

Full Length Research Paper

Study of the ventral prostate of Wistar rats treated with *Heteropterys tomentosa* (A. Juss.)

Karine Moura de Freitas^{1*}, Juliana Castro Monteiro², Marcos de Lucca Moreira Gomes³,
Sebastião Roberto Taboga⁴ and Heidi Dolder¹

¹Department of Anatomy, Cell Biology, Physiology and Biophysics, Institute of Biology, State University of Campinas, UNICAMP, Brazil.

²Department of Agrarian and Biological Sciences, Federal University of Espírito Santo, CEUNES, Brazil.

³Department of Health Sciences, Federal University of Espírito Santo, CEUNES, Brazil.

⁴Department of Biology, Institute of Biosciences, Language and Science, State University of São Paulo, UNESP, Brazil.

Accepted 6 April, 2012

***Heteropterys tomentosa* 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. tomentosa* 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. tomentosa* roots infusion (treated group, n=6) or water (control group, n=6) for 56 days. Morphological, morphometrical and stereological analyses were employed to study 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 analyses. The pattern of androgen receptor expression and the apoptotic index were identical in the control and treated group. Ultrastructural analysis showed no alterations caused by *H. tomentosa*. These results suggested that treatment with *H. tomentosa* infusion, although considered a strong aphrodisiac, did not cause any major damage nor benefit to the prostate tissue.**

Key words: Aphrodisiac, *Heteropterys aphrodisiaca*, medicinal plants, phytotherapy, reproduction.

INTRODUCTION

Studies describing the potential benefits of native Brazilian plants used as phytotherapeutic substance by the traditional medicine are constantly increasing. Among medicinal plants, the ones used as aphrodisiacs are of great interest to researchers (Chieregatto, 2005; Monteiro et al., 2008, 2010; Sbervelheri et al., 2009; Gomes et al., 2011). *Heteropterys tomentosa* A. Juss. (sin. *Heteropterys aphrodisiaca* O.Mach.) 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. Its roots are widely used to prepare an alcoholic drink and infusions consumed as

an aphrodisiac and a stimulant (Rizzini, 1983). According to Marques et al. (2007) *H. tomentosa* 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. Gomes and colleagues (2011) observed that *H. tomentosa* infusion administration increased both spermatogenic yield and spermatogonial mitosis, as well as the plasmatic testosterone levels. Previous studies showed that long term intake of such infusion caused significant body weight gain possibly due to the increased muscle anabolic activity provoked by higher plasmatic testosterone levels (Chieregatto, 2005; Monteiro, 2007). Monteiro et al. (2008) observed that *H. tomentosa* infusion would be a remarkable protective potential against testicular damage caused by Cyclosporine A

*Corresponding author. E-mail: karinemfreitas@gmail.com. Tel: 55 19 35216116. Fax: 55 19 35216185.

(CsA). This drug has been associated with severe nephrotoxicity, hepatotoxicity (Rezzani, 2004) and testicular damage (Monteiro et al., 2008); the main side effects of CsA are associated with increased reactive oxygen species (Cid et al., 2003). Moreover, a *H. tomentosa* 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. tomentosa* infusion, could affect the prostatic tissue. With this study, we investigated the effects of the infusion in normal prostate tissue, as a basis for future studies on the effects of *H. tomentosa* in damaged tissue.

MATERIALS AND METHODS

Medicinal plant

H. tomentosa 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, protected from sunlight and rain, and were ground. According to Marques et al. (2007), water is the best extracting liquid from *H. tomentosa* roots regarding extractable solids. So, the infusion was prepared by pouring 100 ml of boiling water over 25 g of ground roots, allowing it to steep for 4 h until filtering, using filter paper. The yield of this infusion was 68.66 mg of dry extract (6.866% w/v) (Monteiro et al., 2008). *H. tomentosa* infusion preparation was identical to that used previously by other authors (Chieriegatto, 2005; Monteiro et al., 2008; Monteiro et al., 2010; Sbervelheri et al., 2009; Gomes et al., 2011).

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 were maintained under standard conditions with 12 h light/12 h 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. tomentosa* infusion (0.5 ml), both by gavage. The treatment was administered daily, for 56 days (Chieriegatto, 2005; Monteiro et al., 2008, 2010; Sbervelheri et al., 2009; Gomes et al., 2011).

