca;* Group IV - *H. aphrodisiaca* followed by CsA; Group V- *H. aphrodisiaca.*

The values are the means ± SEM; n = 6 for each group;

[a-b] In each row, values with different superscripts are significantly different ($p < 0.05$).

Table 3- Volumetric proportion (%) of the components of the intertubular compartment in adult Wistar treated with CsA and/or *Heteropterys aphrodisiaca*

Parameters	Group I	Group II	Group III	Group IV	Group V
Lymphatic space	54.47 ± 3.53	65.51 ± 2.47	62.20 ± 4.95	56.55 ± 4.40	51.21 ± 2.85
Connective tissue	8.38 ± 2.31^{a}	13.09 ± 0.94^{ab}	11.80 ± 1.24^{ab}	15.94 ± 1.82^{b}	10.10 ± 1.33^{ab}
Blood vessels	13.69 ± 1.85^{b}	7.82 ± 1.30^{ab}	6.07 ± 1.00^{a}	8.12 ± 1.07^{ab}	12.14 ± 2.50^{ab}
Leydig Cell	23.46 ± 1.53^{b}	13.58 ± 1.18^{a}	19.93 ± 3.64^{ab}	19.40 ± 1.97^{ab}	26.27 ± 2.05^{b}

Group I - control; Group II – CsA; Group III - CsA + *H. aphrodisiaca;* Group IV - *H. aphrodisiaca* followed by CsA; Group V- *H. aphrodisiaca.*

The values are the means ± SEM; n = 6 for each group;

[a-b] In each row, values with different superscripts are significantly different ($p < 0.05$).

Table 4- Leydig cell morphometry in adult Wistar rats treated with CsA and/or *Heteropterys aphrodisiaca*

Parameters	Group I	Group II	Group III	Group IV	Group V
Nucleus Diameter LC (μm)	6.27 ± 0.07[ab]	5.66 ± 0.16[a]	5.80 ± 0.11[a]	6.02 ± 0.20[a]	6.91 ± 0.20[b]
Volume of a LC (μm3)	432.55 ± 28.11[ab]	338.79 ± 38.86[a]	318.20 ± 29.91[a]	402.06 ± 38.14[ab]	503.65 ± 47.09[b]
Nucleus Volume (μm^3)	129.11 ± 3.99[a]	96.30 ± 8.25[a]	102.87 ± 6.15[a]	115.94 ± 10.92[a]	174.65 ± 14.50[b]
Cytoplasm Volume (μm^3)	306.44 ± 24.13	242.45 ± 32.63	215.33 ± 24.25	286.12 ± 29.06	329.00 ± 33.75
Number LC/testis (10^6)	189.90± 37.96[a]	181.72 ± 21.36[a]	299.05 ± 28.81[b]	220.19 ± 20.30[ab]	156.40 ± 15.50[a]
Number LC/g testis (10^6)	117.70± 18.54[a]	122.59 ± 8.99[a]	190.95 ± 14.30[b]	134.19 ± 12.30[ab]	102.33 ± 13.48[a]

Group I - control; Group II – CsA; Group III - CsA + *H. aphrodisiaca;* Group IV - *H. aphrodisiaca* followed by CsA; Group V- *H. aphrodisiaca.*

The values are the means ± SEM; n = 6 for each group;

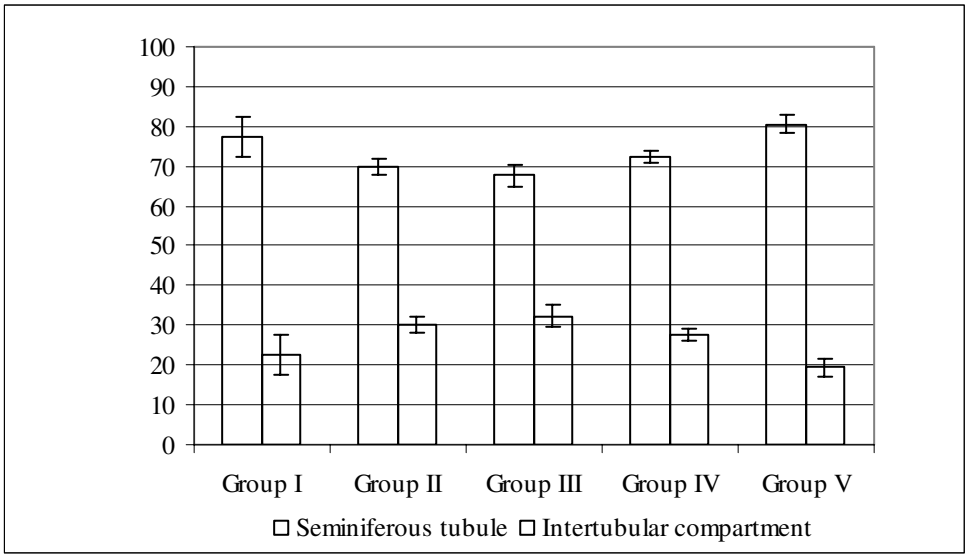
[a-b] In each row, values with different superscripts are significantly different ($p < 0.05$).

Figure Legends

Figure 1- Volumetric proportion (%) of testis compartments in adult Wistar treated with CsA and/or *Heteropterys aphrodisiaca* (mean ± SEM; n = 6 for each group). Group I - control; Group II – CsA; Group III - CsA + *H. aphrodisiaca*; Group IV - *H. aphrodisiaca* followed by CsA; Group V- *H. aphrodisiaca*.

Figure 2- Light microscopy of the testicular tissue of Wistar rats: A - control; B – CsA; C – *H. aphrodisiaca;* D - CsA + *H. aphrodisiaca*; E - *H. aphrodisiaca* followed by CsA. ST = seminiferous tubule; Ls = lymphatic space; L = Leydig cell; v = blood vessels; → = Sertoli cell. Toluidine Blue. Scale bar = 30 μm.

