UNIVERSIDADE ESTADUAL DE CAMPINAS
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"EFEITO DA CICLOSPORINA A ASSOCIADA À INFUSÃO DE NÓ-DE-CACHORRO (*Heteropterys aphrodisiaca*, O. MACH, 1949) NA PRÓSTATA DE RATOS WISTAR"

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Dedico esta dissertação
aos meus pais
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ÍNDICE

1. RESUMO

A Ciclosporina A (CsA) é um imunossupressor amplamente utilizado no tratamento pós-transplante de órgãos e contra doenças auto-imunes. Entretanto, diversos efeitos colaterais estão relacionados ao tratamento com esta droga, dentre eles nefrotoxicidade, hepatoxicidade, tremor, hipertensão, hiperlipidemia, hipercalcemia, hipertricose e hiperplasia gengival. Além destes, danos à reprodução masculina como redução na produção de testosterona e disfunção testicular são extensivamente documentados. *Heteropterys aphrodisiaca* é uma planta arbustiva típica do Cerrado brasileiro. É conhecida como nó-de-cachorro, sendo utilizada popularmente como tônica e afrodisíaca. Estudos anteriores confirmam a eficiência da infusão de *H. aphrodisiaca* contra os efeitos colaterais da CsA nos testículos. Visto isso, o objetivo do presente trabalho foi determinar os possíveis efeitos colaterais da CsA na próstata ventral de ratos Wistar e verificar se a infusão de *H. aphrodisiaca* seria eficiente em minimizá-los. Trinta ratos Wistar adultos (90 dias) foram divididos em cinco grupos (n=6 em cada): Grupo I (controle): tratado com água; grupo II: tratado com CsA; grupo III: tratado com infusão de *H. aphrodisiaca*; grupo IV: tratamento simultâneo de CsA e *H. aphrodisiaca*; e grupo V: tratamento com CsA e *H. aphrodisiaca* em dias alternados. A CsA foi administrada na dose de 15 mg/kg/dia e a infusão de nó-de-cachorro no volume de 0,5 ml por animal (infusão preparada com 25 g de raiz seca e moída em 100 ml de água fervida). Todos os tratamentos foram realizados diariamente, durante 56 dias. Após este período os animais foram pesados e eutanasiados. Testículo, epidídimo, glândula vesicular, glândula de coagulação e próstata ventral foram coletados e pesados. A próstata ventral foi dividida em três porções. Uma foi fixada em Karnovsky e processada para inclusão em metacrilato; este material foi utilizado para análises morfológicas, morfométricas e estereológicas do tecido. A segunda porção, também fixada com Karnovsky, foi processada para inclusão em Epon; este material foi utilizado para realização de análises em microscopia eletrônica de transmissão. Por fim, a terceira porção foi fixada em Metacarn e incluída em parafina para realização de imunohistoquímica para detecção de receptor de andrógeno e técnica para detecção de apoptose (TUNEL). As análises morfológicas, morfométricas, estereológicas e ultraestruturais mostraram que o tratamento com a infusão de *H. aphrodisiaca* não causou nenhuma alteração na próstata ventral. Por outro lado, o tratamento com CsA causou severos danos a este tecido como redução nos volumes de lumen, epitélio, estroma muscular e não muscular, além de atrofia do epitélio. A análise ultraestrutural mostrou que nas células do epitélio atrofiado havia redução das organelas relacionadas à secreção de proteínas (retículo endoplasmático rugoso e complexo de Golgi), o que provavelmente leva à diminuição de sua atividade secretora. O

tratamento conjunto de CsA e *H. aphrodisiaca* aparentemente reduziu os danos causados na próstata, o que foi evidenciado pela normalização nos parâmetros analisados que haviam sido prejudicados pela administração somente da CsA. Nenhuma alteração entre os grupos experimentais foi observada no padrão de marcação imunohistoquímica para receptores de andrógeno e no índice apoptótico. Os resultados obtidos mostram que o tratamento com CsA causa danos na estrutura prostática que provavelmente refletem em danos funcionais ao tecido. Além disso, foi confirmada a ação protetora da infusão de *H. aphrodisiaca* contra os efeitos colaterais da CsA na próstata ventral de ratos Wistar.

2. ABSTRACT

Cyclosporin A (CsA) is an immunosuppressive drug widely used in the post-operative treatment after organ transplants and against auto-immune diseases. However, various side effects have been associated with the use of this drug, including nephrotoxicity, hepatotoxicity, tremors, hypertension, hyperlipidemy, hypercalcicity, hypertrichosia and hyperplasticity of the gums. Besides these, damage to the male reproductive system, such as diminished testosterone production and testicular dysfunction, have been extensively documented. *Heteropterys aphrodisiaca* is a bush, typical of the Brazilian "Cerrado" region. It is known as "nó-de-cachorro" and used in traditional medicine as a tonic and an aphrodisiac drink. Previous studies confirm the efficiency of *H. aphrodisiaca* against the side effects of CsA in rat testicles. As a consequence, this research was undertaken with the aim of determining the possible collateral effects of CsA in the ventral prostate of Wistar rats and to verify whether the infusion of *H. aphrodisiaca* would be efficient to diminish the possible collateral effects of CsA in this organ. Thirty adult Wistar rats (90 days old) were divided into five groups (n=6 in each). Group I (control) was treated with water, Group II with CsA, group III received the *H. aphrodisiaca* infusion, group IV was treated simultaneously with CsA and *H. aphrodisiaca* infusion and group V received CsA or *H. aphrodisiaca* infusion on alternate days. CsA was administered in the dose of 15 mg/kg/day and the infusion in a volume of 0.5 ml per animal (infusion prepared with 25 g of dry ground roots in 100 ml of boiling water). All treatments were given daily for 56 days. After this period the rats were weighed and euthanized. Testis, epididymis, vesicular gland, coagulating gland and the ventral prostate were collected and weighed. The ventral prostate was divided into three portions. One was fixed in Karnovsky fixative and processed for inclusion in methacrylate. This portion was used for morphological, morphometrical and stereological analysis of the tissue. A second portion, fixed in the same manner, was processed for inclusion in Epon and analyzed with the transmission electron microscope. The last portion was fixed in Metacarn and included in paraffin to be used for immunohistochemistry of the androgen receptor and the TUNEL technique for apoptosis. The morphological, morphometrical, stereological and ultrastructural analyses showed that the *H. aphrodisiaca* infusion did not cause any alteration of the prostatic tissue. On the other hand, CsA treatment resulted in severe damage to the tissue, reducing the lumen, epithelium, muscular and non-muscular stroma. Atrophied epithelium was observed after CsA administration. Ultrastructural analysis showed that the atrophied epithelium had reduced organelles involved in protein secretion (rough endoplasmic reticulum and Golgi complex) which probably causes decreased secretory activity of the cells. The

simultaneous treatment of CsA and *H. aphrodisiaca* apparently reduced the prostatic damage, since these were less damaged when compared to the prostate of animals that received only CsA. No alteration was found in the pattern of immunohistochemical labeling for androgen receptors and the apoptotic index, for all experimental groups. The results showed that CsA treatment caused structural damage to the prostate which probably reflected functional damage to the organ. The protective action of the *H. aphrodisiaca* infusion against the collateral effects of CsA was also confirmed for this organ.

3. INTRODUÇÃO

3.1. Próstata

O sistema reprodutor masculino de ratos é composto pelos testículos, ductos genitais (epidídimo, ducto deferente e uretra), glândulas acessórias (próstata, glândulas vesiculares e glândulas bulbouretrais) e o pênis (Komárek, 2000).

A próstata produz um líquido fino de aspecto leitoso que aumenta o volume do ejaculado e favorece a neutralização do meio onde ocorre a fecundação, além de contribuir para o aumento da motilidade e fertilidade dos espermatozóides (Guyton, 1984; Risbridger e Taylor, 2006).

Em roedores da superfamília Muroidea, que inclui ratos e camundongos, a próstata é geralmente dividida em quatro pares de lobos de acordo com sua posição anatômica, são eles: anterior (conhecido como glândula de coagulação), ventral, lateral e dorsal (Risbridger e Taylor, 2006; Setchell e Breed, 2006).

Em animais adultos, a glândula de coagulação está ligada à superfície côncava da glândula vesicular. Cada lobo da próstata lateral está localizado exatamente abaixo da glândula vesicular e glândula de coagulação. Os lobos da próstata dorsal estão abaixo e atrás da glândula vesicular e glândula de coagulação, além disso, está na posição "ínfero-posterior" à bexiga e posterior (dorsal) à próstata lateral. Por fim, a próstata ventral se localiza imediatamente abaixo da bexiga (Hayashi et al., 1991).

A próstata é constituída por glândulas secretórias túbulo-alveolares compostas por epitélio pseudoestratificado, sendo identificados três tipos celulares: as células basais, secretórias e neuroendócrinas (Miki, 2010). As células secretórias são responsáveis pela secreção exócrina da próstata. São células colunares que produzem e secretam proteínas. Estas células expressam elevados níveis de receptores de andrógenos e necessitam de testosterona para sua manutenção e função. As células basais podem dar origem a células-tronco e células "amplificadoras de transição"

(*transit amplifying cells* – TA) (Isaacs e Coffey, 1989). As células basais não são secretoras e andrógeno-independentes, mas são andrógeno-responsivas, ou seja, não necessitam de andrógenos para sua sobrevivência, mas necessitam do estímulo destes para seu crescimento e diferenciação (Risbridger e Taylor, 2006).

O estroma prostático é constituído por músculo liso, fibroblastos, vasos sanguíneos e uma população variável de macrófagos e mastócitos. As células musculares lisas periacinares e perivasculares apresentam marcação positiva para receptores de andrógenos com intensidade similar àquela apresentada pelas células epiteliais. Já as células endoteliais e a maioria dos fibroblastos não apresentam marcação para estes receptores (Prins et al., 1991). Na próstata ventral diferenciada, o músculo liso é fundamental para a expulsão das secreções das glândulas em direção dos ductos prostáticos durante a ejaculação. A incidência de apoptose e proliferação celular sofre variações regionais nas glândulas prostáticas e estão associadas às diferenças na organização do estroma. Aparentemente, a sinalização realizada por fibroblastos promove proliferação celular. Sinais advindos do músculo liso ou ausência de sinais proliferativos oriundos dos fibroblastos promovem a morte celular (Risbridger e Taylor, 2006).

O desenvolvimento da próstata no período embrionário não está relacionado ao sexo genético do animal, mas sim à exposição de andrógenos (Vilamaior et al., 2006). Há formação de próstata em machos e fêmeas caso ocorra estimulação por andrógeno no período adequado (Takeda et al., 1986). O desenvolvimento e diferenciação do músculo liso e epitélio prostático são inteiramente dependentes um do outro, não havendo o desenvolvimento de um, na ausência do outro (Hayward e Cunha, 2000). O crescimento normal, diferenciação e manutenção da integridade funcional e estrutural da próstata, assim como dos demais órgãos do sistema reprodutor masculino de ratos, dependem dos níveis de andrógenos circulantes (Spaziani, 1975).

A próstata ventral é o lobo da próstata que apresenta a maior concentração de receptores de andrógeno, sendo a mais responsiva a variações de andrógenos. Por isso, é considerada o órgão

mais indicado para estudos de ação de andrógenos e a cada ano vem crescendo o número de trabalhos sobre morfologia, fisiologia, bioquímica e biologia molecular desta glândula na literatura (Cordeiro et al., 2008; García-Flórez et al., 2005; Hayashi et al., 1991; Santos et al., 2006; Scarano et al., 2009; Scarano et al., 2006; Vilamaior et al., 2006).

3.2. Ciclosporina A

A Ciclosporina A (CsA) é um polipeptídeo neutro, hidrofóbico, cíclico com 11 aminoácidos, isolado de micélios dos fungos *Tolypocladium inflatum* Gams e *Cylindrocarpon lucidum* (Borel e Kis, 1991). Possui potentes propriedades imunossupressoras e tem sido amplamente usado na terapia após o transplante de órgãos, aumentando as taxas de sobrevivência dos enxertos, e no tratamento de algumas doenças auto-imunes.

A atividade imunossupressora da CsA está relacionada ao efeito citotóxico seletivo às subpopulações de linfócitos T citotóxicos e supressores (Hess e Tutschka, 1980). Porém, não interfere com a função das células fagocitárias e células-tronco hematopoiéticas (Borel et al., 1989).

A CsA liga-se a uma proteína intracelular específica, ciclofilina, formando um complexo que inibe a atividade calcineurina, que então fica impossibilitada de desfosforilar o fator de transcrição NFAT (Fator nuclear de ativação de células T). Uma vez desfosforilado, o NFAT não é transportado para o núcleo deixando de transativar genes envolvidos na ativação dos linfócitos T, como o da interleucina 2 (IL-2) que é essencial para a proliferação de linfócitos (Langone e Helderman, 2004; Matsuda et al., 1976; Rezzani, 2004).

Apesar de sua ampla utilização como imunossupressora, a CsA tem efeitos colaterais tanto em animais experimentais quanto em pacientes. A severa nefrotoxicidade associada ao tratamento com CsA é um fator dose-limitante para sua administração (Mason, 1990). Além disso, hepatoxicidade, tremor, hipertensão, hiperlipidemia, hipercalcemia, hipertricose, hiperplasia

gengival (Lee e Canafax, 1996; Ready, 2004), disfunção testicular (Seethalakshmi et al., 1987) e ginecomastia (Rajfer et al., 1987) são documentadas.

O tratamento com CsA está relacionado à disfunção e deterioração renal prematura em pacientes com rins transplantados e em pacientes com tratamento pós-transplante de coração, ou contra doenças auto-imunes. O tratamento progressivo com CsA pode levar a um estágio final de falência renal (Fellstrom, 2004). A nefrotoxicidade causada pela CsA parece estar associada ao aumento do estresse oxidativo (Cid et al., 2003; Fellstrom, 2004).

A hepatotoxicidade induzida pelo tratamento com CsA ocorre normalmente após 90 dias de pós transplante (Rezzani, 2004). O aumento da atividade de diferentes enzimas hepáticas (fosfatase alcalina, lactato desidrogenase e NADPH-diaforase) indica que o estresse oxidativo deve estar relacionado à hepatotoxicidade induzida pela CsA (Rezzani, 2004).

Muitos são os efeitos prejudiciais da CsA na fertilidade de ratos machos encontrados na literatura, com ampla discussão sobre os efeitos colaterais desta droga nos testículos (Krueger et al., 1991; Masuda et al., 2003; Rajfer et al., 1987; Seethalakshmi et al., 1988; Seethalakshmi et al., 1990a; Seethalakshmi et al., 1990b; Seethalakshmi et al., 1987; Srinivas et al., 1998a). Dentre eles, já foi documenta degeneração de células nos túbulos seminíferos (Masuda et al., 2003; Seethalakshmi et al., 1987), redução da contagem espermática e da fertilidade (Seethalakshmi et al., 1987).