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, and 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's fixative (4% glutaraldehyde and 4% paraformaldehyde in sodium phosphate buffer of 0.1 M, pH 7.4), for 24 h. Some fragments were routinely processed for further hydroxyethyl methacrylate embedding. Thin sections (2 μ m) were obtained and stained with hematoxylin and eosin for light microscopy observation. Other fragments were post-fixed in 1% osmium tetroxide, and then were processed using the routine technique for epoxy resin embedding, for transmission electron microscopy.

The other portion of the ventral prostate was fixed in methacarn (Methanol: chloroform: acetic acid, 6:3:1) for 4 h (4°C) and was processed by routine paraffin embedding technique. They were sectioned (5 μ m) and used for androgen receptor (AR) immunohistochemistry and transferase dUTP nick end labeling (TUNEL) assay.

Transmission electron microscopy (TEM)

Ultra thin sections (60 nm) were obtained and stained with 3% uranyl acetate (30 min) and 2% lead citrate (5 min) and were 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 were 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 for each image. The absolute volumes of the aforementioned 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 mm³. The perimeter and area of 30 epithelial cells per animal (and their nuclei) were estimated using images at 1000x 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 within the epithelial cell. Epithelium and muscular stroma thickness were estimated measuring 10 thicknesses per image at 1000x magnification. Ten images were used per animal.

Immunohistochemistry and terminal TUNEL

Immunohistochemical technique was used for AR detection. All dilutions were prepared following the manufacturer's kit data sheet.

The slides were maintained in citrate buffer and kept at a high temperature (100°C) for 45 min 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 4°C, and were incubated with biotinylated secondary antibody, at 37°C, for 45 min (goat, anti-rabbit, SC-2018, Santa Cruz Biotechnology, USA). Afterwards, as

Table 1. Biometrical parameters of control adult Wistar rats and those treated with *H. tomentosa* (Ht) infusion.

Weight (g)	Control	Ht
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

There were no significant differences among the experimental groups. The values are means ± SD.

recommended by the kit's manufacturer, the slides were incubated with peroxidase-conjugated avidin–biotin complexes and the reaction stained by diaminobenzidine (DAB) (SC-2018, Santa Cruz Biotechnology, USA) for 5 min. The sections were counterstained with Harris's hematoxylin for 3 s. In all reactions, a negative control was made following the same protocol described earlier, except for the primary antibody, which was substituted by phosphate buffered saline (PBS) buffer (pH 7.4) at 37° for 45 min.

The TUNEL assay was applied for the detection of apoptotic nuclei using the kit for fragmented DNA detection (US1QIA33-1EA; TdT FragEL™ 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 5 min. They were submitted to the equilibrium reaction, labeled and stained. Harris' hematoxylin was used for counterstaining (3 s). 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

The 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 significantly 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. tomentosa* treated group (Table 1). Considering all quantitative parameters of the prostatic tissue, no significant alteration was observed for the *H. tomentosa* treated group (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 TEM (Figures 1 and 2). Besides, epithelial cells TEM revealed the cytoplasm filled with a well developed rough endoplasmic reticulum that was enlarged in most of the cells analyzed (Figure 2).

AR immunohistochemistry did not show any variation of nuclear androgen receptor expression pattern in the epithelial and stromal nuclei of the *H. tomentosa* 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 with the control.

DISCUSSION

H. tomentosa is a traditional aphrodisiac plant and according to previous results, the infusion of this plant roots could increase testosterone levels (Gomes et al., 2011). The rat prostate, particularly the ventral lobules, has 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. tomentosa* infusion administration. Sbervelheri et al. (2009), using the same preparation method and dose of *H. tomentosa* infusion observed that it did not cause any toxic effect to the liver and kidneys of Wistar rats. Contrary to Chieriegatto (2005) observations, the body and testicular weight did not increase in the *H. tomentosa* treated group, when compared with the control group. The weights of the body, testis, epididymis, coagulating gland and seminal vesicles did not vary in the *H. tomentosa* treated group in the present study, as observed by Monteiro et al. (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

Table 2. Morphometric and stereological parameters of the ventral prostate of control adult Wistar rats and those treated with *H. tomentosa* (Ht) infusion.

Parameter	Control	Ht
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 (µm²)		
Epithelial cell	158.94 ± 14.79	164.60 ± 20.85
Epithelial cell nucleus	35.21 ± 2.27	36.24 ± 3.47
Epithelial cell cytoplasm	123.73 ± 12.91	128.36 ± 19.63
Form factor	0.68 ± 0.04	0.73 ± 0.02*
Epithelium thickness	23.21 ± 2.06	23.87 ± 3.46
Muscular stroma thickness	5.01 ± 0.75	5.03 ± 0.56

The values are means ± SD. *Indicates significant difference ($P < 0.05$), according to t-test.