Figure 3- Light microscopy of testis interstitium of Wistar rats: A - control; B – CsA; C – *H. aphrodisiaca;* D - CsA + *H. aphrodisiaca*; E - *H. aphrodisiaca* followed by CsA. ST = seminiferous tubule; Ls = lymphatic space; L = Leydig cell; M = Macrophage; v = blood vessels; → = Sertoli cell. Toluidine Blue. Scale bar = 10 μm.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Group I
Group II
Group III
Group IV
Group V
Seminiferous tubule
Intertubular compartment

Figure 1

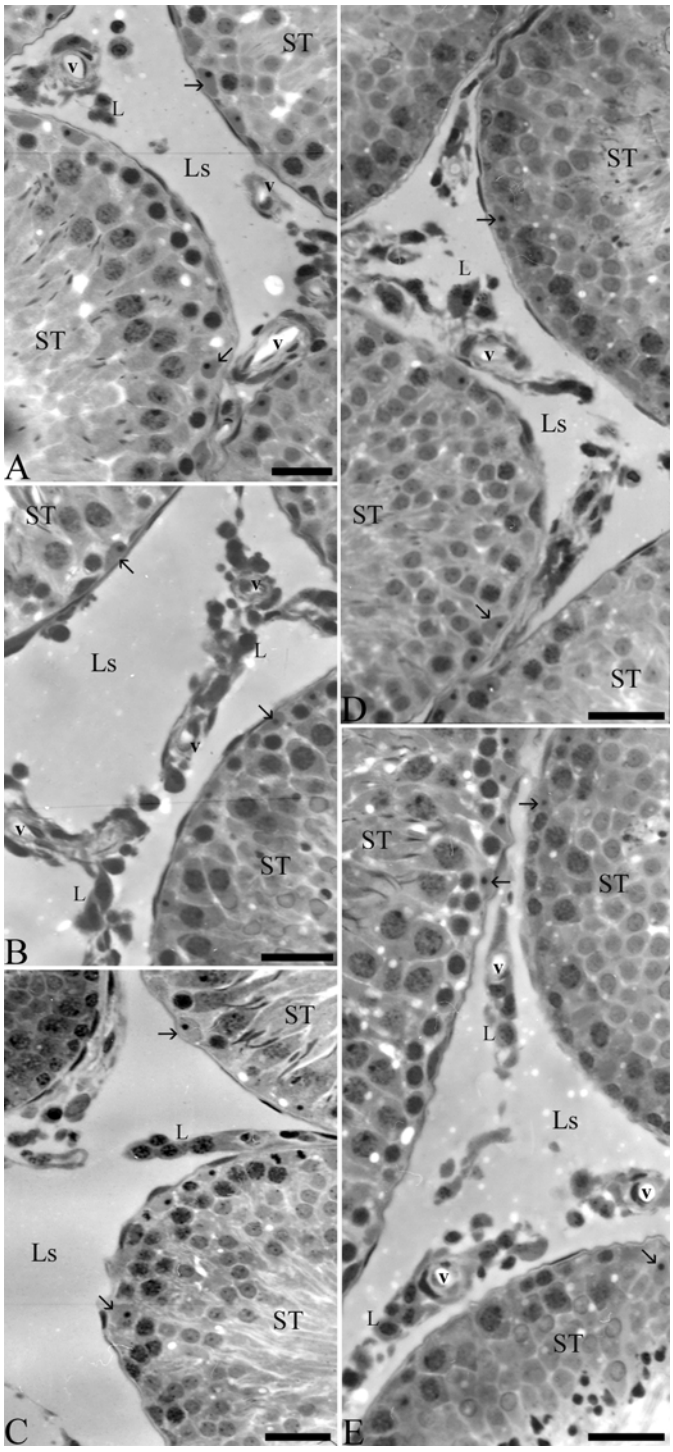
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Figure 2

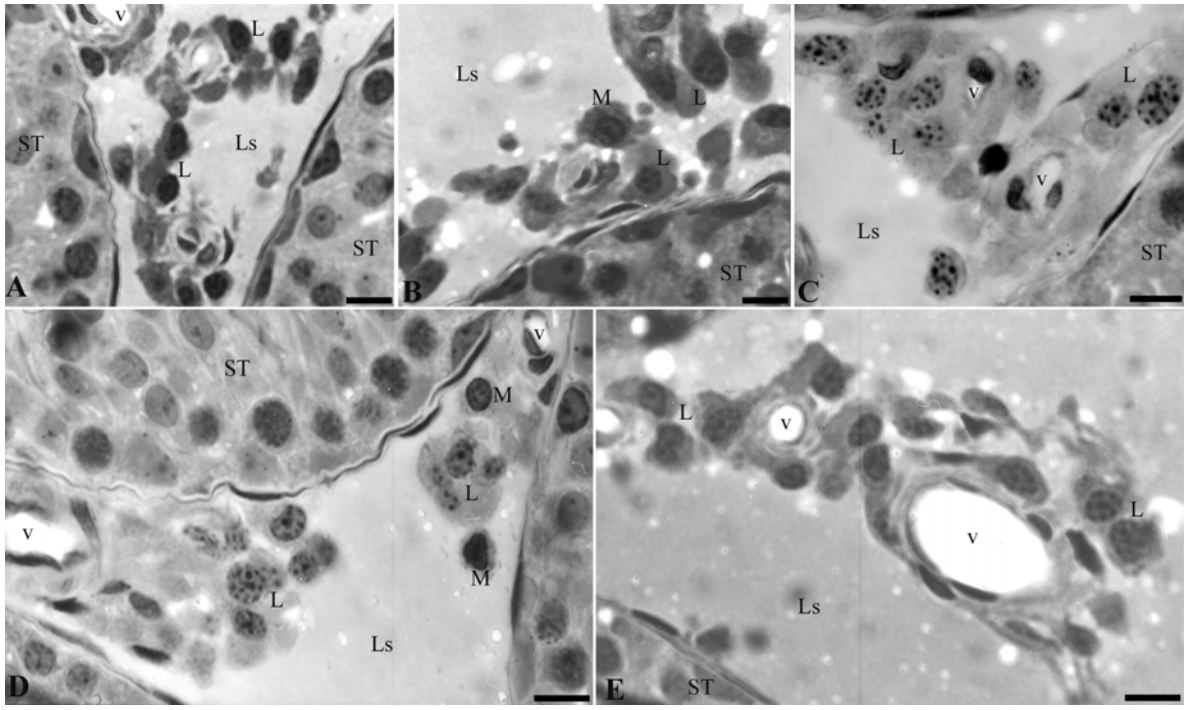

Figure 3

Could *Heteropterys aphrodisiaca* Infusion Help Maintain Testicular Ultrastructure Altered by Cyclosporine Use? *

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ABSTRACT

Cyclosporine A (CsA) is known to have testicular toxicity, leading to male infertility. Stimulant and aphrodisiac properties have been attributed to the plant, *Heteropterys aphrodisiaca*. Data from the literature suggest that the root extract can increase the volume of Leydig cells in rat testis. Thus, the present work was undertaken to study the association of the drug and the medicinal herb in adult Wistar rats, evaluating testicular ultrastructure. Thirty rats were used, divided into five groups: I- control (sham); II– use of CsA; III– simultaneous use of CsA and *H. aphrodisiaca* infusion; IV- *H. aphrodisiaca* for 30 days and CsA sequentially for another 26 days; and V- only *H. aphrodisiaca* infusion. CsA was administered daily, by gavage for 56 days, at a dose of 15 mg/kg/day and *H. aphrodisiaca* at a dose of 0.5 ml of the infusion prepared with 25 g of roots/100 ml boiling water. CsA caused degeneration of germ cells, Sertoli cell vacuolization, abnormal round spermatids and Leydig cells with atrophy of the organelles involved in steroid synthesis. *H. aphrodisiaca* caused Sertoli cell vacuolization, loss of germ cell attachment, expanded intercellular spaces between germ cells and Leydig cells with more mitochondria and smooth endoplasmic reticulum. The simultaneous and sequential treatment with *H. aphrodisiaca* and CsA effectively maintained Leydig cell ultrastructure. The modifications found for the seminiferous epithelium were a combination of the alterations identified for each individual treatment. However, *H. aphrodisiaca* protected the seminiferous epithelium of rats submitted to CsA treatment, since the seminiferous tubules observed following its use, showed less severe modifications than the testicles treated only with CsA.