Em estudo realizado por Rajfer e colaboradores (1987), a administração de CsA em doses maiores ou iguais que 15 mg/Kg/dia em ratos, por um mês, levou à diminuição nos níveis séricos e intratesticulares de testosterona, sem terem sido observadas alterações nos pesos das glândulas vesiculares, nem da próstata ventral (órgãos andrógeno-dependentes). 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 de um desequilíbrio na razão de andrógenos periféricos em relação ao estrógeno.

Embora alguns estudos relatem que o tratamento com CsA não altera o peso da próstata ventral, outros associam o tratamento com CSA à redução no peso deste órgão (Seethalakshmi et al., 1988; Seethalakshmi et al., 1990a; Seethalakshmi et al., 1987). Apesar de serem muito estudados os efeitos prejudiciais da CsA nos testículos e na fertilidade de ratos (Krueger et al., 1991; Masuda et al., 2003; Rajfer et al., 1987; Seethalakshmi et al., 1988; Seethalakshmi et al., 1990a; Seethalakshmi et al., 1990b; Seethalakshmi et al., 1990c; Seethalakshmi et al., 1990d; Seethalakshmi et al., 1992; Seethalakshmi et al., 1987; Seethalakshmi et al., 1989; Srinivas et al., 1998a), nenhum estudo abordou os efeitos deste tratamento nos padrões teciduais da próstata normal. A redução dos níveis de testosterona associados ao tratamento com esta droga (Krueger et al., 1991; Rajfer et al., 1987), pode causar danos estruturais e funcionais à próstata, que é um órgão andrógeno-dependente.

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

A planta *Heteropterys aphrodisiaca* foi descrita por Hoehne em 1920, como tendo propriedades afrodisíacas e estimulantes. Foi atribuída à família Malpighiaceae por Othon X. B. Machado em 1949 (Pio Corrêa, 1984). É um arbusto de 0,6 a 2,0 metros de altura, típica do Cerrado de Mato Grosso, Goiás e norte de Minas Gerais. Normalmente, a população utiliza as raízes dessa planta como tônico ou estimulante e para o tratamento de debilidades do sistema nervoso (Guarim Neto, 1996; Pio Corrêa, 1984; Pott e Pott, 1994). *H. aphrodisiaca*, 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 (Guarim Neto, 1996; Pott e Pott, 1994).

Rizzini (1983) descreve *H. aphrodisiaca* como estimulante e a inclui em um elenco de 65 espécies de plantas com princípios psicoativos conhecidos 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 desta planta é empregado como depurativo do sangue, dentre outras indicações. Entretanto, o emprego mais difundido é sob a forma de garrafadas, reconhecidas como afrodisíacas.

Chieregatto (2005) observou que ratos tratados com *H. aphrodisiaca* apresentaram aumento no peso corporal, inferindo um possível aumento dos níveis plasmáticos de testosterona como potencial causador deste efeito anabolizante.

Experimentos com homogenato de cérebro de rato mostraram que o extrato liofilizado de *H. aphrodisiaca* possui componentes que inibem a interação de radicais livres com biomoléculas presentes no cérebro. Além disso, o tratamento com este extrato aumentou a quantidade das enzimas antioxidantes SOD (Superóxido Dismutase), MnSOD (Superóxido Dismutase Manganês-dependente) e CuZnSOD (Superóxido Dismutase Cobre e Zinco-dependente) no cérebro de ratos velhos (Mattei et al., 2001). Por fim, o tratamento de ratos velhos com extrato liofilizado de *H. aphrodisiaca* se mostrou eficiente em restaurar os danos relativos à memória nestes animais (Galvão et al., 2002).

No trabalho realizado por Monteiro e colaboradores (2008), o tratamento com *H. aphrodisiaca* foi efetivo em manter a estrutura testicular de ratos tratados com CsA, utilizando-se dados histológicos, morfométricos e ultraestruturais. Não foram observadas alterações significativas no peso da próstata dorsolateral, ventral e glândula de coagulação, nos grupos do estudo que foram tratados com: CsA, *H. aphrodisiaca* e CsA + *H. aphrodisiaca*. Apesar disso, nenhuma análise histológica da próstata ventral foi realizada.

Tendo sido comprovada atividade antioxidante da infusão da raiz de *H. aphrodisiaca* no cérebro de ratos (Mattei et al., 2001) e a sua eficiência no combate aos danos testiculares causados pela CsA, surgiu o interesse em estudar se a infusão desta planta seria também eficiente em combater os possíveis efeitos colaterais da CsA à próstata.

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5. OBJETIVOS

5.1. GeraI

Analisar se o tratamento com a infusão de *Heteropterys aphrodisiaca* causa algum efeito na próstata ventral de ratos Wistar; determinar se o tratamento com Ciclosporina A causa algum efeito colateral nesta glândula e, investigar se a infusão de *H. aphrodisiaca* pode minimizar os possíveis efeitos colaterais causados pela CsA na próstata ventral de ratos Wistar.

5.2. Específicos

- Determinar, com o uso de análises morfométricas, estereológicas, morfológicas e ultraestruturais os efeitos do tratamento crônico com CsA e *H. aphrodisiaca* no tecido prostático de ratos Wistar;
- Determinar, com o uso de imunohistoquímica, os efeitos do tratamento crônico com CsA e *H. aphrodisiaca* na expressão de receptores de andrógeno na próstata ventral de ratos Wistar;
- Determinar as possíveis alterações no índice apoptótico causadas pelo tratamento crônico com CsA e *H. aphrodisiaca* na próstata ventral de ratos Wistar;
- Determinar, com o uso de todas as ferramentas acima citadas a possível proteção conferida pela infusão das raízes de *H. aphrodisiaca* contra os possíveis danos causados pela CsA na próstata ventral de ratos Wistar.

6. RESULTADOS

A partir dos resultados obtidos com este trabalho, foram redigidos os seguintes manuscritos:

6.1. "Study of the ventral prostate of Wistar rats treated with *Heteropterys aphrodisiaca* (O. Mach.)."

6.2. "Cyclosporin A causes impairment to the ventral prostate tissue of Wistar rats."

6.3. "*Heteropterys aphrodisiaca* (O. Mach) infusion: Relieving Cyclosporin A side effects on the ventral prostate."

Study of the ventral prostate of Wistar rats treated with *Heteropterys aphrodisiaca* (O. Mach.)

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Running Title: Prostate of rats treated with *H. aphrodisiaca*

Key words: aphrodisiac, medicinal plants, phytotherapy, reproduction.

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ABSTRACT

Heteropterys aphrodisiaca is a Brazilian plant traditionally used as an aphrodisiac and stimulant. Previous studies suggested possible androgenic and antioxidant effects after long term administration of *H. aphrodisiaca* infusion. The aim of the present study was to evaluate the effects of this plant infusion on the rat ventral prostate – an androgen responsive organ. Wistar rats were treated, by gavage, with *H. aphrodisiaca* roots infusion (treated group, n=6) or water (control group, n=6) during 56 days. Morphological, morphometrical and stereological analyses were employed on the ventral prostate tissue, as well as androgen receptor and apoptotic cell staining. The ultrastructure of the prostatic epithelium was also analyzed. No alteration was observed in the stereological and morphometrical analyzes. The patter of AR expression and the apoptotic index were identical in the control and *H. aphrodisiaca* treated group. Either the ultrastructure had no alterations among the experimental groups. These results suggested that the treatment with *H. aphrodisiaca* infusion did not cause any major damage or benefit to the prostate tissue.

INTRODUCTION

Several Brazilian plant species are used as phytotherapics in traditional medicine, and studies using these plants are increasing numerically every year. Among medicinal plants, the ones used as aphrodisiacs are of great interest to researchers (Chieregatto, 2005; Gomes, 2007; Melo, 2007; Monteiro et al., 2008). *Heteropterys aphrodisiaca* is a native plant species from the Brazilian "Cerrado", a savanna-like biome, commonly known as "nó-de-cachorro", "raiz-de-Santo-Antônio" and "cordão-de-São-Francisco" (Pio Corrêa, 1984; Pott and Pott, 1994). It was described by Hoehne (1920) as a stimulant and aphrodisiac plant. The *H. aphrodisiaca* roots are commonly used as an alcoholic drink and infusions consumed as aphrodisiac and stimulant drinks (Guarim Neto, 1996; Rizzini and Mors, 1976).

According to Marques et al. (2007) *H. aphrodisiaca* roots contain anthracene and steroidal substances, as well as tannins and high levels of flavonoids. In addition, Galvão et al. (2002) detected flavonoid glycosides, cardiac glycosides with a steroidal nucleus and pentagonal lactonic rings, aromatic glycosides, saponins, hydrolysable and condensed tannins and aliphatic nitro-compounds.

According to Chieregatto (2005) and Monteiro (2007) long term treatments using *H. aphrodisiaca* infusion caused significant body weight gain, which could be related to higher plasma testosterone levels, possibly leading to anabolic effects on the muscles of Wistar rats.

Monteiro et al. (2008) observed that *H. aphrodisiaca* infusion had a protective potential against testicular damage caused by Cyclosporine A. This drug has been associated to severe nephrotoxicity, hepatotoxicity (Rezzani, 2004) and testicular damage (Monteiro et al., 2008), and the main side effects are associated with increased reactive oxygen species (Cid et al., 2003). Moreover, the *H. aphrodisiaca* extract (BST 0298) increased the total antioxidant enzyme activity: superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD) and copper and zinc

superoxide dismutase (CuZnSOD) in brains of old rats (Mattei et al., 2001). It also improved the memory of aged rats (Galvão et al., 2002).

Since the prostate depends on testosterone for its maintenance (Spaziani, 1975), alterations of testosterone levels, caused by *H. aphrodisiaca* infusion, would probably affect the prostatic tissue. With this study we determined the effects of the infusion in normal prostate tissue, as a basis for future studies on the effects of *H. aphrodisiaca* in damaged tissue.

MATERIAL AND METHODS

Medicinal Plant

H. aphrodisiaca roots were collected in Nova Xavantina, Mato Grosso, Brazil, and identified by comparison with voucher samples kept in the herbarium of the Federal University of Mato Grosso (record number 23928).

The roots were dried at room temperature (approximately 23ºC) and protected from sunlight and rain. Then, they were ground and the infusion was prepared pouring 100mL of boiling water over 25g of ground roots, allowing it to steep for 4 hours until filtering, using filter paper. The yield of this infusion was 68.66 mg of dry extract (6.866% w/v). *H. aphrodisiaca* infusion preparation was identical to that used previously by other authors (Chieregatto, 2005; Monteiro et al., 2008; Sbervelheri et al., 2009; Monteiro et al., 2010).

Experimental Animals and Treatment Protocol

Twelve adult male Wistar rats (90 days old) were obtained from the Center of Biological Investigation – CEMIB (State University of Campinas, Campinas, SP, Brazil) – and maintained

under standard conditions with 12h light: 12h darkness. The animals received commercial rat chow and water *ad libitum*.

The animals were randomly divided into two groups (n=6 in each). The control group received distilled water (0.5 mL), whereas the treated group received *H. aphrodisiaca* infusion (0.5 mL), both by gavage. The treatment was administrated daily, during 56 days (Chieregatto, 2005; Monteiro et al., 2008; Sbervelheri et al., 2009; Monteiro et al., 2010).

The experimental protocol followed the rules employed by the Committee for Ethical Treatment in Animal Experimentation used by the Brazilian College of Animal Experimentation (COBEA) and was approved by the latter (protocol number 1805-1).

Euthanasia, Biometry and Tissue Samples

The animals were weighed and anesthetized with xylazine and ketamine (5 and 80 mg/kg body weight, respectively). The abdominal cavity was opened to reach the testis, epididymis, seminal vesicle, coagulating gland and ventral prostate, which were excised and weighed.

Preparation of Tissue for Microscopy

After dissection, the ventral prostate was divided into two portions. One of them was fixed in Karnovsky fixative (4% glutaraldehyde and 4% paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4), for 24 hours. Some fragments were routinely processed for further hydroxyethyl methacrylate embedding. Thin sections (2µm) were obtained and stained with hematoxilin and eosin for light microscopy observation. Other fragments were post-fixed in 1% osmium tetroxide and then processed using the routine technique for epoxy resin embedding, for transmission electron microscopy.

The other portion of the ventral prostate was fixed in Metacarn (Methanol: chloroform: acetic acid – 6:3:1) during 4 hours (4ºC) and processed by routine paraffin embedding technique. They were sectioned (5µm) and used for androgen receptor (AR) immunohistochemistry and TUNEL assay.

Transmission Electron Microscopy (TEM)

Ultra thin sections (60nm) were obtained and stained with 3% uranyl acetate (30 min) and 2% lead citrate (5 min) and photodocumented with the transmission electron microscope (Zeiss; Leo 906).

Morphometry and Stereology

All morphometrical and stereological analyzes were employed in the intermediary region of the ventral prostate.

Digital images were taken with a camera coupled to a light microscope (Olympus BX-40), and then used to perform all morphometrical and stereological analyzes using the software Image-Pro Plus 6.0.

Ten random images (200x magnification) were used to estimate the volumetric proportion of the prostate tissue elements (lumen, epithelium, muscular and non muscular stroma) per animal. A 130-point grid system was used per image. The absolute volumes of the above elements were estimated multiplying the volumetric proportions based on the weight of the prostate (Cordeiro et al., 2008), considering that the volume of 1 mg of ventral prostate is approximately 1 mm^3 (DeKlerk e Coffey, 1978; Huttunen et al., 1981).

The perimeter and area of 30 epithelial cells per animal (and their nuclei) were estimated using images at 1,000x magnification. The cytoplasmic area was estimated subtracting the nuclear area from the cellular area. The calculated areas were used to estimate the proportion of nucleus and cytoplasm in the epithelial cell. The form factor (4π X nuclear area / [nuclear perimeter]^2) was calculated, based on both the perimeter and area of the nuclei (Santos et al., 2006).

Epithelium and muscular stroma thickness were estimated measuring 10 thicknesses per image at 1,000x magnification. Ten images were used per animal.

Immunocytochemistry and TUNEL

An immunocytochemical technique was used for the detection of androgen receptor (AR).

The slides were maintained in citrate buffer and kept at a high temperature (100ºC) during 45 minutes for antigenic retrieval. Hydrogen peroxide (0.3%) in methanol was used for blocking endogenous peroxidase activity. The slides were incubated with the primary antibody anti-AR (1:150, rabbit polyclonal IgG, SC-816, Santa Cruz Biotechnology, USA) overnight, at 4ºC, and incubated with biotinylated secondary antibody, at 37°C, for 45 minutes (goat, anti-rabbit, SC-2018, Santa Cruz Biotechnology, USA), the dilution was according to manufacturer. Afterwards, as recommended by the kit's manufacturer, the slides were incubated with peroxidase-conjugated avidin–biotin complexes and then the reaction stained by diaminobenzidine (DAB) (SC-2018, Santa Cruz Biotechnology, USA) during 5 minutes. The sections were counterstained with Harris's hematoxylin for 3 seconds. In all the reactions a negative control was made following the same protocol described above, except for the primary antibody that was substituted by PBS buffer (pH7.4), at 37º, for 45 minutes.

The TUNEL assay was applied for the detection of apoptotic nuclei using the kit for fragmented DNA detection (US1QIA33-1EA; TdT FragEL TM DNA Fragment. Detect, Merck).