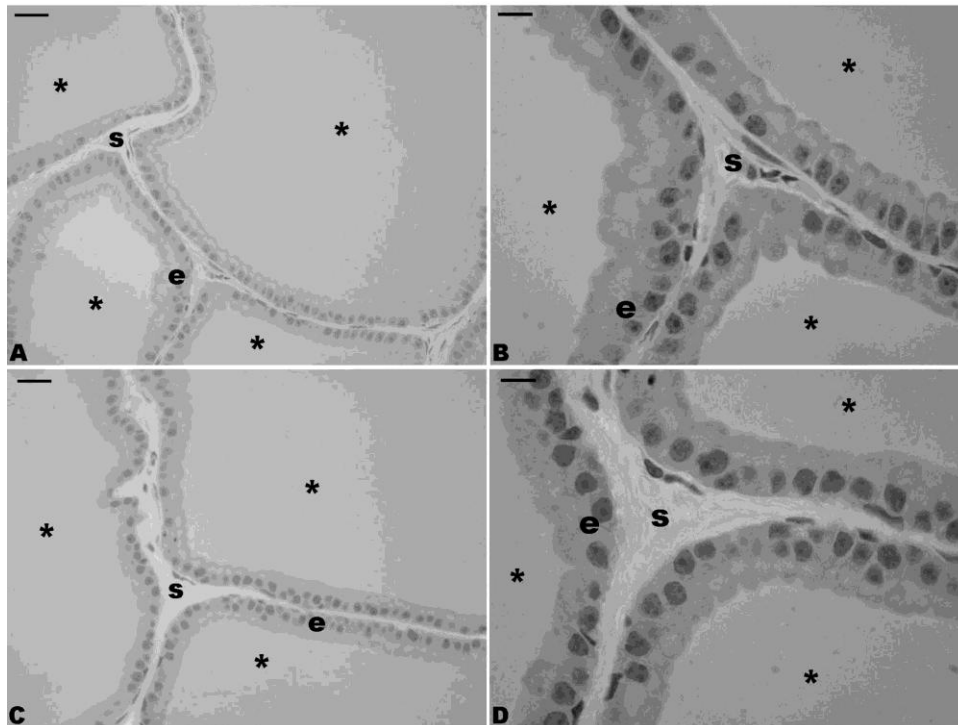


Figure 1. Histological sections of Wistar rats' ventral prostate stained with hematoxylin and eosin. The images represent the control (A and B) and *H. tomentosa* treated (C and D) animals. Notice the basal nuclei and the clear supranuclear region that corresponds to the Golgi complex region in the epithelial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

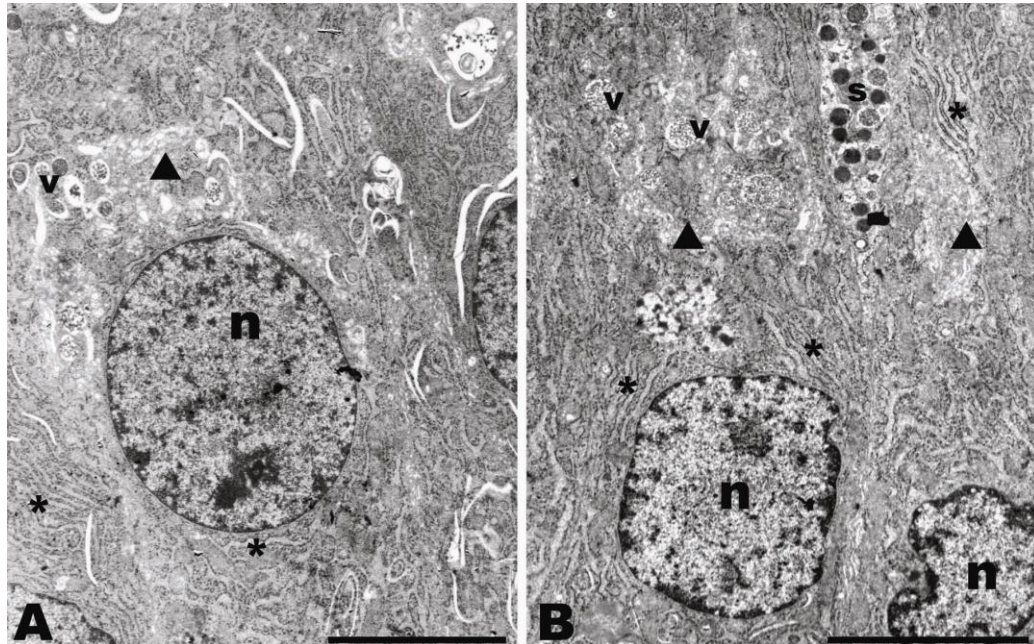


Figure 2. TEM of typical control (A) and *H. tomentosa* treated (B) ventral prostate epithelium in adult Wistar rats. Observe the region occupied by the Golgi complex (black triangle) above the nucleus (n). The secretion vesicles (v) are near the Golgi area. The large amount of rough endoplasmic reticulum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