Key Words: *Heteropterys aphrodisiaca*, Cyclosporine, ultrastructure, testes, Wistar rat

INTRODUCTION

Cyclosporine A (CsA) is a metabolite isolated from fungus *Tolypocladium inflatum* Gams and *Cylindrocarpon lucidum* Booth. This agent has been demonstrated to have powerful immunosuppressive characteristics due to selective action on the T-lymphocytes by inhibiting the plasmatic membrane signal needed for the formation of lymphokine mRNA transcripts [1]. Its application in organ transplantation has resulted in remarkably improved graft survival. In addition, this drug is used in the treatment of a variety of autoimmune diseases such as idiopathic nephritic syndrome, inflammatory bowel disease, psoriasis and rheumatoid arthritis [2]. However, CsA has certain adverse side-effects, including renal and hepatic cytotoxicities and testicular dysfunction. Seethalakshmi et al. [3] were the first to report that CsA administration caused degenerative changes of seminiferous tubules and a decrease in sperm count and motility, resulting in male sterility. Morphological changes induced by CsA included degeneration and atrophy of the seminiferous tubules and anomalous sperm in the epididymal ducts. Thereafter, several groups examined the action of CsA, focusing mainly on endocrinological disturbances of the Sertoli cell or Leydig cell [4-8], because spermatogenesis and spermiogenesis are maintained by proper function of these cells controlled through the hypothalamus-pituitary-gonadal axis. A decrease in serum luteinizing hormone and elevated serum follicle-stimulating hormone in CsA-treated rats, and recovery of sperm counts by treatment with testosterone or gonadotropic hormones in those rats, seemed to support the impaired function of Sertoli or Leydig cells [9,10].

Heteropterys aphrodisiaca O. Mach. (Malpighiaceae) was described by Hoehne in 1920 as a plant with stimulant and aphrodisiac properties and its botanical name was given by Othon Machado in 1949 [11]. The plant is found mainly in the “cerrado” regions of the states of Mato Grosso and Goiás in central Brazil, and is also known as ‘nó-de-cachorro’, ‘nó-de-porco’ and ‘cordão-de-São-Francisco’ [12,13]. Among the general population, *H. aphrodisiaca* roots are used as an aphrodisiac, tonic or stimulant, as well as treatment of nervous debility and nervous breakdown [12,13]. Data from the literature suggest that the

root extract can increase the body and testicular weight, the diameter of seminiferous tubule and individual and total volume of Leydig cells in the testis of rats [14].

The present study aimed to investigate the ultrastructure of the testis in groups of rats treated with CsA and *H. aphrodisiaca* simultaneous and sequentially.

MATERIALS AND METHODS

Experimental animals

Adult Wistar rats, 90 days old and weighing 310-380g, were obtained from the Multidisciplinary Center for Biological Investigation (State University of Campinas/UNICAMP, Campinas, SP, Brazil). The rats were housed, three per cage, under standard conditions with 12hrL:12hrD. Animals were provided with rat chow and water *ad libitum*. The Institutional Animal Care and Use Committee of this University approved the experimental protocol.

Medicinal plant

Roots of *H. aphrodisiaca* were collected in February, 2005, in Mato Grosso State, Brazil. *H. aphrodisiaca* was identified by comparison with the voucher herbarium specimens of the plant in the Herbarium of the Federal University of Mato Grosso, Brazil (number 23928). The roots were crushed and powdered using a grinding mill. The infusion of *H. aphrodisiaca* was routinely prepared by pouring the boiling water over the powdered roots, that were allowed to steep for 4 hours and filtered using filter paper. This produced an infusion of 68.66 mg of extract dry weight per ml of infusion (6.866% w/v) and a yield 6.835% (w/w) in terms of initial crude dry weight plant material.

Treatment protocol

Thirty animals were used, divided into five groups (six animals each):

I - control (sham);

II – CsA treatment for 56 days;

III - simultaneous use of CsA and *H. aphrodisiaca* infusion for 56 days;

IV- *H. aphrodisiaca* infusion for 30 days and CsA sequentially for another 26 days;

V- only *H. aphrodisiaca* infusion for 56 days.

Cyclosporine A (Sandimmun Neoral – Oral Solution, 100mg/ml - Novartis Pharma AG, Switzerland) was administered at a dose of 15 mg/kg/day dissolved in 0.5 mL of distilled water and *H. aphrodisiaca* at a dose of 0.5 ml of the infusion prepared with 25 g of roots/100 ml in distilled boiling water. All animals were weighed weekly and the CsA dose was calculated accordingly. The treatments and water (sham) were administered daily by gavage, during 56 days, this interval representing the duration of spermatogenesis according to Russell (1990).

Preparation of tissue for transmission electron microscopy

Rats were anesthetized with Xylazine and Ketamine (5 and 80 mg/Kg body weight, respectively) and the abdomen and thoracic cavities opened. The animals were fixed by whole body perfusion. Briefly, after a saline wash to clear the blood vessels, the animals were perfused with glutaraldehyde 2.5% and paraformaldehyde 4% in sodium phosphate buffer 0.1 M, with pH 7.2 for 25-30 min, and further the testis were immersed in the same solution overnight at 4ºC. Testis blocks destined for transmission electron microscopy were post fixed with 1% osmium tetroxide in the same buffer at 4ºC, dehydrated in acetone and embedded in epoxy resin. Ultra thin sections (20-60 nm) were stained with 2% uranyl acetate (25 min) and 2% lead citrate (7 min) prior to observation with a transmission electron microscope (Zeiss, Leo 906).