The slides were incubated with Proteinase K for permeabilization followed by blocking the endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 5min. They were submitted to the equilibrium reaction, labeled and stained. Counterstaining used Harris' hematoxylin for 3 sec. The epithelium of 10 randomly selected areas, at 400x magnification, was counted per animal to establish the normal and apoptotic nuclei (Shabisgh et al., 1999). The apoptotic index was the percentage of apoptotic nuclei within the epithelium.

Statistical Analysis

Results were compared for the experimental groups with the t-test, using the software STATISTICA 5.1. A value of $p<0.05$ was considered significant. All the results were presented as mean ± standard deviation.

RESULTS

Final body weight and body weight gain did not vary among experimental groups (Table 1). Similarly, there was no variation in the testis, epididymis, seminal vesicle, coagulating gland and ventral prostate weight in the *H. aphrodisiaca* treated group (Table 1).

Considering all quantitative parameters of the prostatic tissue, no significant alteration was observed for the *H. aphrodisiaca* treated group, except for the form factor that was approximately one in the treated group ($p=0.026$) (Table 2).

There was no difference caused by the treatment as shown by morphology and ultrastructure of the ventral prostate tissue of treated animals (Figures 1 and 2). Both groups showed epithelial cells with basal nuclei and clear supranuclear regions corresponding to the Golgi apparatus area, which was confirmed by transmission electron microcopy (TEM) (Figures 1 and 2).

Also, transmission electron microscopy (TEM) of the epithelial cells revealed the cytoplasm rich in rough endoplasmic reticulum that was enlarged in most of the cells analyzed (Figure 2).

AR immunohistochemistry did not show any variation pattern of nuclear androgen receptor expression in the epithelial and stromal nuclei of the *H. aphrodisiaca* treated group (Figure 3). Both groups showed most of the epithelial cells expressing androgen receptors (Figure 3). Even some smooth muscle cell nuclei expressed a discrete positive reaction (Figure 3). When the reaction intensity (dark or light brown) for AR positive nuclei was analyzed, differences were not observed.

The apoptotic index did not vary in the treated group when compared to the control (Figure 4).

DISCUSSION

Heteropterys aphrodisiaca is a traditional aphrodisiac plant that could stimulate testosterone production (Chieregatto, 2005; Monteiro et al., 2008). The rat prostate, particularly the ventral lobules, have been the preferred organ for studies of androgen action (Hayashi et al., 1991). This was the main reason why the tissue was chosen to evaluate the effects of long term *H. aphrodisiaca* infusion administration.

According to Marques and colleagues (2007) water is the best extracting liquid from *H. aphrodisiaca* roots regarding extractable solids. The infusion preparation and the dose of *H. aphrodisiaca* administered were employed according to previous studies (Chieregatto, 2005, Sbervelheri et al., 2009; Monteiro et al., 2008, 2010). It is also important to point out that Sbervelheri and colleagues (2009) proved that *H. aphrodisiaca* root infusion did not cause any toxic effect on the liver and kidneys of Wistar rats.

Contrary to Chieregatto (2005) observations, the body and testicular weight did not increase in the *H. aphrodisiaca* treated group, when compared to the control. Both body and all organ

weights (testis, epididymis, seminal vesicle, coagulating gland and seminal vesicles) did not increase in the *H. aphrodisiaca* treated animals in the present study, as observed by Monteiro and colleagues (2008). Prostate and seminal vesicles are closely dependent on testosterone for their structural integrity and normal function (Spaziani, 1975). Therefore, the lack of alterations of the prostate and seminal vesicle weights suggests that there was no significant alteration of the testosterone levels in the treated animals.

Previous studies performed by Hayashi and colleagues (1991) showed that ventral prostate tissue in rats consisted in paired right and left lobes and the ducts of the ventral prostate had few enfoldings. Similar structure was observed in both groups of the present study. The histology of the ventral prostate in the control and the *H. aphrodisiaca* treated group was identical to that described by García-Flórez et al. (2005) for control animals: "the ventral prostate had a simple, cylindrical epithelium with basal nuclei, scarce stroma, with few smooth muscle cells and fibroblasts".

The expression of androgen receptors was similar in the control and *H. aphrodisiaca* ventral prostate, and these results were according to those of Shabisgh et al. (1999), who observed the presence of androgen receptors in most epithelial and smooth muscle cells (periductal and perivascular) and in some fibroblast cells of the rat ventral prostate. The reaction intensity of AR-positive cells could be related to an increase in AR expression (Scarano et al., 2009). Therefore, the treatment with *H. aphrodisiaca* probably did not alter the pattern of AR expression on the ventral prostate of Wistar rats.

The programmed cell death (apoptosis) is very important in normal cell turnover of adult tissue (Wijsman et al., 1993). A complicating factor to recognize apoptotic cells by their morphology is the low frequency of these cells and the short duration of the morphological changes related to apoptosis (Wijsman et al., 1993). Therefore a specific technique to detect DNA fragmentation (TUNEL assay) was applied to identify apoptotic cells. Shabisgh et al. (1999) observed that there was virtually no apoptosis in the ventral prostate of control Wistar rats and

according to Wijsman et al. (1993) the apoptotic index in the ventral prostate of rats is less then 0.1%. The ventral prostate apoptotic index observed in the control and treated group of the present study were similar to the above findings, which indicated that *H. aphrodisiaca* infusion did not cause any effect that altered the normal programmed cell death rate of the ventral prostate.

An anabolic effect of *H. aphrodisiaca* caused by an increase of testosterone levels had been suggested by some authors (Chieregatto, 2005; Monteiro 2007). They did not observe any increase in testosterone plasmatic levels, weight of the accessory sex organs and other testicular parameters that could prove higher testosterone levels. The stability of the testosterone levels is supported by various findings. There was no increase in the weight of male reproductive accessory organs, with maintenance of stereological and morphometrical parameters of the prostate. Also, the identical pattern of androgen receptor expression in the ventral prostate of animals treated with *H. aphrodisiaca* when compared to the controls indicated that there was no increase in testosterone levels. Moreover, the normal apoptotic index and absence of histopathological alterations in the ventral prostate of Wistar rats treated with *H. aphrodisiaca* indicated that this treatment did not cause any injury to the ventral prostate tissue.

The route, period and dose of *H. aphrodisiaca* root infusion administration to adult Wistar rats did not result in benefits to their ventral prostate. However, future studies would be important in order to elucidate the apparent antioxidant potential of this plant on prostate tissue.

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TABLE 1. Biometrical parameters of control adult Wistar rats and those treated with *Heteropterys aphrodisiaca* (HA) infusion. There were no significant differences among the experimental groups. The values are means ±SD

Weight (g)	Control	HA
Body	493.33 ± 28.23	496.50 ± 29.62
Body gain	87.56 ± 20.35	94.33 ± 21.74
Testis	1.72 ± 0.12	1.77 ± 0.14
Epididymis	0.61 ± 0.02	0.63 ± 0.03
Seminal Vesicle	0.97 ± 0.10	0.93 ± 0.27
Coagulation Gland	0.21 ± 0.03	0.21 ± 0.04
Ventral Prostate	0.50 ± 0.13	0.48 ± 0.07

TABLE 2. Morphometric and stereological parameters of the ventral prostate of control adult Wistar rats and those treated with *Heteropterys aphrodisiaca* (HA) infusion. The values are means ± SD.

Parameters	Control	HA
Volumetric proportions (%)		
Lumen	55.23 ± 8.65	55.74 ± 6.85
Epithelium	27.77 ± 6.97	27.13 ± 8.05
Muscular stroma	6.69 ± 1.70	6.51 ± 1.55
Non-muscular stroma	10.31 ± 1.20	10.61 ± 2.79
Absolute volume (mL)		
Lumen	0.29 ± 0.08	0.26 ± 0.03
Epithelium	0.14 ± 0.04	0.13 ± 0.05
Muscular stroma	0.03 ± 0.01	0.03 ± 0.01
Non-muscular stroma	0.05 ± 0.01	0.05 ± 0.01
Area (μm^2)		
Ephitelial cell	158.94 ± 14.79	164.60 ± 20.85
Ephitelial cell nucleus	35.21 ± 2.27	36.24 ± 3.47
Ephitelial cell cytoplasm	123.73 ± 12.91	128.36 ± 19.63
Form Factor	**0.68 ± 0.04**	**0.73 ± 0.02***
Ephitelium thickness	23.21 ± 2.06	23.87 ± 3.46
Muscular stroma thickness	5.01 ± 0.75	5.03 ± 0.56

* Indicates significant difference ($p<0.05$), according to t-test.

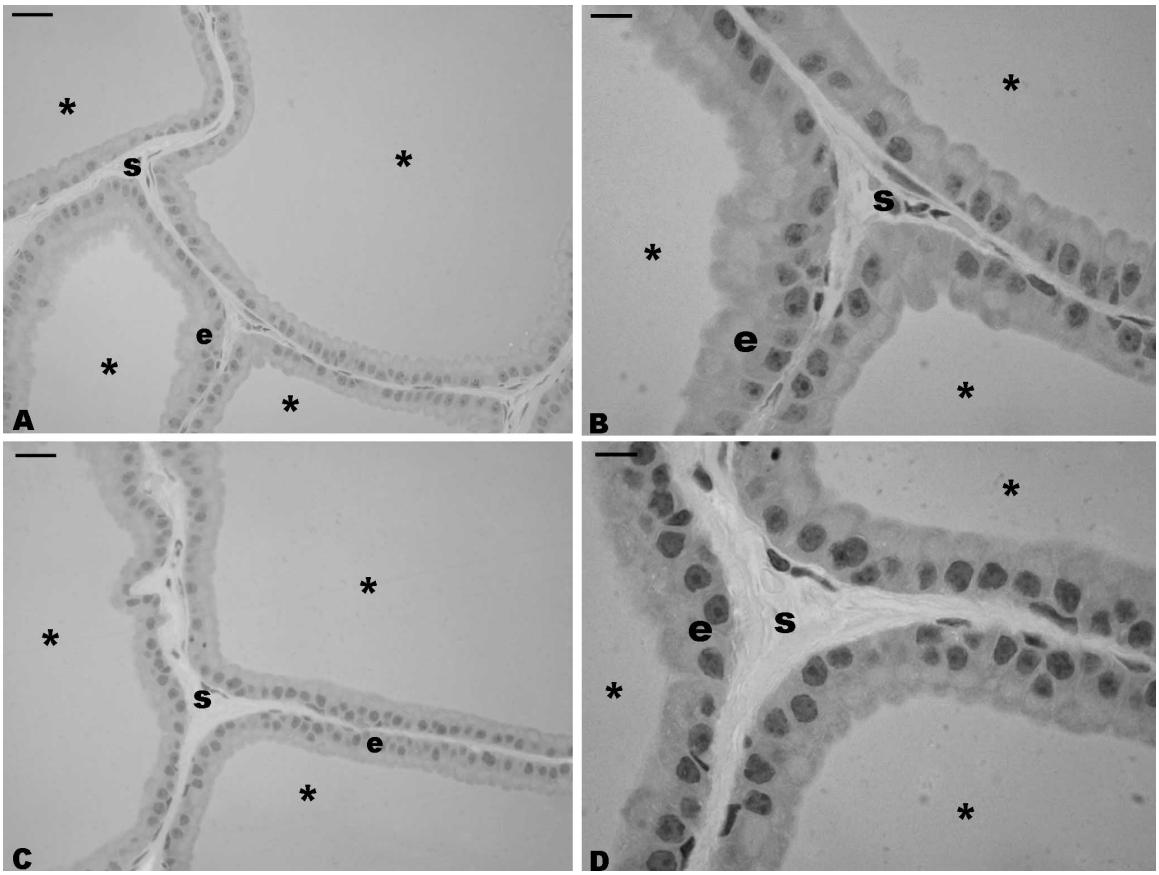

Figure 1. Histological sections of Wistar rats' ventral prostate stained with Hematoxilin and Eosin. The images represent the control (A and B) and *H. aphrodisiaca* treated (C and D) animals. Notice the basal nuclei and the clear supranuclear region that corresponds to the Golgi complex region in the epithelial cytoplasm (e). Abbreviations: e = secretory epithelium; s = stroma; asterisks = lumen. Bars: A and C = 24µm; B and D = 10µm

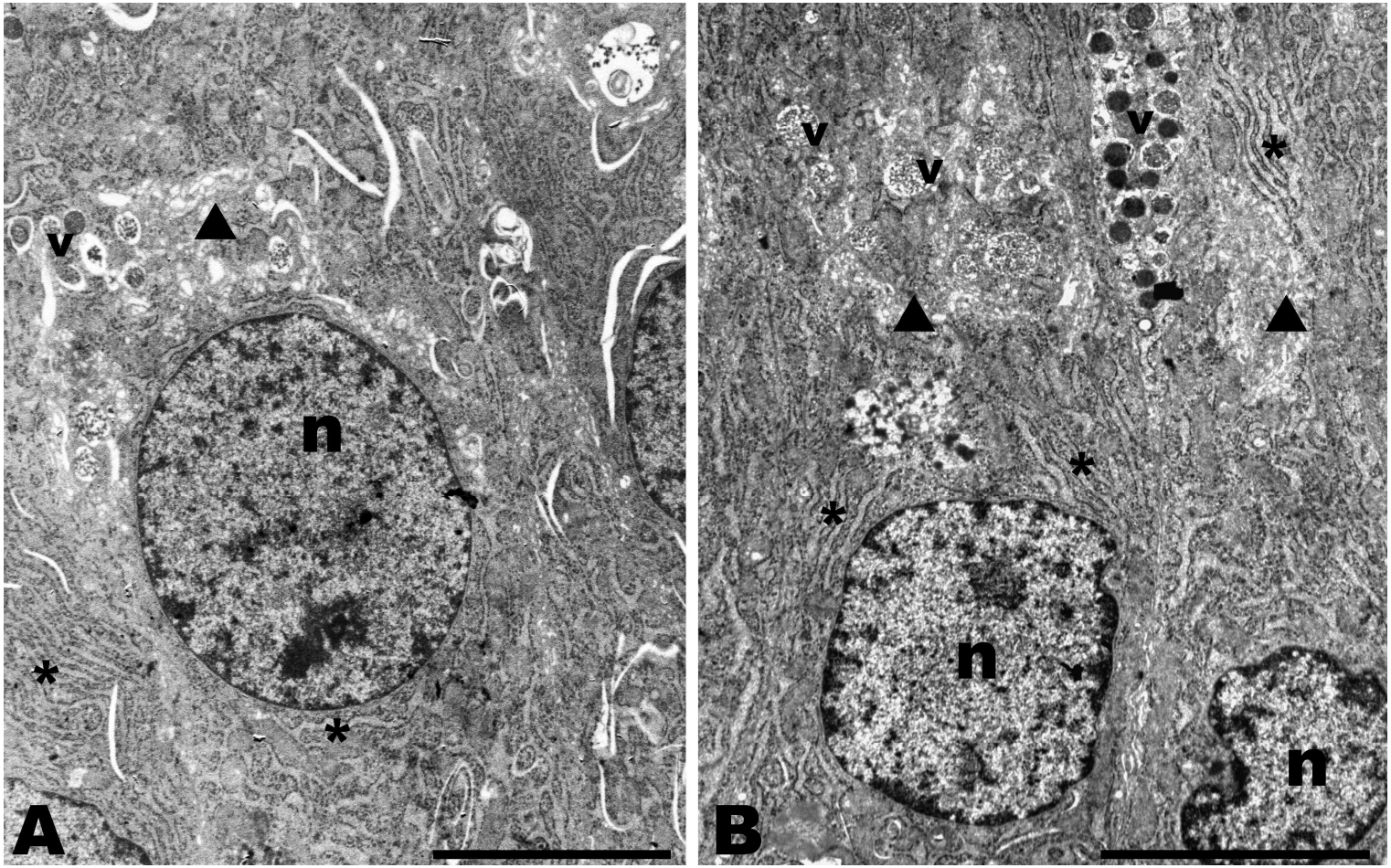

Figure 2. Transmission electron micrographs of typical control (A) and *H. aphrodisiaca* treated (B) ventral prostate epithelium in adult Wistar rats. Observe the region occupied by the Golgi complex (black triangle) above the nucleus (n). The secretion vesicles (v) are near the Golgi area. Note the large amount of rough endoplasmic reticulum in all regions of the cytoplasm (asterisks). Bars A and B = 5µm.

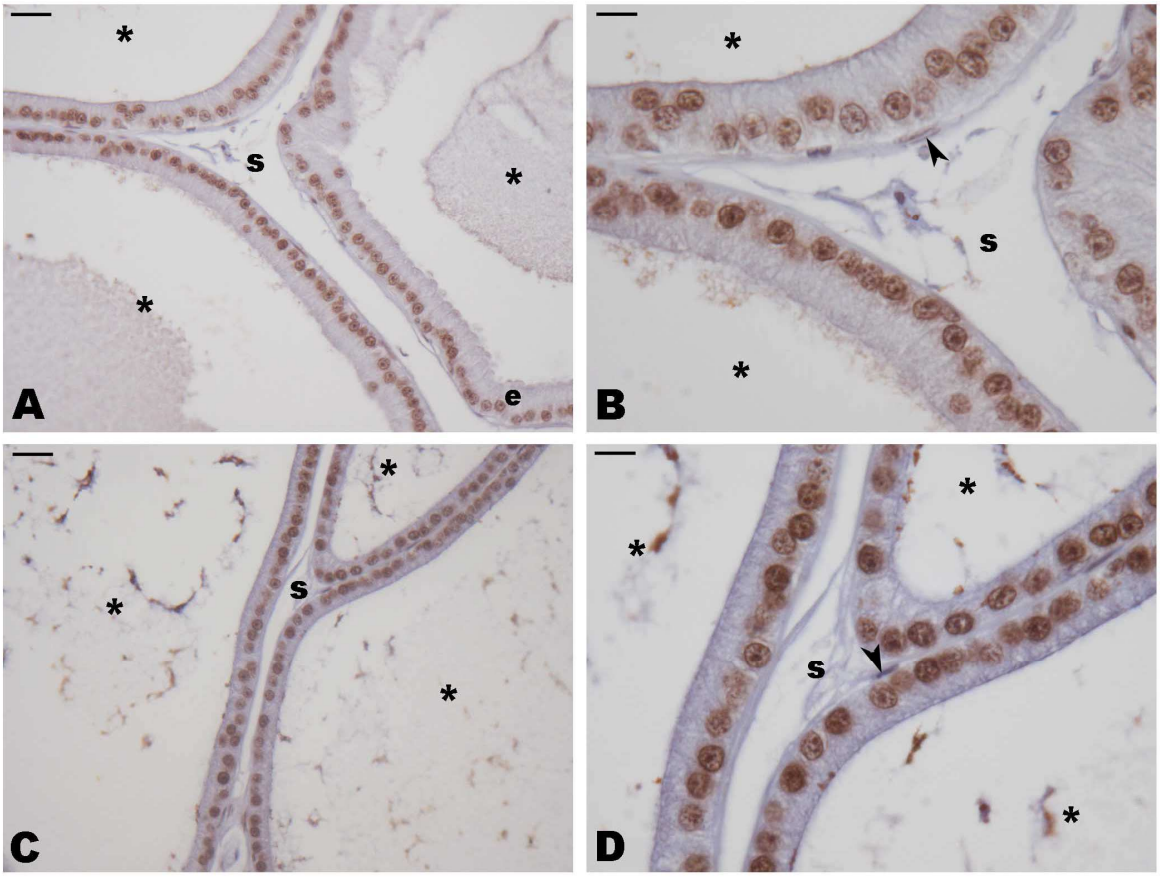

Figure 3. Histological sections of Wistar rats' ventral prostate submitted to AR immunocytochemistry. Brown nuclei indicate positive reaction. Control animals (A and B) showed a reaction similar to the *H. aphrodisiaca* treated animals (C and D). Abbreviations: e = secretory epithelium; s = stroma; asterisks = lumen; arrowheads = smooth muscle cell nuclei. Bars A and B = 24µm; C and D = 10µm.

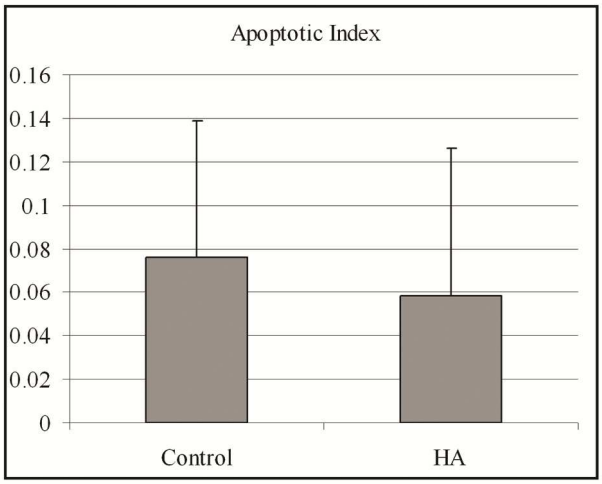

Figure 4. Apoptotic indexes in control and *Heteropterys aphrodisiaca* (HA) treated groups. The values are means ± SD.

Cyclosporin A causes impairment of the ventral prostate tissue of Wistar rats.

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Running title: CsA and rat ventral prostate

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ABSTRACT

Cyclosporin A is an immunosuppressive drug widely used in medicine. However, many side effects can be related to its use, such as, reduction of serum testosterone levels, damage to testis structure, as well as male infertility. The evaluation of the effects of chronic CsA administration on the ventral prostate tissue was studied, using stereological, morphometrical, morphological and ultra-structural observations. The plasmatic testosterone levels were measured and the AR immunohistochemical method applied. Apoptosis was detected with the TUNEL technique. The CsA treatment caused reduction of plasmatic testosterone levels and ventral prostate weight. The volume of all ventral prostate tissue components (lumen, epithelium, muscular and non-muscular stroma) was reduced. The epithelium of treated animals was atrophied, which was also confirmed with transmission electron microscopy. There was no alteration of AR expression or apoptotic index. In conclusion, CsA caused severe damage to the prostate tissue, probably as a consequence of reduced plasmatic testosterone levels.

INTRODUCTION

Cyclosporin A (CsA) is a hydrophobic, cyclic undecapeptide isolated from the fungus *Cylindrocapon lucidum* Booth and *Tolypocladium inflatum* Gamn (Rezzani, 2004; Shevach, 1985); it exerts a variety of biological and physiological effects such as antiparasitic, fungicidal, anti-inflammatory and mainly immunosuppressive agent (Monteiro et al., 2008). This is an immunosuppressive drug widely used after organ transplants and against auto-immune diseases (Heusler e Pletscher, 2001; Rezzani, 2004; Shevach, 1985). CsA exerts its major therapeutic effects by inhibiting T-lymphocyte activation (Shevach, 1985).

CsA is selectively cytotoxic and a suppressor of the lymphocyte subpopulation but does not interfere with phagocytes' functions or hematopoietic stem cells (Borel et al., 1989; Hess e Tutschka, 1980). Also, many side effects, such as immunological, renal, hepatic, neurological and hypertensive complications are caused by CsA administration (Rezzani, 2004). CsA-induced nephrotoxicity and hepatotoxicity are related to the production of increased reactive oxygen species (Cid et al., 2003; Türk et al., 2007). Also, reproductive organs dysfunctions induced by CsA administration are extensively reported in the literature (Krueger et al., 1991; Masuda et al., 2003; Rajfer et al., 1987; Seethalakshmi et al., 1988; Seethalakshmi et al., 1990a; Seethalakshmi et al., 1992; Seethalakshmi et al., 1987; Seethalakshmi et al., 1989; Sikka et al., 1988; Srinivas et al., 1998b). CsA reduces testis, epididymis, seminal vesicle and prostate weight (Seethalakshmi et al., 1987), causes atrophy of seminiferous tubules with impairment of Sertoli cell function (Masuda et al., 2003) and reduced testicular and epididymal sperm counts, sperm motility and fertility (Seethalakshmi et al., 1987; Seethalakshmi et al., 1989). CsA also impairs steroidogenesis (Krueger et al., 1991; Rajfer et al., 1987; Seethalakshmi et al., 1989; Srinivas et al., 1998b), which probably occurs due to a suppression of the hypothalamic-pituitary axis rather than direct inhibition at the testicular level (Krueger et al., 1991; Rajfer et al., 1987; Seethalakshmi et al., 1990a; Seethalakshmi et al., 1992).

Prostate weight reduction caused by CsA administration is commonly reported in related studies (Heusler e Pletscher, 2001; Seethalakshmi et al., 1988; Seethalakshmi et al., 1990a). The prostate provides the bulk of the seminal fluid, adding proteins and ions. The absence of a prostate leads to fertility impairment (Hayward e Cunha, 2000). Its development and functioning are androgen dependent (García-Flórez et al., 2005; Marker et al., 2003; Spaziani, 1975; Vilamaior et al., 2006). Androgen receptors (AR) are present in most cells of the prostatic epithelium and smooth muscle cells and in some fibroblasts, but not endothelial cells (Shabisgh et al., 1999). ATPases could be possible sites for testosterone action in the prostate and affect the transport of water, ions and organic metabolites through blood vessels, reaching the target cells (Spaziani, 1975). Prostatic response to reduction of testosterone levels, in castrated animals, begins with a decline in blood flow followed by epithelial cell apoptosis (Hayward e Cunha, 2000; Shabisgh et al., 1999).

The prostate is divided in three lobes: anterior (coagulating gland), lateral, dorsal, and ventral. Despite the fact that this latter part has been the preferred tissue for studies of androgen action (Hayashi et al., 1991), no previous research has shown the effects on the prostate tissue of CsA administration. Therefore, the present study undertook the evaluation of CsA effects on the ventral prostate of Wistar rats as measured by morphology and ultrastructure.

MATERIAL AND METHODS

Animal Protocol

Twelve adult male Wistar rats (90 days old) were obtained from the Center of Biological Investigation - CEMIB (State University of Campinas, Campinas, SP, Brazil) and maintained under standard conditions with 12h light : 12h dark and free access to food and water.

Six animals received Cyclosporin A (Sandimmun Neoral – Oral Solution; 100mg/mL; Novartis Pharma Ag, Switzerland) daily, by gavage (15 mg/kg per day) (Chung et al., 2005; Kotolová et al., 2006; Monteiro et al., 2008; Türk et al., 2007) dissolved in 0.5 mL distilled water.

Control rats (n=6) received 0.5 mL of distilled water daily by gavage. The animals were weighed weekly to calculate the CsA dose. The animals were treated during 56 days (Chung et al., 2005; Kotolová et al., 2006; Monteiro et al., 2008; Türk et al., 2007). The experimental protocol was approved by the Institutional Committee for Animal Care and Use of the State University of Campinas (protocol number 1805-1).

The animals were weighed, anesthetized with xylazine and ketamine (5 and 80 mg/kg body weight, respectively) and the abdomen and thoracic cavities were opened. The testis, epididymis, seminal vesicle, coagulating gland and ventral prostate were excised and weighed.

Testosterone assay

Blood samples were collected by cardiac puncture (left ventricle) immediately before death, and stored in Vacuette® tubes under refrigeration. Plasma was obtained by blood centrifugation (1,400 G, for 5 minutes at 4ºC). Plasma testosterone levels were estimated by chemiluminescence method in the Modular E170 analyser (Roche), using triplicates for each sample. All samples were sent to a specialized laboratory (Laboratório Álvaro, Cascavel, Brazil).

Morphometrical and Stereological Analysis

Fragments of one lobe of the ventral prostate were fixed by immersion in Karnovsky fixative (4% paraformaldehyde, 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.2) for 24h. After fixation, the tissue was routinely prepared for embedding in 2-hydroxyethyl methacrylate. Two micrometer sections were obtained, stained with hematoxylin-eosin and observed with an Olympus BX-40 light microscope.

All morphometrical and stereological measurements were employed in the intermediary region of ventral prostate. The Image Pro Plus software was used for all the following measurements. The volume density of the tissue compartments (lumen, epithelium, muscular and

non-muscular stroma) was determined employing a modified methodology according to Weibel (Weibel, 1963) using a 130-point grid system. Ten microscope fields were randomly chosen (200x magnification). Absolute volumes were estimated multiplying volume density by prostate weight (Cordeiro et al., 2008) since the ventral prostate density could be considered as approximately 1.0 (DeKlerk e Coffey, 1978; Huttunen et al., 1981). Morphometric analysis included the determination of epithelial cell and muscular stroma thickness (μm), nuclear area (μm^2) and perimeter (μm), cytoplasmic area (μm^2) and form factor (4π X nuclear area / [nuclear perimeter]^2).

Ultrastructural Analysis

Ventral prostate fragments were fixed by immersion in Karnovsky fixative (2.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer, pH 7.4) for 24h. The materials were post fixed in 1% osmium tetroxide for 2 h, dehydrated in acetone and embedded in epoxy resin. Ultra thin sections (60 - 70 nm) were stained with 3% uranyl acetate (30 min) and 2% lead citrate (5 min) and then observed with a transmission electron microscope (Zeiss; Leo 906).

Androgen Receptor Immunohistochemistry and Detection of Apoptosis

The other ventral prostate lobe was fixed by immersion in Metacarn (Methanol: chloroform: acetic acid – 6:3:1), routinely prepared for paraffin embedding and cut into 5μm sections for Androgen receptor (AR) immunohistochemistry and TUNEL assays.

The antigen retrieval was carried out by incubating sections in citrate buffer (pH 6.0) and kept at high temperature (100ºC) for 45 minutes. Endogenous peroxidase was blocked with hydrogen peroxide (0.3%) in methanol for 20 minutes. After rinsing with phosphate saline buffer (PBS) for 5 minutes, the slides were incubated with the primary antibody anti-AR (1:150; rabbit, SC-816, Santa Cruz Biotechnology, USA), at 37ºC, for 1 hour and incubated with hrp-conjugated biotinylated antibody (goat, anti-rabbit; SC 2018, Santa Cruz Biotechnology, USA) at 37◦C for 45

minutes, at the dilution indicated by the kit's manufacturer. The slides were then incubated, as indicated by the kit´s manufacturer, with peroxidase-conjugated avidin–biotin complexes and diaminobenzidine (DAB) for 5 minutes, and counterstained with Harris's hematoxylin for 10 sec. All the reactions had a negative control, which followed the same protocol described above, except for the primary antibody that was substituted by PBS buffer (pH7.4), at 37º, for 45 minutes.