RESULTS

Transmission electron microscopy confirmed the healthy appearance of spermatogenesis from the seminiferous tubule in the control group (Fig. 1). The Sertoli cells were located above the basal lamina of the seminiferous tubule with cytoplasm extending up to the lumen. Sertoli cell nuclei were located in the basal zone of the seminiferous epithelium, directly adjacent to the basal lamina and they showed normal

ultrastructural features. Typically, the nuclei showed patchy chromatin material with a deep indentation of the nuclear envelope and well-defined nucleolus. The ultrastructure of the cytoplasmic organelles was typical. The plasma membrane was conspicuous, tortuous and showed closer association with adjacent spermatocytes and spermatids (Fig. 1A). Specialized intercellular tight junctions between the adjacent Sertoli cells, enveloping germ cells other than spermatogonia, were present. Primary and secondary spermatocytes were rounded and had a prominent nucleus that contained distinct chromatin undergoing condensation, and a well-defined nuclear membrane. Round spermatids were characterized by a well-defined nucleus with distinct nuclear envelope and chromatin network and cytoplasm predominantly occupied by mitochondria (Fig. 1B). Leydig cells in control animals had a large spherical or slightly elongated nucleus, which usually possessed a prominent nucleolus and frequently showed deep indentations of the nuclear envelope (Fig. 1C). The cytoplasm contained small Golgi complexes, round or oval lysosome-like structures, mitochondria containing tubular or lamellar cristae, small patches of rough endoplasmic reticulum (RER) as short cisternae, and a network of interconnecting tubular smooth endoplasmic reticulum (SER).

In the CsA-treated rats, the Sertoli cells showed numerous cytoplasmatic vacuoles and abundant lipid droplets (Fig. 2, A and B). Round spermatids exhibited nuclear prrotusions or swellings because of an extensive enlargement between the nuclear membranes and vacuoles apparently due to swelling of the endoplasmic reticulum (Fig. 2B). Observations of the apical part of the seminiferous epithelium showed the presence of a large accumulation of residual cytoplasm released by spermatids (Fig. 2C). The Leydig cells showed a conspicuous atrophy, mainly due to a decrease in mitochondria and SER, organelles involved in steroid synthesis (Fig. 2D). They had spherical, oval or elongated nuclei, which often had numerous deep infoldings of the nuclear envelope. Mitochondria were smaller in size than other groups. SER was composed of vesicles and short tubules or a loose network of interconnecting tubules.

In the seminiferous epithelium ultrastructural changes induced by *H. aphrodisiaca* infusion were observed in both germ and Sertoli cells. The Sertoli cell cytoplasm contained many vacuoles (Fig. 3A). Loss of germ cell attachment and expanded intercellular spaces

were observed between spermatogonia and spermatocytes and also between spermatids (Fig. 3, A and B). Some late spermatids exhibited irregular acrosomes and were deformed (Fig. 3A). In the tubule lumen, round spermatids were found, which were prematurely released from the Sertoli cells near the spermatozoa. Leydig cells of animals that received only *H. aphrodisiaca* infusion had a large spherical nucleus, which usually possessed a prominent nucleolus and, frequently, deep indentations of the nuclear envelope (Fig. 3C). The cytoplasm contained Golgi complexes, round or oval lysosome-like structures, some mitochondria with tubular or lamellar cristae, small patches of RER forming short cisternae, and a large dense network of interconnecting tubular SER.

In the simultaneous treatment with CsA and ***H. aphrodisiaca*** infusion, the Sertoli cell cytoplasm was fragmented and contained vacuoles (Fig. 4, A and B). Loss of germ cell attachment and appearance of expanded intercellular spaces were observed between spermatocytes and spermatids (Fig 4B). Late spermatids appeared with normal morphology. The Leydig cells appeared with some mitochondria containing tubular or lamellar cristae, small patches of RER forming short cisternae and a network of interconnecting tubular SER (Fig. 4C).

The pretreatment with ***H. aphrodisiaca*** infusion and sequential administration of CsA also showed loss of germ cell attachment and the appearance of expanded intercellular spaces between them (Fig. 5A). The cytoplasm of Sertoli cells was fragmented and contained vacuoles (Fig 5, A and B). The some round spermatids had abnormal chromatin condensation (Fig. 5C), while others exhibited normal chromatin condensation and acrosomes (Fig. 5B). In the interstitial space, the Leydig cells were similar to the control group, with some mitochondria and a network of tubular SER (Fig. 5D).

DISCUSSION

Spermatogenesis is a complex process in which the interaction of diverse cells such as Sertoli, Leydig and germ cells is tightly regulated by hormonal signals and receptor stimulation. If one specific cell type is damaged by toxicants, then the whole process could be disrupted leading to reproductive failure, infertility or sterility [15].

Seethalakshmi et al [3] described markedly altered tubular architecture in rats that received CsA daily at doses of 20 mg/Kg of body weight. Degeneration of primary spermatocytes, spermatids and Sertoli cells increased and a number of vacuoles were seen in the primary spermatocytes. Moreover, the association between spermatids and Sertoli cells was lost and the round spermatids fused with each other [3]. In the present study, we observed expanded nuclei, myelinic figures and vacuoles in the germ cells, which could indicate degeneration of these cells. The Sertoli cells were also vacuolated and contained larger lipid droplets. The accumulation of lipid droplets in Sertoli cells suggested a loss of efficiency, or that these cells are being overly taxed by a larger quantity of degenerating germ cells, phagocyted by Sertoli cells [16].

This is the first ultrastructural study of the testis in rats treated with *H. aphrodisiaca*. Ultrastructural alterations in seminiferous tubules of animals that received *H. aphrodisiaca* infusion include Sertoli cell cytoplasmic vacuolization and fragmentation. Sertoli cell vacuolization, a non specific lesion, is a condition often observed in several different pathological conditions [17]. It appears before extensive germ cell degeneration, and it is believed to be an early indicator of damage to the Sertoli cell [18]. Subsequent to vacuolization, germ cell degeneration, disorganization, or exfoliation is generally seen [15]. In the present study, many exfoliated germ cells appear morphologically normal, suggesting a primary effect on the cell-to-cell junctions between Sertoli and germ cells. Therefore, the mechanisms by which *H. aphrodisiaca* infusion causes disorganization of the seminiferous epithelium could be due to disruption of cytoeskeletal elements. The Sertoli cell has a well-developed cytoskeleton with microtubules, actin microfilaments and intermediate filaments [19]. A cytoskeletal function is central to many Sertoli cell activities, including Sertoli-germ cell attachment, germ cell movement from tubular base to lumen, and Sertoli cell secretory functions [15]. The participation of Sertoli cells in the process of premature loss of germ cells is essential. The germ cells in the adluminal compartment are held in position by characteristic inter-Sertoli cell junctions and the Sertoli cell-germ junctions [20]. These junctional complexes are maintained by cytoskeletal elements and the ectoplasmic specialization of the Sertoli cell cytoplasm [15]. Once a germ cell has arrived at the adluminal compartment, it is released only at spermiation, in the normal course of

events [21]. During toxic manifestations, as in the present case, the Sertoli cells may release the germ cells prematurely, and this would necessarily involve alterations in the junctional complexes and the cytoskeletal framework of the Sertoli cells [21]. The microtubule-disrupting agents, colchicine and vinblastine, result in sloughing of the adluminal portion of the Sertoli cells [22]. Moreover, when the rat testis was injected with cytochalasin D and cytochalasin B, the actin layer of the Sertoli cell–spermatid junction was dissolved and disorientation of spermatids was observed [23]. The disorganization of cytoskeletal elements also explained the appearance of deformed elongated spermatids as in this study. Clearly, further work is needed to elucidate this issue and to determine the underlying mechanisms of *H. aphrodisiaca* effects on the Sertoli cell.