The detection of apoptotic nuclei was obtained using a kit for fragmented DNA detection (US1QIA33-1EA; TdT FragEL TM DNA Fragment. Detect, Merck), all the reagents were diluted as suggested by the kit's manufacturer. The slides were incubated with Proteinase K for permeabilization followed by endogenous peroxidase activity blocking with 3% hydrogen peroxide in methanol (1:1) for 5min. The slides were submitted to the equilibrium reaction. Detection was performed as indicated by the kit's manufacturer. The positive reaction was developed by DAB/H_2O_2. Finally, the sections were counterstained with Harris hematoxylin for 10 sec.

Apoptotic nuclei (brown colored) were quantified in 10 different high magnification fields (x400), for each animal, and compared to the total number of epithelial cells in the field to obtain the apoptotic index (Shabisgh et al., 1999).

Statistics

Statistical tests were performed with Statistica® 5.1 software. Results were expressed as mean ± standard deviation. The means were compared using t-test and a value of $p<0.05$ was considered significant.

RESULTS

Biometric Analysis

All biometrical data appear in Table 1. The body weight gain in CsA animals was 49.64% lower than in the control group, but still not significant ($p=0.052$). The ventral prostate, coagulating

gland and epididymis weight was significantly reduced in the animals treated with CsA (p= 0.006; 0.002 and 0.026 respectively). Testicular and seminal vesicle weight did not differ significantly among the experimental groups.

Plasmatic testosterone levels

The plasmatic testosterone levels reduced 60.76% in CsA treated group when compared to the control (p=0.025) (Figure 1).

Morphometric and Stereological Parameters

The volumetric proportion of prostate tissue elements did not differ between the experimental groups. The absolute volume of lumen, epithelium, muscular and non-muscular stroma was significantly reduced in the CsA treated group (p= 0.03, 0.01, 0.005 and 0.001 respectively) (Figure 2).

The nuclear, cytoplasmic and total areas of prostate epithelial cells, as well as epithelial and muscular thicknesses, were significantly reduced in CsA treated animals (Table 2).

Morphological and Ultrastructural Aspects

The ventral prostate of control animals showed normal morphology. The epithelium had cubic/columnar secretory cells with basal nuclei and a clear supra-nuclear region that corresponded to the Golgi complex region (Figure 1A and B and 2A). Most of the epithelial cell cytoplasm was occupied by enlarged rough endoplasmic reticulum in the control animals (Figure 2A). Secretion vesicles could be observed near the Golgi complex (Figure 2A).

Despite occurring in the whole organ, the atrophy observed in the prostatic epithelium was variable: while some parts showed severe damage due to CsA treatment, other regions were clear, with less damage. The ventral prostate epithelium varied from normal, cubic/columnar cells to

completely atrophied squamous epithelium (Figure 1 and 2). The ultra-structural analyses confirmed epithelial atrophy with reduction of secretory organelles (rough endoplasmic reticulum and Golgi complex), or their total absence (Figure 2).

AR Immunohistochemistry and Apoptosis Detection

No change was observed in androgen receptor (AR) immunocytochemistry in CsA treated ventral prostate animals: even atrophied epithelial cells nuclei were positive to AR assay (Figure 3).

No variation of the apoptotic index was found between experimental groups (Figure 4).

DISCUSSION

Many side effects are related to the chronic treatment with CsA. There are extensive studies on the side effects of testicular structure, sperm production and testosterone production (Krueger et al., 1991; Masuda et al., 2003; Monteiro et al., 2008; Rajfer et al., 1987; Seethalakshmi et al., 1988; Seethalakshmi et al., 1990a; Seethalakshmi et al., 1992; Seethalakshmi et al., 1987; Seethalakshmi et al., 1989; Sikka et al., 1988; Srinivas et al., 1998b; Türk et al., 2007). However, even after a wide search of specialized journals, no previous study determining the effects of chronic treatment with Cyclosporin A (CsA) on the ventral prostate tissue was found.

The dose of CsA administered to the animals in this study was according to that specified by the manufacturer for treatment after organ transplantation and was the same used by other authors (Chung et al., 2005; Kotolová et al., 2006; Monteiro et al., 2008; Türk et al., 2007).

In general, body weight is evaluated in order to determine the effect of CsA treatment on the general health of the animals (Seethalakshmi et al., 1990b; Seethalakshmi et al., 1987). Body weight gain was 49.03% lower in the CsA-treated group, although this was not statistically significant, probably due to large differences in the animals' sensibility to the treatment. A similar

result was observed by Monteiro et al. (2008) in Wistar rats treated with the same CsA dose, administered for the same period of time.

Some reproductive organs, such as ventral prostate, coagulating gland and epididymis, showed significant weight reduction after CsA treatment which probably occurred due to reduction of plasma testosterone levels, since they are testosterone dependent (Risbridger e Taylor, 2006; Spaziani, 1975). On the other hand, testis and seminal vesicle gland weight did not alter in the treated group. In a previous study using animals treated with the same CsA dose no alteration was observed in ventral prostate weight, coagulating gland, seminal vesicle, epididymis or testis (Monteiro et al., 2008). However, Türk et al. (2007) observed the maintenance of testis, epididymis and prostate weights while the seminal vesicle weight was reduced in animals treated daily with 15mg/kg of CsA.

CsA treatment caused significant reduction in volume of all the prostate components (epithelium, lumen and stroma – muscular and non muscular), leading to decreased prostate weight. Also, prominent epithelial atrophy was observed in CsA-treated animals. Prostate atrophy has been described as an adaptive or secondary response in a normal developed of the organ; and is characterized by shrinkage due to loss of cellular substance, leading to diminished organ size (Shappell et al., 2004), corroborating the results that we presented. Atrophy of the ventral prostate epithelium can be related to reduction of cellular secretory capacity, since there was significant reduction of nuclear and cytoplasmic volume. The organelles directly involved in cellular secretion, mainly the rough endoplasmic reticulum and Golgi complex, were also greatly reduced, as observed by transmission electron microscopy. Despite the fact that atrophy is considered a common histological alteration in the human prostate, it is apparently uncommon in wild-type mouse species (Shappell et al., 2004). Atrophy showed by CsA treated animals was probably due to the reduction of the testosterone levels (60.78% less testosterone in the treated group). Reduction of testosterone levels in animals treated with CsA was extensively reported in other studies (Krueger et al., 1991;

Rajfer et al., 1987; Seethalakshmi et al., 1989; Sikka et al., 1988; Srinivas et al., 1998b). In castrated animals, the epithelium was reduced to half within two days after castration (Huttunen et al., 1981). According to Huttunen et al. (1982) the epithelium is especially sensitive to androgen deprivation.

The alloxan-induced diabetes in medium-term cause reduction of testosterone levels and atrophy of the ventral prostate epithelium (Arcolino et al., 2010), similar to that observed in the present study. According to the authors (Arcolino et al., 2010) the epithelial alterations that occur in alloxan-induced, medium-term diabetes could be caused by the joint action of insulin and other factor associated to diabetes, such as high glucose levels, as well as reduced testosterone levels. Nielsen and colleagues (1986) concluded that CsA has a direct inhibitory effect on insulin secretion. So the prostate damage caused by CsA could either be consequence of a reduction of insulin secretion and/or increase of glucose levels, besides the reduction on testosterone levels.

In ventral prostate of adult rats, AR receptors are concentrated in the epithelial cell nuclei, while in stoma cell nuclei they occur in moderate levels or are absent (Prins et al., 1991). The pattern of AR expression suggests that epithelial cells are the major target of androgen action in the ventral prostate (Prins et al., 1991). There is a dramatic loss of nuclear androgen receptor 12h after castration and it appears to be an important (not the only) initiator of apoptosis activation within the prostate (Kyprianou e Isaacs, 1988). The atrophy of the prostatic epithelium is associated with a large increase of apoptotic index, which reaches its maximum range 72 hours after castration. After this, the apoptotic index decreases, going back to normal after 7 days (García-Flórez et al., 2005; Shabisgh et al., 1999). The absence of alteration of the AR immunohistochemical test and the apoptotic index in the present study can be due to the chronic treatment with CsA (56 days).

Many antiandrogenic drugs are used in the treatment of the prostate cancer. Cyproterone acetate and megestrol acetate are steroidal antiandrogenic drugs that inhibit testosterone production by Leydig cells as a result of partial suppression of the luteinizing hormone from the anterior

pituitary lobe (McLeod, 1993). According to Marks et al. (1999), the patients with symptomatic benign prostate hyperplasia, that were treated with finasteride for 6 months, presented progressive epithelial atrophy. This appears to be the basic mechanism of this drug and may explain the important benefits of the long-term therapy with finasteride (Marks et al., 1999). CsA treatment reduced testosterone production, acting on the hypothalamic-pituitary axis (Sikka et al., 1988), and causing epithelial atrophy. However, this drug is not a good candidate for the treatment of prostate cancer, since it was shown by Pollard (1997) that the treatment with CsA enhanced metastasis of prostate adenocarcinoma cells (Pollard, 1997).

The present study showed one more side effect of CsA long-term administration. The damage caused to the prostate tissue could increase the male fertility damage caused by CsA.

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Table 1. Biometric data of Wistar rats treated with Cyclosporin A (mean ± SD).

Parameter	Groups	
	Control	**CsA**
Final Body Weight (g)	493.33 ± 28.23	463.43 ± 29.57
Body Weight Gain (g)	87.56 ± 20.35	47.29 ± 45.03
Weight (g)		
Prostate	0.52 ± 0.10	0.33 ± 0.04*
Seminal Vesicle	0.99 ± 0.11	0.79 ± 0.27
Coagulating Gland	0.21 ± 0.04	0.15 ± 0.02*
Testis	1.96 ± 0.11	1.88 ± 0.23
Epididymis	0.61 ± 0.018	0.55 ± 0.05*

Asterisks indicate significant difference ($p<0.05$) according to t-test.

Table 2. Prostate morphometrical data obtained from Wistar rats treated with Cyclosporin A (mean ± SD).

Parameter	Groups	
	Control	**CsA**
Epithelial cell nuclear area (μm^2)	35.21 ± 2.27	29.75 ± 2.02*
Epithelial cell cytoplasmic area (μm^2)	123.73 ± 12.91	91.67 ± 11.79*
Epithelial cell area (μm^2)	158.94 ± 14.79	121.42 ± 11.16*
Epithelium thickness (μm)	23.21 ± 2.06	16.77 ± 2.78*
Muscular Stroma thickness (μm)	5.01 ± 0.75	4.14 ± 0.37*

Asterisks indicate significant difference ($p<0.05$) according to t-test.

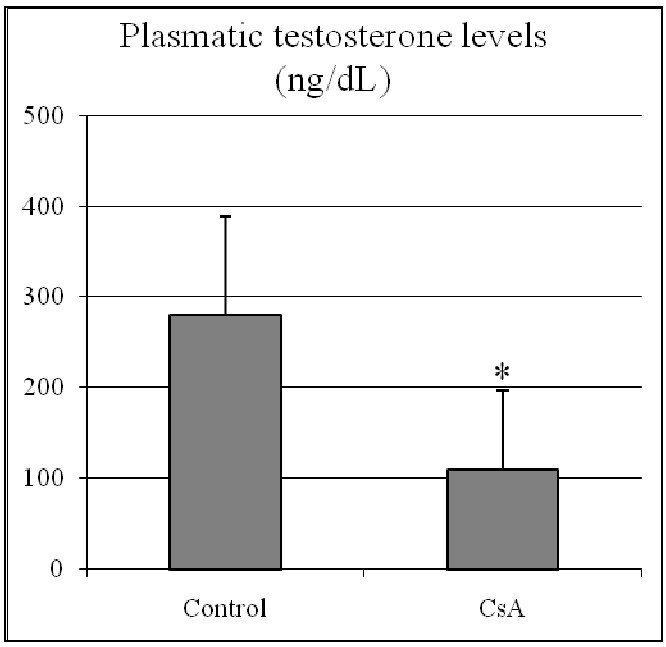

Figure 1. Plasmatic testosterone levels. Asterisks indicate significant difference ($p<0.05$) according to t-test. The values are means ±SD.

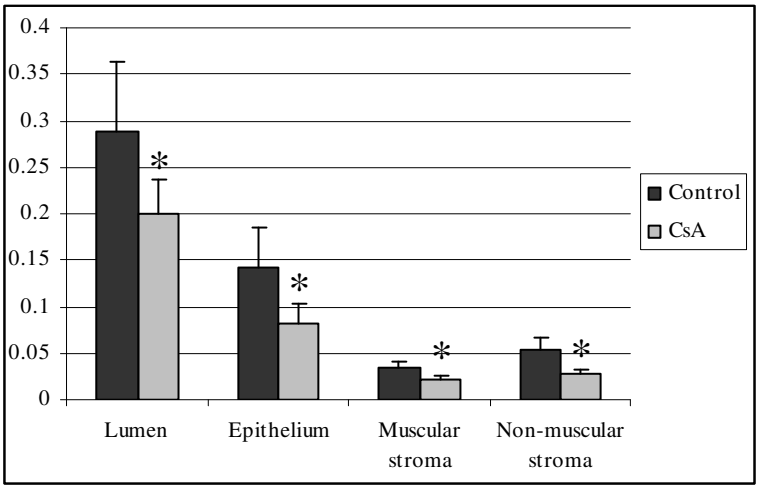

Figure 2. Volumes (mL) of prostate tissue components from control and CsA-treated groups. Asterisks indicate significant difference ($p<0.05$) according to t-test. The values are means ± SD.