The most important findings of the present study are the results of the treatment of adult Wistar rats with *H. aphrodisiaca* infusion in relation to the Leydig cell ultrastructure. It is interesting to note that the treatment did not cause specific changes in plasma testosterone levels (Monteiro et al, unpublished data). However, the treatment was able to increase the nuclear volume and the quantity of mitochondria and smooth endoplasmic reticulum, which are the organelles involved in the production of the enzymes for steroid biosynthesis. Zirkin et al [24] demonstrated that there was a positive linear correlation between testosterone secretion and volumetric proportion of the smooth endoplasmic reticulum of the Leydig cells in different species. Therefore, we believe that steroid biosynthesis in these animals is increased. Further work will be done to investigate this issue. The discrepancy of the plasma testosterone levels measured in this experiment is probably due to differences in the time of day when the blood samples were extracted for the different groups. The plasma levels of testosterone normally appear as small pulses, throughout the day, according to peaks of LH liberation by the pituitary gland [25]. Also, the plasma level of testosterone follows a superimposed tendency, whereby it is 25% lower at 8 pm than it is at 8 am [25].

Cavallini et al [6] showed that, after 30-day administration of CsA (20 mg/Kg day) there was conspicuous atrophy of Leydig cells, mainly due to a decrease in mitochondria and smooth endoplasmic reticulum, which are the organelles containing the enzymes for testosterone biosynthesis. They concluded that CsA inhibits the growth and steroidogenic

capacity of rat Leydig cells, causing a decrease in serum testosterone levels [6]. Seethalakshmi et al [3] found decreased serum testosterone levels and increased LH and FSH levels in CsA-treated rats. They suggested that this finding was due to either degeneration of Leydig cells or to inhibition of testicular steroidogenesis as either mechanism can decrease androgen production [3]. In another study, histological observations also demonstrated that Leydig cell damage occurred in a dose-dependent manner in CsA-treated rats [26].

In the present study, a decrease in plasma testosterone was not found (Monteiro et al, unpublished data). However, the ultrastructure of Leydig cells showed a characteristic atrophy of the organelles involved in steroid synthesis and of nuclei with deep indentations, and very dense cytoplasm. Possibly the discrepancy in the plasma testosterone levels is due to differences in the time of collection of blood samples of the CsA-treated and control animals.

The simultaneous and sequential treatment with *H. aphrodisiaca* and CsA was efficient in maintaining the Leydig cell ultrastructure. The seminiferous epithelium modifications were a combination of the modifications caused separately by each treatment. However, *H. aphrodisiaca* apparently protected the seminiferous epithelium, since the alterations observed for this group were less severe than those encountered in the CsA treated material. Large lipid droplets in Sertoli cells, degenerated cells and vacuoles in germ cells were not observed in these treatments.

Thus, in conclusion, the treatment with CsA and *H. aphrodisiaca* alone or combined, altered the testicular ultrastructure. The testicular changes reported for the CsA in the present study are similar to those reported by other researchers [3,6,26,27]. Since this is the first study of testis ultrastructure of in rats treated with *H. aphrodisiaca*, important findings were reported, such as Sertoli cell fragmentation and vacuolization and increased in the organelles containing the enzymes for steroid biosynthesis, in Leydig cells. However, further studies are warranted to substantiate these views, and are currently in progress. But, the association of CsA and *H. aphrodisiaca* was effective in maintaining Leydig cell ultrastructure while the seminiferous epithelium alterations were less severe than resulting from CsA treatment.

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FIGURE LEGENDS

Fig. 1- Control group. **A**- Seminiferous epithelium showing Sertoli cell (Se) and spermatogonia (Go) in the basal compartment. **B**- Round spermatids (Rs), in the adluminal compartment, beginning acrosome (a) development and chromatin condensation. **C**- Leydig cell showing a large nucleus (n) with prominent nucleolus (nu) and cytoplasm rich in mitochondria (m), smooth endoplasmic reticulum (ser) and rough endoplasmic reticulum (rer). Scale bar = 5 μm.

Fig. 2- Group treated with CsA. **A**- Seminiferous epithelium showing Sertoli cell (Se), with vacuoles and lipid droplet (l), spermatogonia (Go) and spermatocyte (Sp) in the basal compartment. **B**- Elongated spermatids (Es) are enclosed in vacuolated Sertoli cell (Se) cytoplasm. Round spermatids (Rs) with expanded nuclei (★) and vacuoles (➔). Note the acrosome (a) in formation. **C**- Large residual bodies (Rb) of spermiated spermatozoa. **D**- Leydig cell showing large irregular nucleus (n) and dense cytoplasm with smooth endoplasmic reticulum (ser), lipid droplets (l) and mitochondria (m). Scale bar A to C = 10 μm.

Fig. 3- Group treated with *H. aphrodisiaca*. **A**- Seminiferous tubule showing basal compartment with vacuolated Sertoli cell (Se), spermatogonias (Go), spermatocytes (Sp) and adluminal compartment with round spermatids (Rs) and altered elongated spermatids (Es). Expanded intercellular space (*). **B**- Round spermatid (Rs) with its developing acrosome (a) and expanded intercellular space (*). Note the Golgi complexes (g). **C**- Leydig cell showing a large nucleus (n) and cytoplasm rich in smooth endoplasmic reticulum (ser), rough endoplasmic reticulum (rer) and mitochondria (m). Scale bar = μm.

Fig. 4- Group treated with CsA and *H. aphrodisiaca*. **A**- Seminiferous tubule showing basal compartment with Sertoli cell (Se), spermatogonia (Go), spermatocyte (Sp) and adluminal compartment with round spermatids (Rs). Expanded intercellular space (*) and Sertoli cell (Se) with vacuolated cytoplasm. **B**- Basal and adluminal compartments with normal

spermatocytes (Sp), round spermatids (Rs) and elongated spermatids (Es) enclosed in Sertoli cell (Se) cytoplasm with some vacuoles. **C**- Leydig cell showing large irregular nucleus (n), evident nucleolus (nu) and cytoplasm rich in smooth endoplasmic reticulum (ser), mitochondria (m) and rough endoplasmic reticulum (rer). Scale bar A and B= 5 μm.

Fig. 5- Group treated with *H. aphrodisiaca* for 30 days and CsA sequentially for another 26 days. **A**- Seminiferous epithelium showing Sertoli cells (Se), spermatogonias (Go) in the basal compartment and round spermatid (Rs) in adluminal compartment. Expanded intercellular space (*). **B**- Round spermatids (Rs), some elongated spermatids (Es) enclosed in vacuolated Sertoli cell (Se) cytoplasm and residual bodies (Rb). **C**- Round spermatids (Rs) with deformed nuclei and acrosomes (a). **D**- Interstitium showing blood vessel (v) and Leydig cells (L) with a nucleus (n) and cytoplasm rich in mitochondria (m) and smooth endoplasmic reticulum (ser). Scale bar A to C= 10 μm.

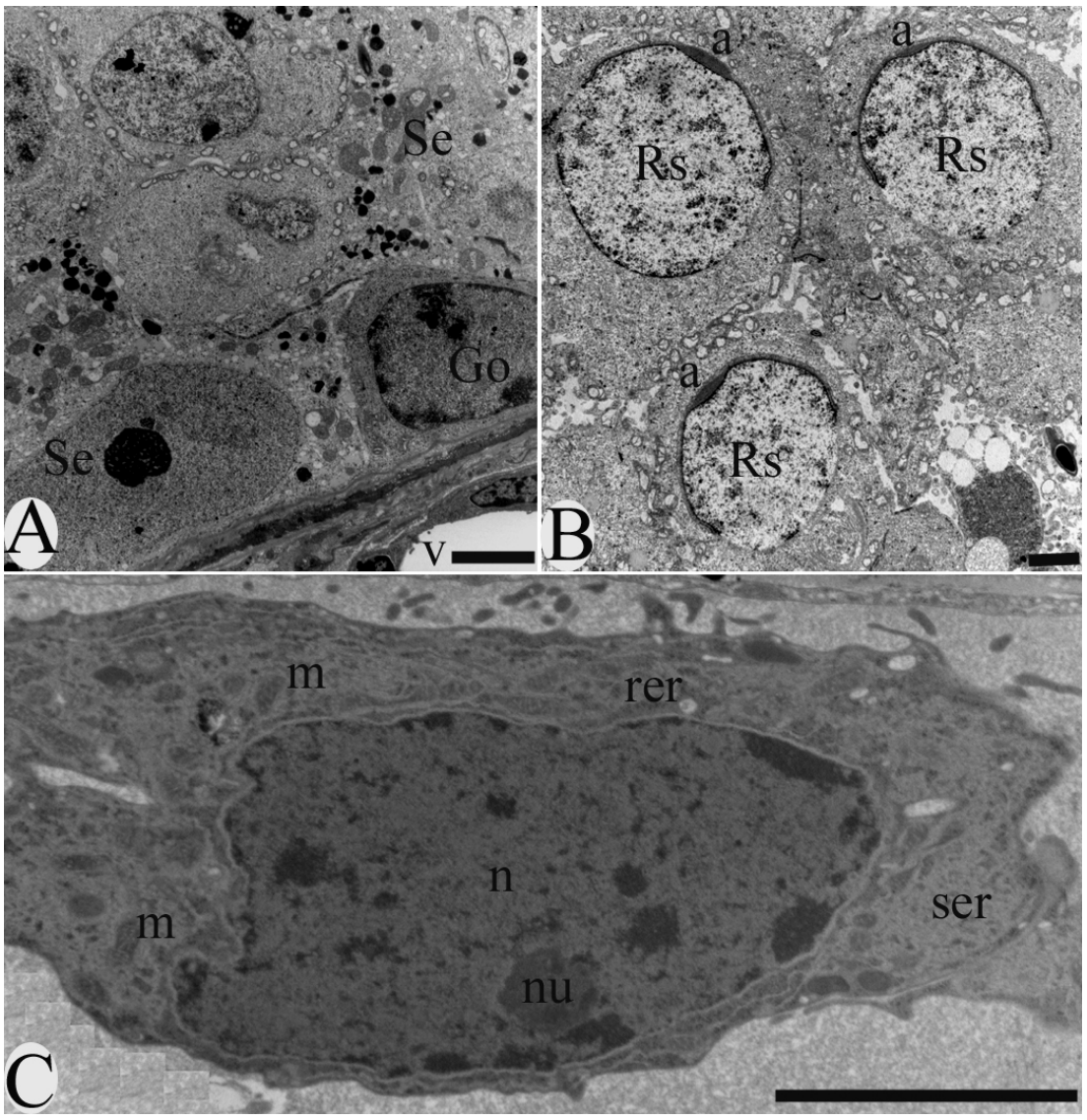

Fig. 1

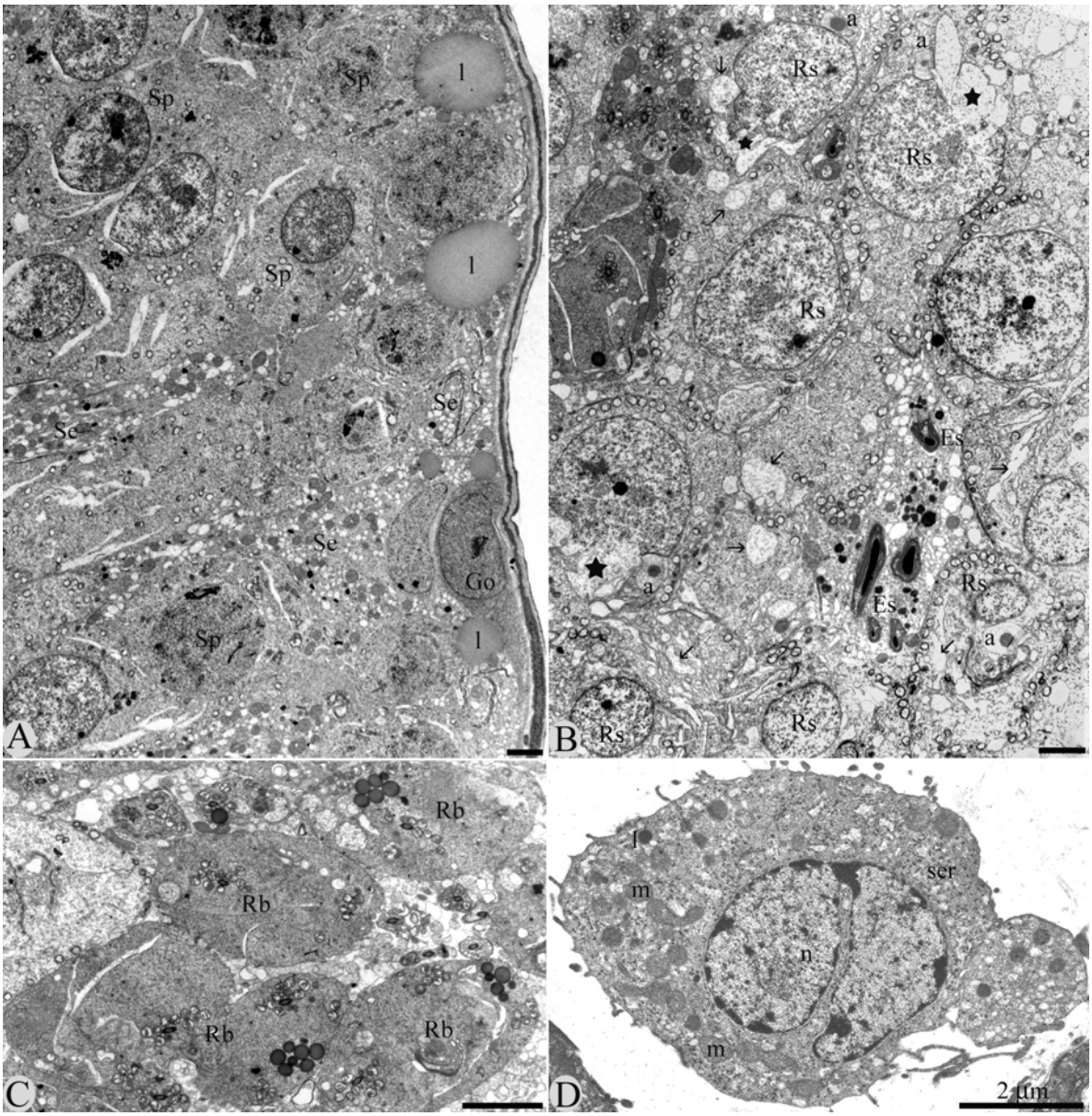
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Fig. 2