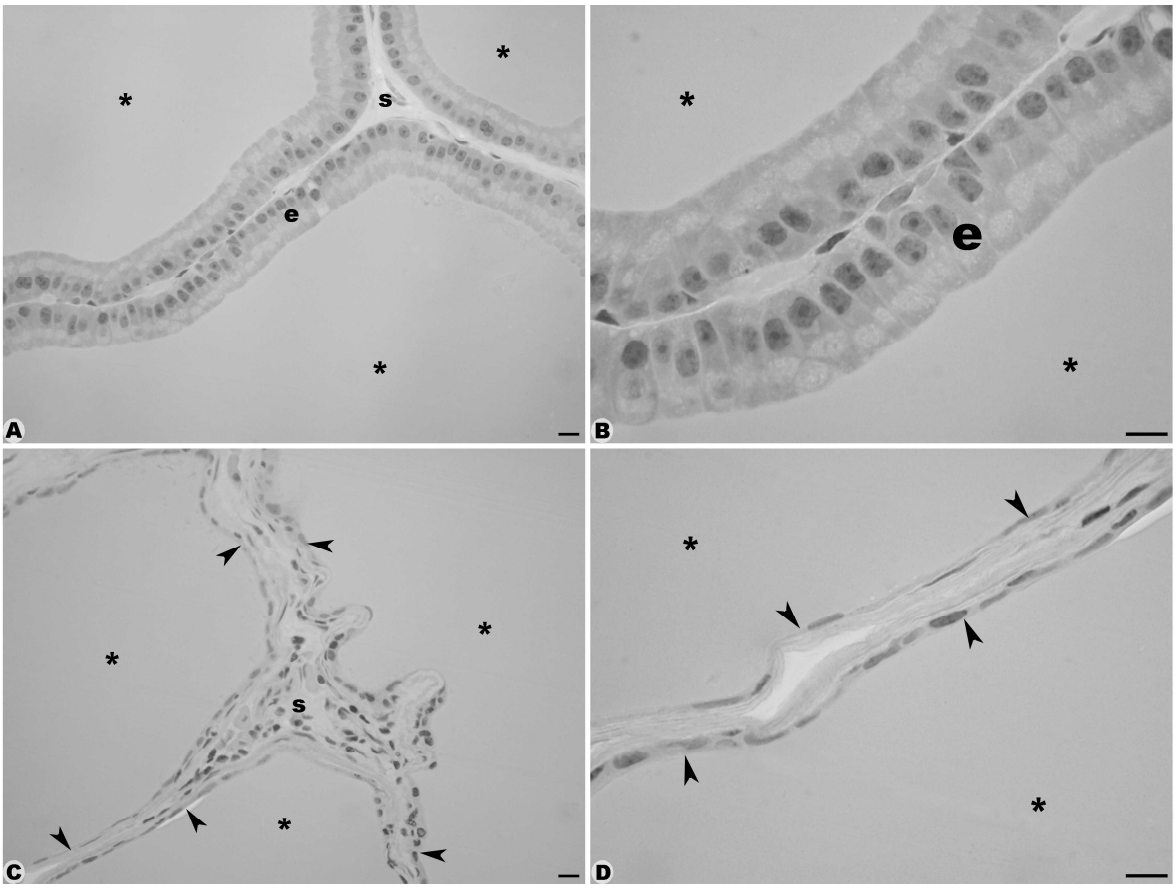

Figure 1. Histological sections of Wistar rats' ventral prostate stained with Hematoxilin and Eosin. A and B represent normal ventral prostate tissue structure (control). CsA-treated prostate (C and D) showing squamous atrophied epithelium (arrowhead). e = secretory epithelium; s = stroma; asterisks = lumen. Bars = 10µm

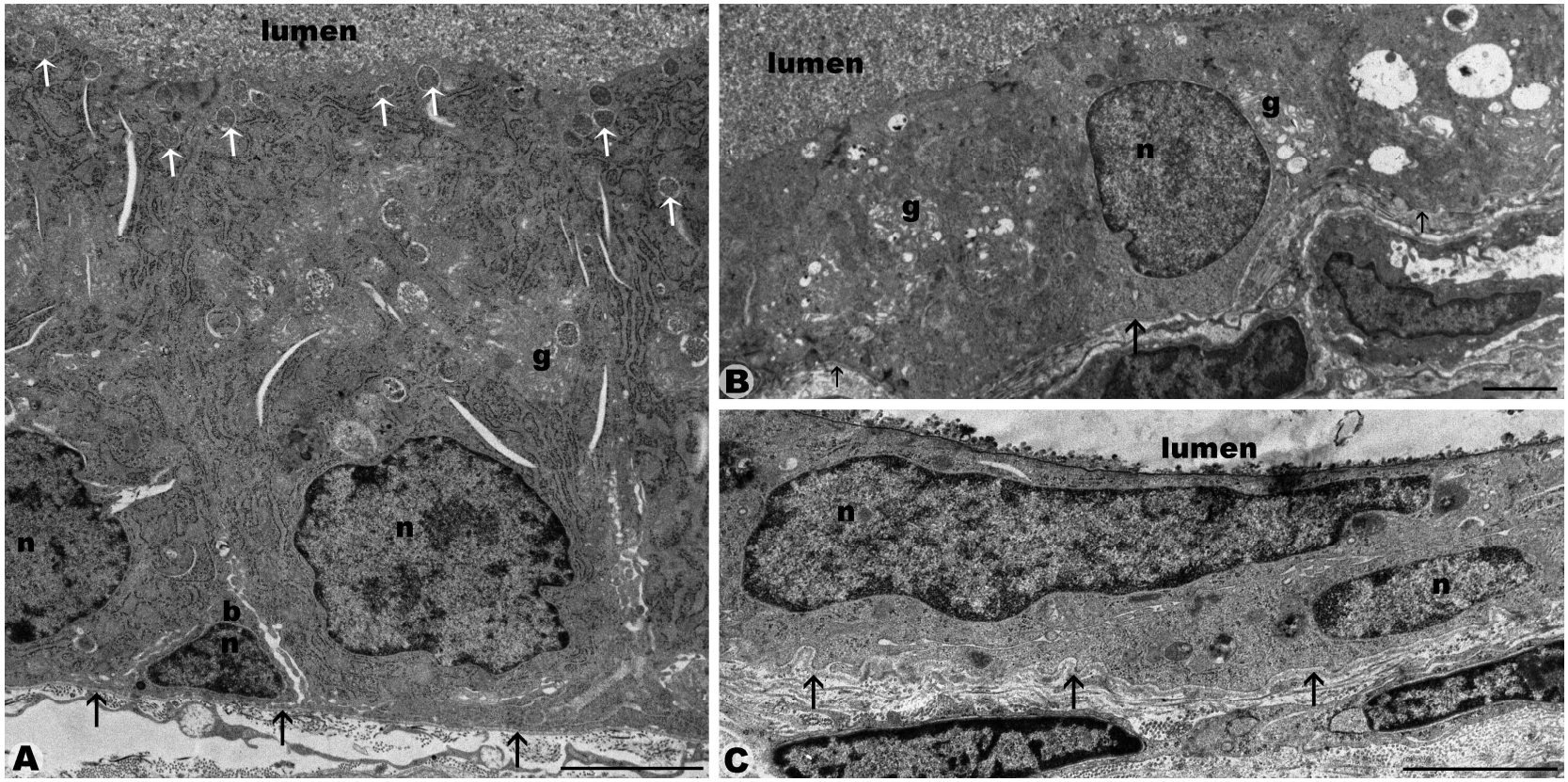

Figure 2. Ultrastructure of the ventral prostate epithelium of control (A) and CsA treated animals (B and C). g = Golgi complex; n = nucleus; b = basal cell; white arrows = secretory vesicles; and black arrows = basal lamina. Bars: A = 3µm and B and C = 2µm.

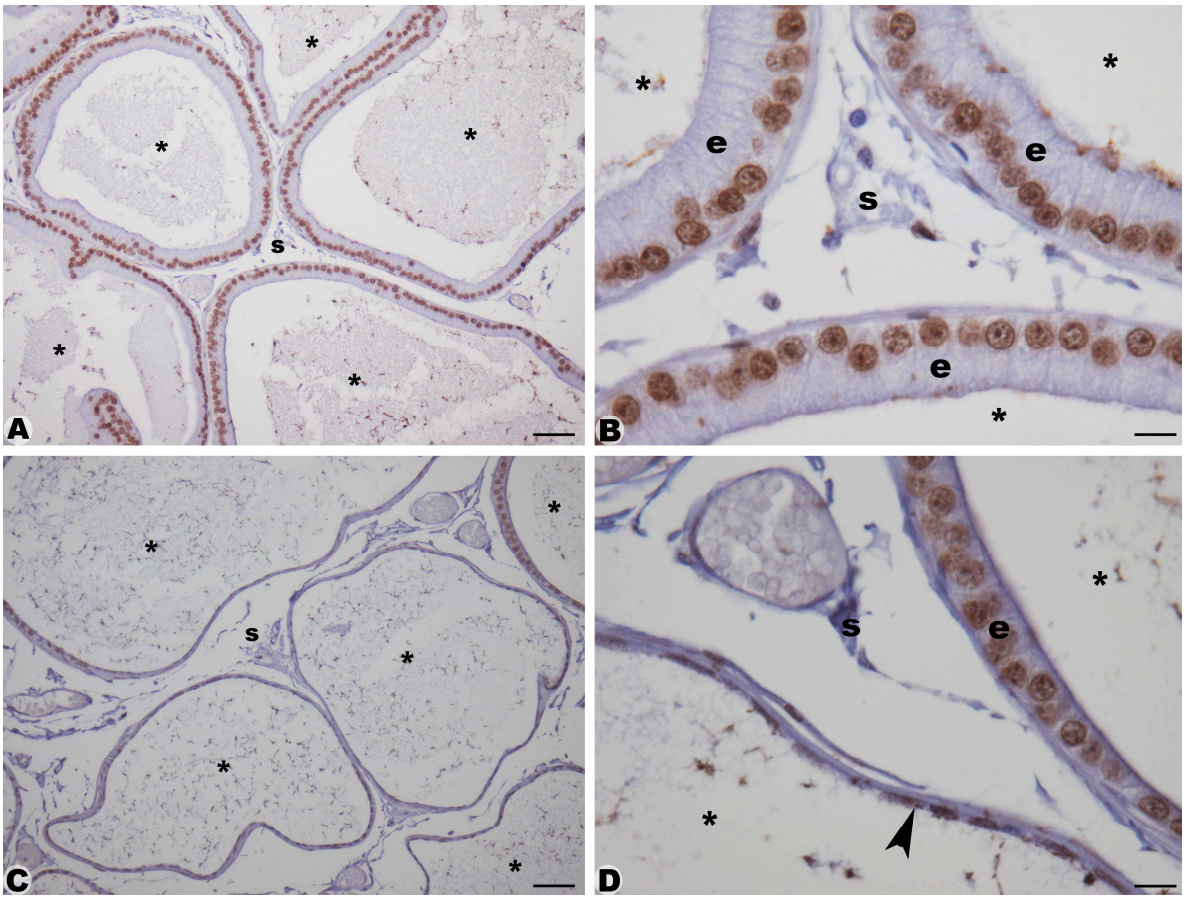

Figure 3. Light micrographs of ventral prostate with AR immunohistochemical method of control (A and B) and CsA-treated (C and D) animals. The arrowheads indicate the atrophied secretory epithelium with positive nuclei staining for AR expression. Asterisks = lumen; s = stroma; e = epithelium. Bars: A and C = 50µm; B and D = 10µm

Heteropterys aphrodisiaca (O. Mach.) infusion: Relieves Cyclosporin A side effects on the ventral prostate

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Running Title: *H. aphrodisiaca* against CsA side effects on prostate

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ABSTRACT

Cyclosporin A (CSA) is an immunosuppressive drug widely used in the post-operative treatment after organ transplant and against auto-immune diseases. However, many side effects are associated with CsA use. Many studies look for substances that could reduce its collateral effects. *Heteropterys aphrodisiaca* is a native Brazilian plant that was efficient to minimize the damage caused by CsA to the testes of Wistar rats. Therefore, the present study aimed to evaluate if the *H. aphrodisiaca* infusion was efficient against the CsA-induced damage of the prostate tissue. Thirty adult Wistar rats (90 days old) were divided into five groups. Group I (control) was treated with water, Group II with CSA, group III received the *H. aphrodisiaca* infusion, group IV was treated simultaneously with CSA and *H. aphrodisiaca* infusion and group V received CsA or *H. aphrodisiaca* infusion on alternate days. All treatments were given daily for 56 days by gavage. After this period the rats were weighed and euthanized. Testicle, epididymis, vesicular gland, coagulation gland and the ventral prostate were collected and weighed. Morphological, morphometrical and stereological analysis of the ventral prostate tissue were employed. Moreover immunohistochemistry for the androgen receptor and the TUNEL technique for apoptosis were applied. The groups treated with *H. aphrodisiaca* and CsA showed normalization of lumen, epithelium, muscular and non-muscular stroma volumes, when compared to those treated only with CsA. The animals treated simultaneously with *H. aphrodisiaca* and CsA had a significant increase in the volume of epithelial cells (nucleus and cytoplasm), epithelium and muscular stroma thicknesses when compared to those treated with CsA. These treatments administered on alternate days either caused a normalization of the above cited parameters when compared to the CsA-treated group, but these differences were not significant, except for the muscular stroma thickness that was significantly increased when compared to the CsA-treated group. These results showed that the association of *H. aphrodisiaca* to CsA treatment apparently reduced the damage to the prostate, since it was less damaged when compared to the prostate of animals that received only CsA. The treatments administered simultaneously were more efficient than when given on alternate days. The results, suggesting a protective action of the *H. aphrodisiaca* infusion against the side effects of CSA, were confirmed also for this organ.

INTRODUCTION

Cyclosporin A (CsA) was originally obtained from the fungi *Cylindrocapon lucidum* Booth and *Tolypocladium inflatum* Gams. It is a peptide composed of 11 amino acid residues, neutral, hydrophobic and cyclic (Shevach, 1985). CsA is a powerful immunosuppressive drug that improved the survival and decreased the rejection episodes and hospitalization days of transplant patients (Borel et al., 1989; Shevach, 1985). The CsA immunosuppressive properties are specific for lymphocytes (cytotoxic and suppressor) and do not interfere with the functions of phagocytes or haemopoietic stem cells (Borel et al., 1989; Hess e Tutschka, 1980).

CsA treatment causes many collateral effects, with histopathological changes in various organs, such as thymus, kidney, liver, heart, pancreas, nervous system (Rezzani, 2004), testis (Krueger et al., 1991; Masuda et al., 2003; Monteiro et al., 2008; Rajfer et al., 1987; Seethalakshmi et al., 1990; Srinivas et al., 1998) and prostate (unpublished results). The reduction of serum testosterone caused by CsA is reported by many authors (Krueger et al., 1991; Rajfer et al., 1987; Srinivas et al., 1998).

Freitas and colleagues (unpublished results) reported that treatment with CsA reduced the ventral prostate weight, the volume of prostate tissue components (epithelium, lumen, muscular and non-muscular stroma) and caused atrophy of the prostate secretory epithelium. These side effects probably are associated to the reduction of serum testosterone levels caused by CsA.

CsA is of fundamental importance in the treatment of patients that received transplanted organs and patients with auto-immune diseases. However, many side effects are related to its use, so, many studies seek substances that decrease CsA side effects (Cid et al., 2003; Kotolová et al., 2006; Kurus et al., 2008; Mohamadin et al., 2005; Monteiro et al., 2008; Türk et al., 2007; Wongmekiat et al., 2008).

H. aphrodisiac is a Brazilian native species; the roots are used by the local population as a tonic or stimulant in the treatment of central nervous system debilities, besides being used as an

aphrodisiac (Guarim Neto, 1996; Pio Corrêa, 1984; Pott e Pott, 1994). A lyophilized extract of *H. aphrodisiaca* was proven to inhibit the interaction among biomolecules and free radicals in the brain. Moreover this treatment increased the quantity of the antioxidant enzymes: SOD (Superoxide Dismutase), MnSOD (Manganese Superoxide Dismutase) e CuZnSOD (Copper-Zinc Superoxide Dismutase) in the brain of old rats (Mattei et al., 2001). The *H. aphrodisiaca* treatment of old rats was efficient to restore the memory damage that had occurred in these animals (Galvão et al., 2002). *H. aphrodisiaca* was confirmed to have antioxidant effects (Galvão et al., 2002; Mattei et al., 2001) and was efficient to reduce the testicular damage caused by CsA (Monteiro et al., 2008). So, the present study analyzed whether *H. aphrodisiaca* infusion is efficient to reduce the damage caused by CsA in prostate tissue.

MATERIAL AND METHODS

Medicinal Plant and CsA

Heteropterys aprhosiaca roots were colleted in Nova Xavantina (Mato Grosso, Brazil) and indentified by comparison with the voucher species samples of the plant in the Federal University of Mato Grosso Herbarium, Brazil (register number: 23928). After collection, the roots were dried at room temperature, protected against rain and direct incidence of the sun. The roots were then crushed and powdered using a grinding mill. The infusion was prepared by adding 100 ml of boiling water poured over 25 g of powdered roots. According to Marques et al. (2007) the best extracting liquid for *H. aphrodisiaca* roots in regard to extractable solids is water. This manner of *H. aphrodisiaca* infusion preparation had a yield of 6.832% (wt/wt) in terms of initial crude dry weight of plant material, and produced 68.66mg of extract dry weight per ml of infusion (Monteiro et al., 2008).

Cyclosporin A (Sandimmun Neoral – Oral Solution; 100 mg/ml; Novartis Pharma AG, Switzerland) was administrated to the animals. The dose of CsA was 15 mg/kg of body weight diluted in 0.5 ml of distilled water.

Experimental Animals and Treatment Protocol

Wistar rats (30 animals), 90 days old, were randomly divided into 5 experimental groups. Group I (control): received distilled water; Group II: received CsA; Group III: received *H. aphrodisiaca* infusion; Group IV: received CsA and *H. aphrodisiaca* simultaneously; and Group V: received CsA and *H. aphrodisiaca* on alternate days. All the treatments described above were administrated by gavage in a total volume of 0.5 ml, during 56 days. The simultaneous treatment with CsA and *H. aphrodisiaca* was employed diluting the dose of CsA in 0.5 ml of the plant infusion.

After the 56 days of treatment, the animals were weighed and anesthetized with xylazine and ketamine (5 and 80 mg/kg body weight, respectively). The abdomen and thoracic cavities were opened. Testis, epididymis, seminal vesicle, coagulating gland and ventral prostate were excised and weighed.

Preparation of Tissue for Microscopy

The two lobes of the ventral prostate were separated. One of them was fixed by immersion in Karnovsky solution (4% paraformaldehyde, 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.2) for 24h at room temperature. After fixation, the tissue was prepared for embedding in hydroxyethyl metacrylate using routine technique. Two micrometer sections were obtained, stained with hematoxylin-eosin and observed with an Olympus BX-40 light microscope. The other ventral prostate lobe was fixed by immersion in Metacarn (Methanol: chloroform: acetic acid – 6:3:1),

prepared for embedding in paraffin using routine technique. This material was cut into 5µm sections to apply the AR immunohistochemical and TUNEL techniques.

Morphometrical and stereological parameters

All morphometrical and stereological measurements were performed using the material included in hydroxyethyl metacrylate. The Image Pro Plus software was used to do all the morphometrical and stereological analyses. The volume density of lumen, epithelium, muscular and non-muscular stroma was determined using a 130-point grid system in ten microscopic fields that were chosen at random. Absolute volumes were estimated multiplying the volume density of the tissues cited above by the prostate weight (Cordeiro et al., 2008), since the ventral prostate density could be considered equal to 1.0 (DeKlerk e Coffey, 1978; Huttunen et al., 1981). The epithelial and muscular stroma thicknesses (µm) were measured. Nuclear area (μm^2), perimeter (µm), and cytoplasmic area (μm^2) were either measured and the form factor (4π X nuclear area / [nuclear perimeter]^2) was calculated.

Androgen Receptor (AR) Immunohistochemistry

The antigenic recuperation was carried out by immersion of the sections in citrate buffer (pH 6.0) and kept at a high temperature (100ºC) during 45 min. Hydrogen peroxide (0.3%) in methanol (during 20 min) was used for blocking the endogenous peroxidase. The slides were incubated with the primary antibody anti-AR (rabbit, 1:150; SC-816, Santa Cruz Biotechnology, USA), at 37ºC, during 1 hour. To obtain the negative controls, sections were incubated PBS buffer (pH 7.4) in stead of the primary antibody. For all the followed reactions, the kit SC-2018 (Santa Cruz Biotechnology, USA) was used, with dilutions made in accordance to the indications of the manufacturer. The sections were incubated with biotinylated secondary antibody (goat, anti-rabbit), at 37°C for 45 min and then incubated with peroxidase-conjugated avidin–biotin complexes. The

positive reaction was revealed by diaminobenzidine (DAB), during 5 minutes. Sections were then counterstained with Harris's hematoxylin for 10 sec.

TUNEL technique

Apoptotic nuclei were determined using a kit for fragmented DNA detection (US1QIA33-1EA; TdT FragEL TM DNA Fragment. Detect, Merck). The sections were incubated with Proteinase K for permeabilization followed by blocking of endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 5 min. Equilibrium reaction, label staining detection and development with diaminobenzidine (DAB) were carried out according to instructions of the kit's manufacturer. The sections were counterstained with Harris' hematoxylin for 10 sec.

The nuclei labeled for apoptosis were counted in the epithelium of 10 different high magnification fields (x400) for each animal and compared to the total number of epithelial cells in the field to obtain the percentage of apoptotic epithelial nuclei (apoptotic index) (Shabisgh et al., 1999).

Statistical Analysis

All averages were compared between the experimental groups by variance analysis and with post hoc Duncan's test, using the software STATISTICA for WINDOWS 3.11. A value of $p<0.05$ was considered significant. All data are presented as mean ± standard deviation (SD).

RESULTS

Biometrical Parameters

Final body weight, body weight gain, ventral prostate and epididymis weight had a non significant increase in the groups treated with the plant infusion and CsA (IV and V) when compared the group that received only CsA (II) (Table 1).

In the group that received alternated treatments with *H. aphrodisiaca* and CsA (V) there was a significant increase in the coagulating gland weight when compared to the group treated only with CsA (II) (Table 1). On the other hand the in the group treated simultaneously with *H. aphrodisiaca* and CsA (IV) the coagulating gland was almost the same as that of group II (Table 1).

Stereology and Morphometry

Regarding the effects of *H. aphrodisiaca* infusion simultaneously administered with CsA (Group IV), we observed that the volume of lumen, epithelium, muscular and non muscular stroma did not have a significant increase when compared to the group treated only with CsA (II). These parameters were not statistically different when compared to the control (I) or the CsA treated (II) groups (Table 2). The effects of *H. aphrodisiaca* and CsA treatment on alternate days showed similar results when considering the volume of lumen, muscular and non muscular stroma (Table 2). On the other hand the epithelium volume remained significantly smaller that in group I (Table 2).

The area of a single epithelial cell, cytoplasm and nuclei were higher in group IV when compared to group II, while in group V only the nuclear volume had a non significant increase (Table 3). The proportion of nuclei and cytoplasm in group IV was very similar to the control, while in group V, it was similar to that observed for group II (Table 3). The epithelium and muscular stroma thickness in group IV were significantly higher than in group II (Table 3). In group V, these parameters were not significantly higher when compared to group II (Table 3).

AR immunohistochemical test and TUNEL

The AR immunohistochemical method showed a positive reaction in most of the epithelial nuclei of all of the experimental groups (Figure 2). Some stromal cells also had a positive reaction to AR (Figure 2).

The apoptotic index did not vary among the experimental groups, and was lower that 0.5 in all the experimental groups.

DISCUSSION

The CsA dose was administered according to that recommended by the manufacturer to patients after the organ transplant and according to that used in previous studies (Chung et al., 2005; Kotolová et al., 2006; Monteiro et al., 2008; Türk et al., 2007). The preparation method for *H. aphrodisiaca* infusion and the dose administered was also according to previous studies (Chieregatto, 2005; Monteiro et al., 2008).

Despite the side effects caused by CsA administration, its use is fundamental for the treatment after organ transplant and against auto-immune diseases. Therefore, many studies look for a substance that could diminish CsA side effects (Chung et al., 2005; Kotolová et al., 2006; Kurus et al., 2008; Mohamadin et al., 2005; Monteiro et al., 2008; Türk et al., 2007; Wongmekiat et al., 2008). Kotolová et al. (2006) demonstrated that carvedilol (a cardiovascular drug with wide therapeutic potential) could protect the renal tissue of Wistar rats from CsA induced nephropathy. Cid et al. (2003) reported some antioxidant substances that could protect against CsA nephrotoxicity, among them Vitamins E and C. Mohamadin et al. (2005) observed a renoprotective potential for Green tea extract in relation to CsA-induced nephropathy. Wongmekiat et al. (2008) showed that the treatment with shallot (*Allium ascalonicum* L.) extract attenuates renal injury following CsA administration. Kurus et al. (2008) working with rat liver observed that oral administration of L-arginine prevent the damaged development caused by CsA administration. Regarding the protection against CsA testicular impairment, Türk et al. (2007) observed that lycopene had a potential protective effect against the CsA-induced oxidative stress that lead to the structural and functional damages in the testicular tissue and sperm quality of rats. Finally,

Monteiro et al. (2008) observed that *H. aphrodisiaca* infusion was efficient to protect the testis against the structural and functional damage caused by CsA.

As shown in previous studies, the CsA caused extensive damage to the ventral prostate tissue, with reduction in the volume of the tissue parameters (lumen, epithelium, muscular and non muscular stroma) and atrophy of some epithelial regions. Ultra-structural analyses showed that in the atrophied cells there was less rough endoplasmic reticulum and Golgi complex. The damage caused by CsA on prostate tissue was probably related to the lower serum testosterone levels (unpublished results).

The body weight was assessed to determine the effects of CsA treatment on general health of the animals (Seethalakshmi et al., 1987). Despite the fact that there was no variation of body weight among the experimental groups in the present study, the body weight gain in the group treated with CsA and *H. aphrodisiaca* simultaneously had a significant increase of 98.52% when compared to those treated only with CsA. While in the group treated with CsA and *H. aphrodisiaca* on alternated days the body weight gain had an increase of 48.45% (not significant) when compared to CsA-treated group. These results indicated that the simultaneous treatment with *H. aphrodisiaca* with CsA was beneficial, and a slight improvement in relation to the treatment on alternated days considering the reduction of body weight gain caused by CsA. Monteiro et al. (2008) did not observe any significant difference among the body weight gains of the animals treated with CsA and simultaneously with CsA and *H. aphrodisiaca*.

In previous study, the treatment with CsA or CsA and *H. aphrodisiaca* (simultaneously) did not alter the ventral prostate, epididymis or coagulating gland weights (Monteiro et al., 2008). While Türk et al. (2007) observed lower seminal vesicle weight in CsA treated rats and unaltered seminal vesicle weight in rats treated with CsA + lycopene, these authors did not observe variation of the testis, epididymis and prostate weights. In the present study, the ventral prostate and epididymis weight increased slightly in the groups treated with CsA and/or *H. aphrodisiaca*. The

coagulating gland weight volume was significantly higher in group V when compared to group II. Although these results were not statistically significant, they showed parameters similar to the control animals, differing from the group treated only with CsA. These tendencies also show an improvement caused by the treatment with *H. aphrodisiaca* in relation to CsA prostate damage.

The morphometrical and stereological analyses showed that CsA and *H. aphrodisiaca* simultaneous administration protected the prostate tissue against the impairment caused by CsA administration. When comparing the group treated simultaneously with CsA and *H. aphrodisiaca* with the one that received only CsA, the volume of prostate tissue components (lumen, epithelium, muscular and non muscular stroma) had a non statistically significant increase while the area of epithelial cell (nucleus and cytoplasm), epithelium and muscular stroma thickness increased significantly. These results showed that the plant infusion could partially restore the tissue or avoid damage caused by CsA. Many of the stereological and morphometrical parameters of the groups treated with CsA and the plant infusion on alternated days were similar to that observed with the same treatment administered simultaneously. However, in this group treated on alternated days, the epithelium volume, cellular and cytoplasm area and epithelial thickness did not improve as observed with the simultaneous treatment. So, the CsA and *H. aphrodisiaca,* administration, on alternated days, also protected the tissue, but was less efficiently.

In a previous report, Shabisgh et al. (1999) showed that there was virtually no apoptosis in the ventral prostate of control Wistar rats. The apoptotic index of all the groups in the present study was less that 0.5, a value that is considered normal for the ventral prostate epithelium of non treated animals (Shabisgh et al., 1999). Freitas et al. (unpublished results) suggested that the absence of alteration of the apoptotic index in animals treated with CsA for 56 days is probably was due to the long term treatment. In castrated animals the apoptotic index reaches its maximum range 72 hours after the surgery, then it starts declining and becames normal after 7 days (García-Flórez et al.,

2005; Shabisgh et al., 1999). Therefore, the apoptotic index was probably not a good parameter to observe possible benefits from *H. aphrodisiaca* treatment associated to CsA administration.

According to Shabisgh et al. (1999) the androgen receptors are present in most epithelial and smooth muscle cells (periductal and perivascular) and in some fibroblast cells of the rat ventral prostate. The AR expression in all the experimental groups was similar to that in the research cited above. Since the reaction intensifying the AR-positive cells could be related to an increase in AR expression (Scarano et al., 2009), the absence of variations suggests that there was no alteration of AR expression.

Mattei et al. (2001) observed that the treatment with a *H. aphrodisiaca* extract increased the total activities of SOD, MnSOD and CuZnSOD in brain of old rats. In this case, the protection provided by *H. aphrodisiaca* against CsA-induced prostatic damage could be related to antioxidant properties of *H. aphrodisiaca.* The higher efficiency of the simultaneous treatment probably was due to the continuous treatment with the infusion that could, little by little, attenuate or avoid the CsA impairment of the ventral prostate.

The present study indicated that *H. aphrodisiaca* infusion protects the prostate tissue against the damage caused by CsA. Investigations whether or not the plant infusion could interfere with the CsA immunosuppressant capacity will be carried out.

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TABLE 1. Biometrical parameters (g) of animals treated with CsA, *H. aphrodisiaca* infusion or both.