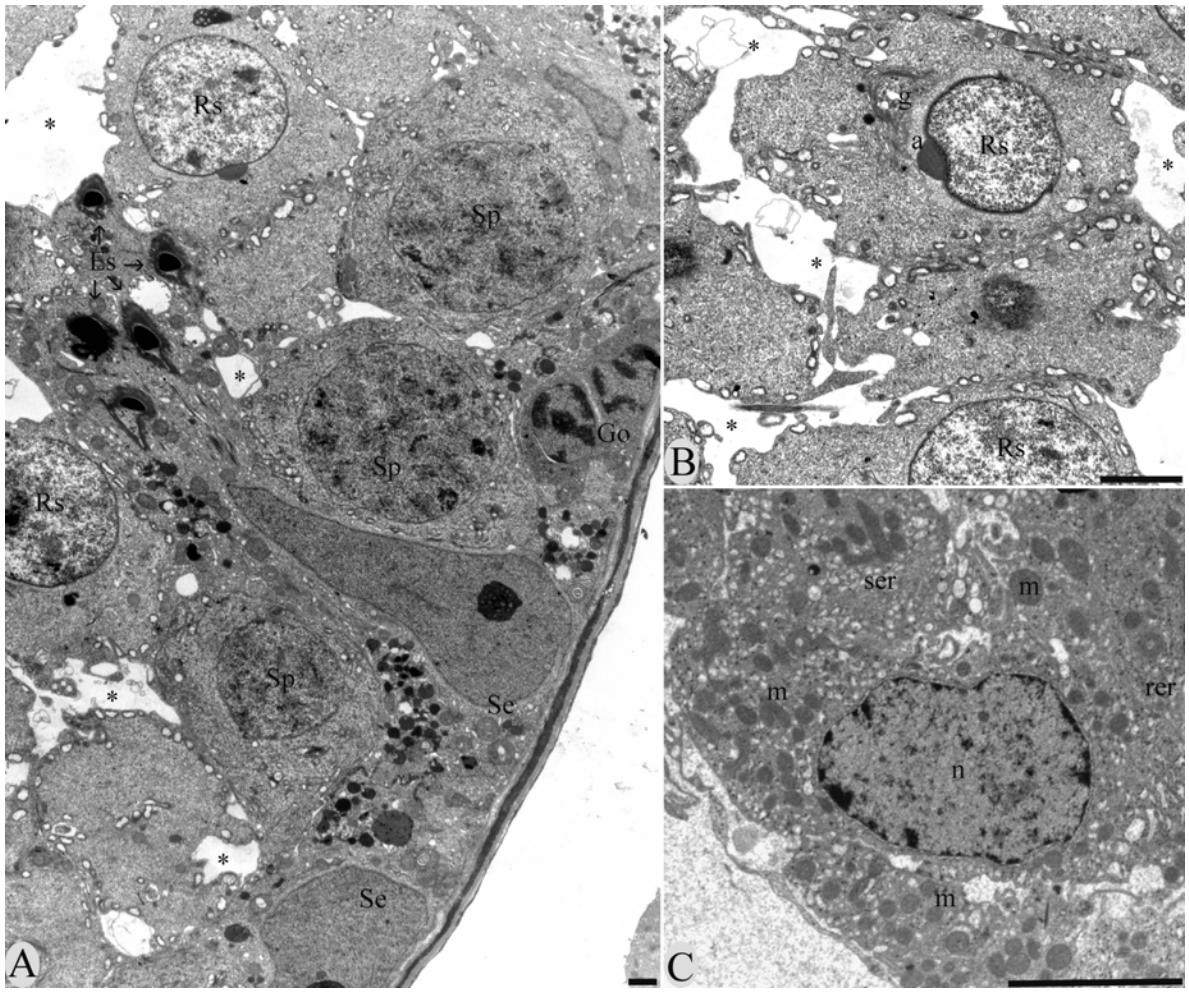

Fig. 3

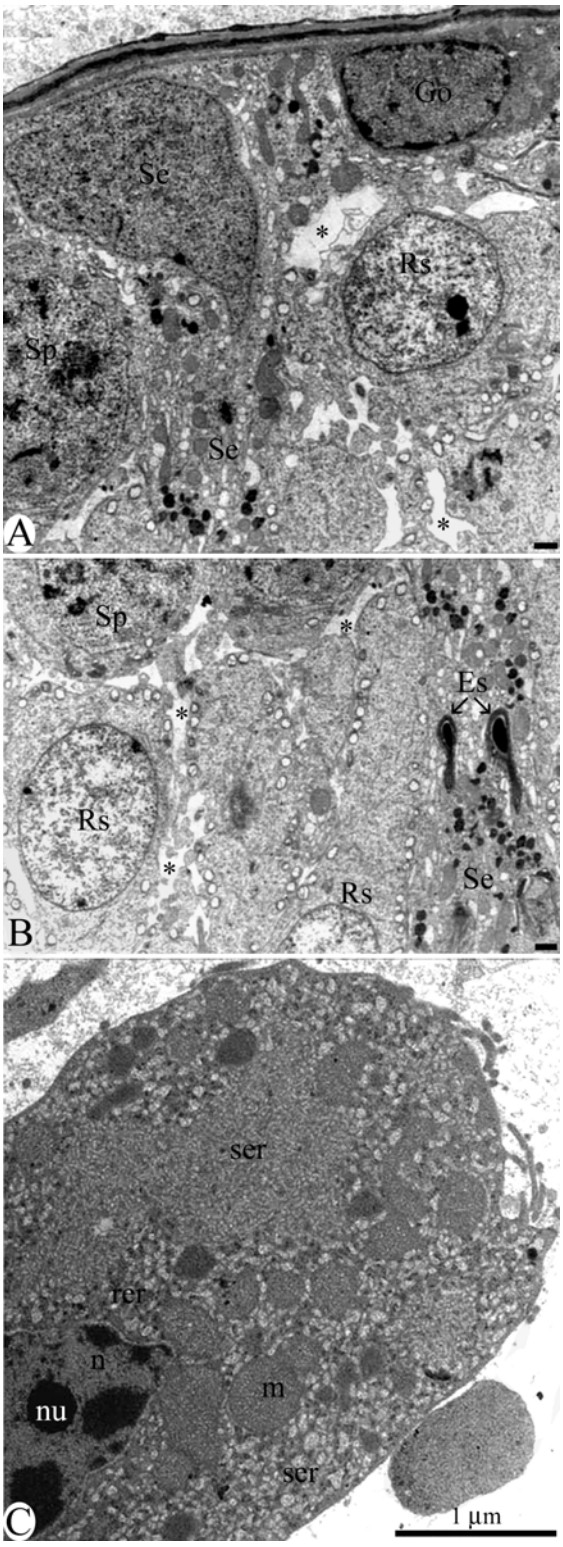

Fig. 4

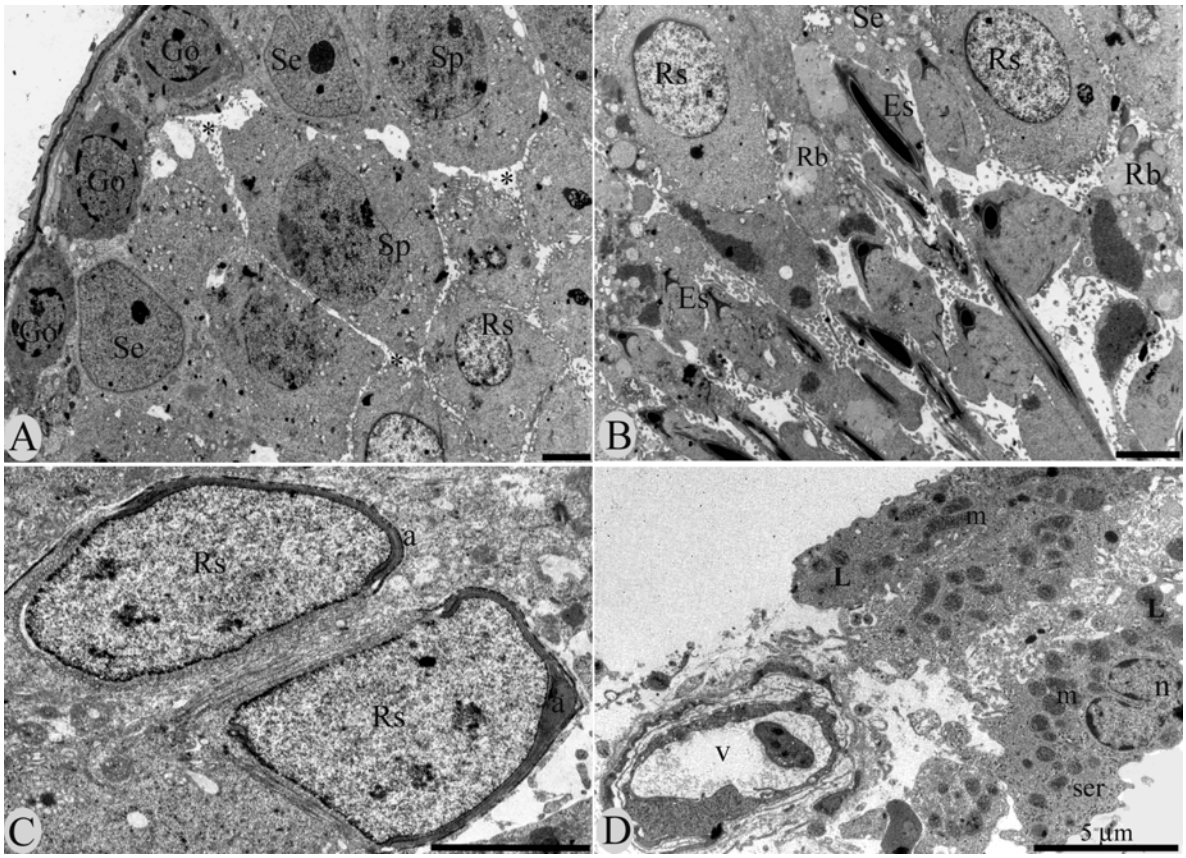
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Fig. 5

7. CONSIDERAÇÕES FINAIS

O objetivo desse trabalho foi avaliar os efeitos da associação da infusão de *H. aphrodisiaca* e CsA nos testículos de ratos Wistar adultos. Os resultados obtidos mostraram que a infusão da planta foi efetiva em manter a morfologia das células de Leydig e do epitélio seminífero dos animais que receberam tratamento com a infusão e CsA, os quais foram menos lesados que animais que receberam somente CsA.

A análise em microscopia eletrônica de transmissão permitiu avaliar minuciosamente os danos causados por CsA e o efeito protetor de *H. aphrodisiaca*, os quais foram pouco visíveis na análise morfológica em microscopia de luz e na morfometria testicular.

Embora os níveis plasmáticos de testosterona não tenham sido inteiramente de acordo com o esperado, sugerimos que a discrepância dos valores obtidos ocorreu devido aos diferentes períodos de coleta do sangue dos animais dos diferentes grupos. Baseado nos dados morfométricos