Parameter	I	II	III	IV	V
Final Body Weight	493.33 ± 28.23	463.43 ± 29.57	496.50 ± 29.62	502.88 ± 33.26	474.90 ± 45.84
Body Weight Gain	87.56 ± 20.35[a]	47.29 ± 45.03[b]	94.33 ± 21.74[a]	93.88 ± 32.12[a]	70.20 ± 19.35[ab]
Weights					
Ventral Prostate	0.499 ± 0.13[a]	0.332 ± 0.04[b]	0.481 ± 0.07[a]	0.398 ± 0.11[ab]	0.375 ± 0.09[ab]
Seminal Vesicle	0.97 ± 0.10	0.788 ± 0.27	0.93 ± 0.27	0.82 ± 0.17	0.88 ± 0.14
Coagulating Gland	0.21 ± 0.03[a]	0.152 ± 0.02[b]	0.21 ± 0.04[a]	0.16 ± 0.03[b]	0.19 ± 0.03[a]
Testis	1.96 ± 0.11	1.88 ± 0.23	1.97 ± 0.14	1.87 ± 0.19	1.88 ± 0.18
Epididymis	0.61 ± 0.018[a]	0.55 ± 0.53[b]	0.64 ± 0.03[a]	0.58 ± 0.05[ab]	0.59 ± 0.05[ab]

Group I: Control; group II: CsA treated; group III: *H. aphrodisiaca* treated; group IV: CsA and *H. aphrodisiaca* simultaneous treatment; group V: CsA and *H. aphrodisiaca* on alternate days. The values are means ± SD. Different superscriptions indicate that the means are statistically different according to Duncan's test ($p<0.05$).

TABLE 2. Ventral prostate stereology of animals treated with CsA, *H. aphrodisiaca* infusion or both.

Parameter	I	II	III	IV	V
Volumetric density (%)					
Lumen	55.23 ± 8.65	60.69 ± 7.04	55.74 ± 6.85	55.13 ± 2.66	61.77 ± 6.58
Epithelium	27.77 ± 6.97	24.69 ± 5.55	27.13 ± 8.05	28.23 ± 5.03	22.33 ± 7.85
Muscular stroma	6.69 ± 1.34	6.33 ± 1.57	6.51 ± 1.55	6.54 ± 0.41	5.95 ± 1.89
Non muscular stroma	10.31 ± 0.10	8.28 ± 1.87	10.61 ± 2.79	10.03 ± 2.39	9.95 ± 3.21
Volume (ml)					
Lumen	0.29 ± 0.08^{a}	0.20 ± 0.04^{b}	0.26 ± 0.03^{ab}	0.23 ± 0.08^{ab}	0.25 ± 0.08^{ab}
Epithelium	0.14 ± 0.04^{a}	0.08 ± 0.02^{b}	0.13 ± 0.05^{a}	0.11 ± 0.05^{ab}	0.08 ± 0.02^{b}
Muscular stroma	0.03 ± 0.007^{a}	0.02 ± 0.005^{b}	0.03 ± 0.01^{a}	0.03 ± 0.008^{ab}	0.02 ± 0.008^{ab}
Non muscular stroma	0.05 ± 0.01^{a}	0.03 ± 0.005^{b}	0.05 ± 0.01^{a}	0.04 ± 0.02^{ab}	0.04 ± 0.02^{ab}

Group I: Control; group II: CsA treated ; group III: *H. aphrodisiaca* treated; group IV: CsA and *H. aphrodisiaca* simultaneously treatment; group V: CsA and *H. aphrodisiaca* on alternate days. The values are means ± SD. Different superscriptions indicate that the means are statistically different according to Duncan's test ($p<0.05$).

TABLE 3. Ventral prostate stereology of animals treated with CsA, *H. aphrodisiaca* infusion or both.

Parameter	I	II	III	IV	V
Epithelial Celular Area (μm^2)	158.94 ± 14.79[a]	121.42 ± 11.16[b]	164.60 ± 20.85[a]	168.97 ± 21.72[a]	135.38 ± 14.98[b]
Epithelial Citoplasmatic Area (μm^2)	123.73 ± 12.91[a]	91.67 ± 11.79[b]	128.36 ± 19.63[a]	134.34 ± 17.66[a]	102.81 ± 13.40[b]
Epithelial Nuclear Area (μm^2)	35.21 ± 2.27[a]	29.75 ± 2.02[b]	36.24 ± 3.47[a]	34.63 ± 2.17[a]	32.57 ± 2.17[ab]
Form Factor	0.68 ± 0.04[a]	0.71 ± 0.03[ab]	0.73 ± 0.02[b]	0.70 ± 0.02[ab]	0.70 ± 0.03[ab]
Volumetric proportion in an ephitelial cell (%)					
Cytoplasm	77.79 ± 1.13[ab]	75.30 ± 3.07[a]	77.78 ± 2.78[ab]	79.50 ± 1.51[b]	75.81 ± 1.74[a]
Nuclei	22.21 ± 1.13[ab]	24.70 ± 3.07[a]	22.22 ± 2.78[ab]	20.50 ± 1.51[b]	24.19 ± 1.74[a]
Thickness (µm)					
Epithelium	23.21 ± 2.06[ac]	16.77 ± 2.78[b]	23.87 ± 3.46[ac]	24.50 ± 3.22[c]	20.08 ± 3.63[ab]
Muscular Stroma	5.01 ± 0.75[ab]	4.14 ± 0.37[a]	5.03 ± 0.56[ab]	5.43 ± 0.46[b]	5.49 ± 1.56[b]

Group I: Control; group II: CsA treated; group III: *H. aphrodisiaca* treated; group IV: CsA and *H. aphrodisiaca* simultaneous treatment; group V: CsA and *H. aphrodisiaca* on alternated days. The values are means ± SD. Different superscriptions indicate that the means are statistically different according to Duncan's test ($p<0.05$).

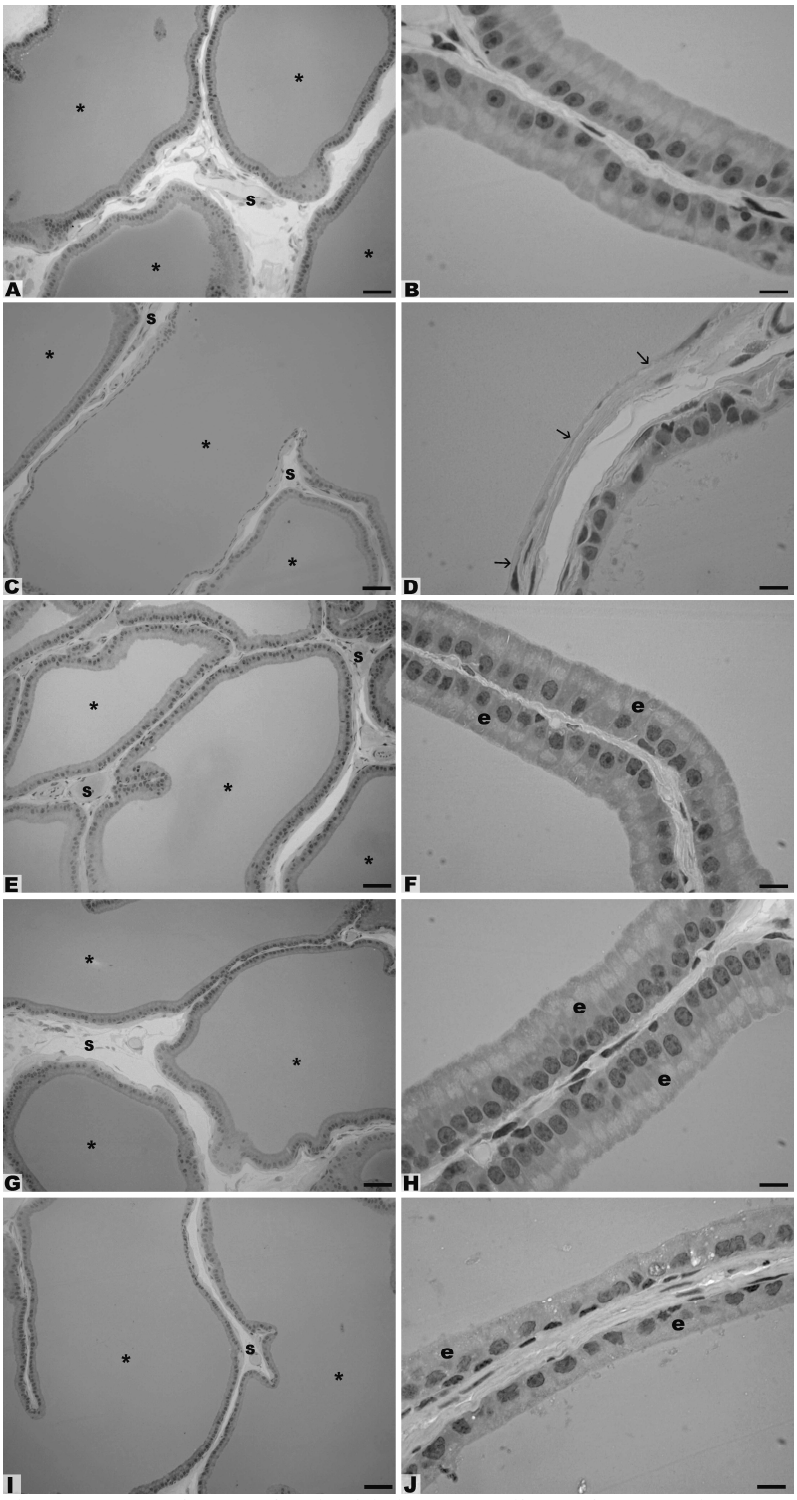

Figure 1. Ventral prostate sections stained with hematoxylin-eosin. A and B are representative of control group (group I). In the images C and D the atrophied epithelium from group II was shown. The following images are representative of groups III (E and F), IV (G and H) and V (I and J). s = stroma; asterisks = lumen; e = epithelium. Bars = 50 µm (A, C, E, G and I) and bars = 10 µm (B, D, F, H and J).

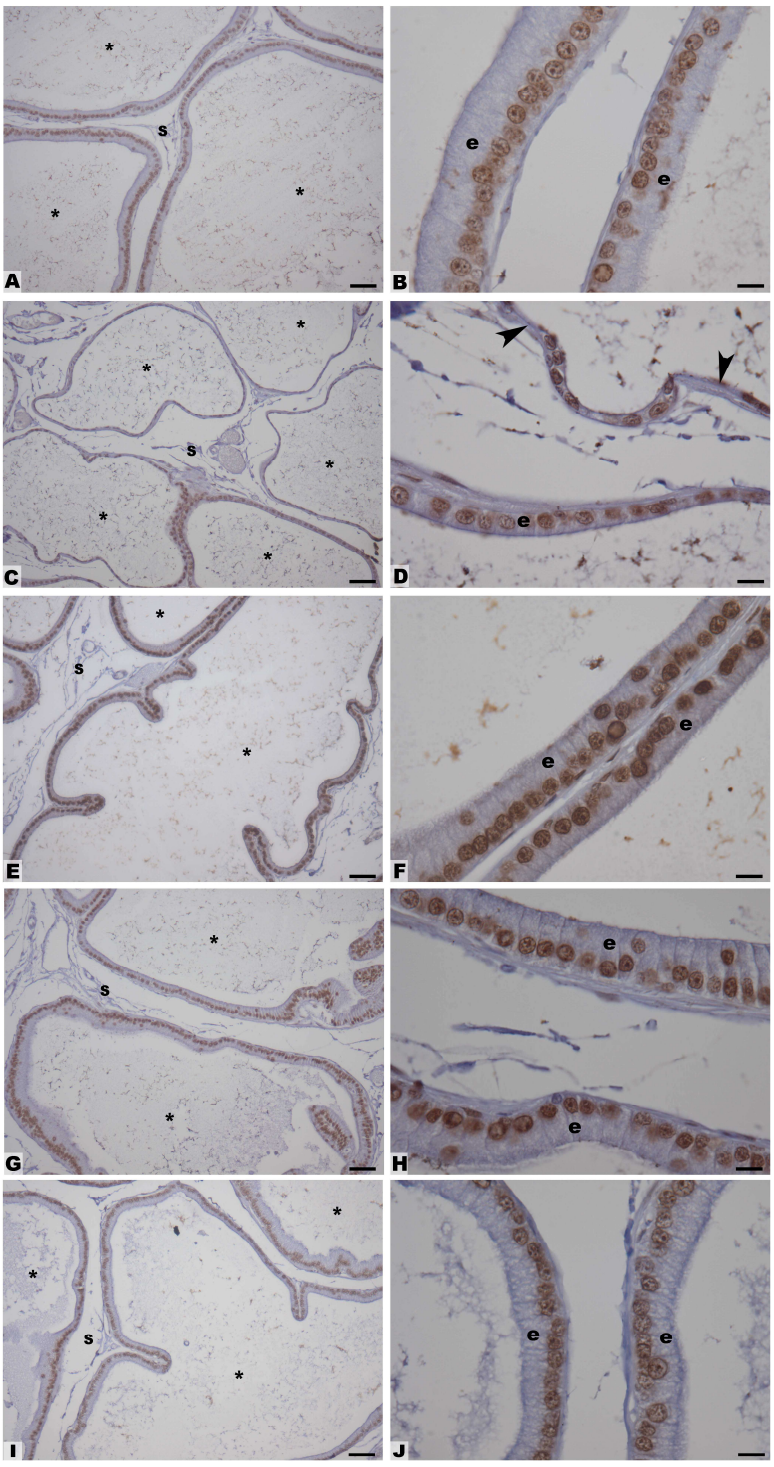

Figure 2. AR immunohistochemical reaction in the ventral prostate sections of groups I (A and B), II (C and D), III (E and F), IV (G and H) and V (I and J). Abreviations: s = stroma; asterisks = lumen; e = epithelium. Bars = 50 µm (A, C, E, G and I) and Bars = 10 µm (B, D, F, H and J).

7. CONCLUSÕES FINAIS

Os resultados obtidos com este trabalho mostraram que o tratamento de ratos Wistar com Ciclosporina A na dosagem de 15mg/kg/dia durante 56 dias causa danos estruturais na próstata ventral. Estes danos provavelmente acarretam alterações funcionais ao tecido, uma vez que, com a atrofia do epitélio secretor, ocorre redução nas organelas relacionadas com a secreção protéica (retículo endoplasmático rugoso e complexo de Golgi), conforme foi observado através de microscopia eletrônica de transmissão. Os efeitos colaterais causados pela CsA na próstata de ratos Wistar, provavelmente são em decorrência da queda dos níveis de testosterona circulante.

O tratamento somente com a infusão de *Heteropterys aphrodisiaca* não causou nenhuma alteração estrutural da próstata ventral de ratos Wistar. A administração da infusão de *H. aphrodisiaca* simultaneamente a CsA amenizou os danos causados por esta na próstata ventral. O tratamento com a infusão da planta em dias alternados à CsA também amenizou os efeitos colaterais desta na próstata ventral, porém foi menos eficiente ou o efeito pode ser dose dependente. As observações morfológicas mostram uma gradação de danos dependendo do tratamento, mas o uso de morfometria e estereologia demonstrou claramente as alterações ocorridas no tecido prostático.

Não houve variação no padrão de marcação para receptores de andrógeno entre os grupos experimentais. Além disso, não foram verificados alterações no índice de apoptose, provavelmente por conseqüência do longo período de tratamento usado.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha Dissertação de Mestrado intitulada "EFEITO DA CICLOSPORINA A ASSOCIADA À INFUSÃO DE NÓ-DE-CACHORRO (*Heteropterys aphrodisiaca*, O. MACH, 1949) NA PRÓSTATA DE RATOS WISTAR":

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

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