e ultra-estruturais, sugerimos que os animais que receberam somente CsA e *H. aphrodisiaca* produzem menores e maiores níveis de andrógenos nos testículos, respectivamente, quando comparados com os animais controles.

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **851-1**, sobre **"ASSOCIAÇÃO DE CICLOSPORINA E *HETEROPTERYS APHRODISIACA* (NÓ-DO-CACHORRO) ADMINISTRADOS CONCOMITANTEMENTES E SEQÜÊNCIALMENTE EM RATOS WISTAR: ESTRUTURA, ULTRA-ESTRUTURA E MORFOMETRIA TESTICULAR"** sob a responsabilidade de **Profa. Dra. Mary Anne Heidi Dolder / Juliana Castro Monteiro** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 03 de agosto de 2005.

CERTIFICATE

We certify that the protocol nº **851-1**, entitled **"ASSOCIATION OF CYCLOSPORINE AND *HETEROPTERYS APHRODISIACA* ADMINISTERED TOGETHER OR SEQUENTIALLY TO WISTAR RATS: TESTICULAR STRUCTURE, ULTRASTRUCTURE AND MORPHOMETRY"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on August 3, 2005.

Campinas, 03 de agosto de 2005.

Profa. Dra. Ana Maria A. Guaraldo
Presidente - CEEA/IB/UNICAMP

Fátima Alonso
Secretária - CEEA/IB/UNICAMP

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