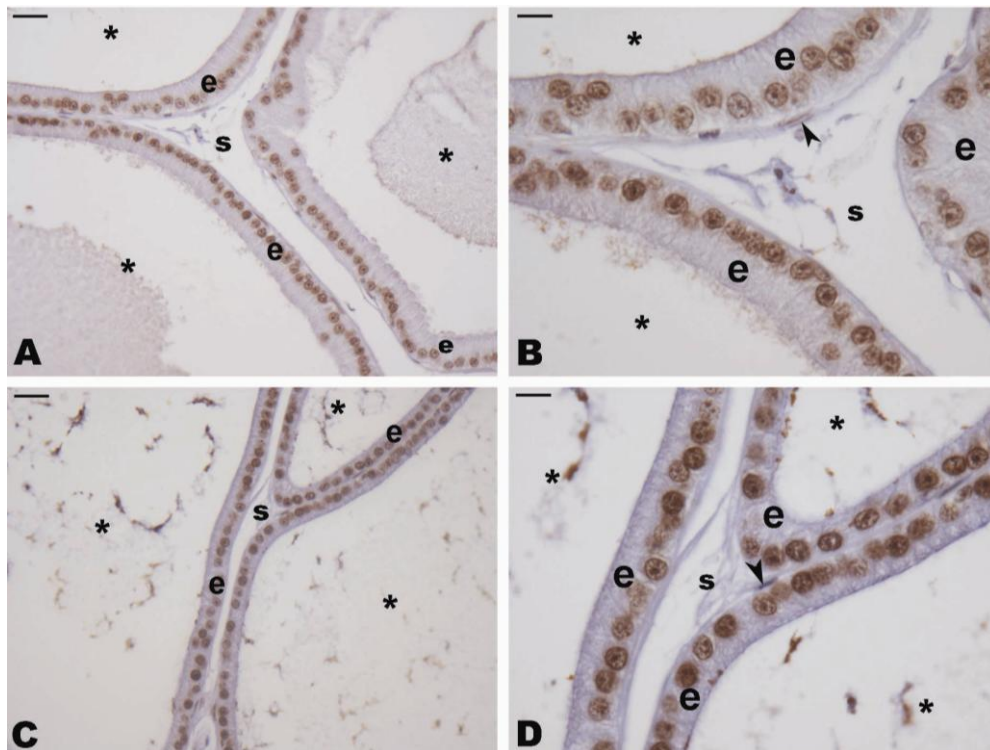


Figure 3. Histological sections of Wistar rats' ventral prostate after AR immunohistochemistry. Brown nuclei indicate positive reaction. Control animals (A and B) showed a reaction similar to the *H. tomentosa* treated animals (C and D). Abbreviations: e = secretory epithelium; s = stroma; asterisks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

seminal vesicle weights suggests that there was no significant alteration of the testosterone levels in the treated animals or that the time of experiment was not long enough to alter these organs.

The ventral prostate in rats consisted of paired right and left lobes and the ducts of this organ had few infoldings (Hayashi et al., 1991). Similar structure was observed in both groups of the present study. The histology of the ventral prostate in the control and the *H. tomentosa* 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 pattern of AR expression was similar in control and *H. tomentosa* ventral prostate, positive staining for androgen receptors was observed in most epithelial and smooth muscle cells (periductal and perivascular) as well as in some fibroblast cells of the rat ventral prostate, as also described by Shabisgh et al., (1999). The reaction intensity of AR-positive cells could be related to an increase in AR expression (Scarano et al., 2009). The absence of intensity alterations could indicate that the treatment with *H. tomentosa* did not modify the AR expression pattern in the ventral prostate of Wistar rats.

Programmed cell death (apoptosis) is very important in normal cell turnover of adult tissue (Wijsman et al., 1993). Low frequency of apoptotic cells and the short duration of their morphological changes are complicating factors for the morphological recognition of cells that undergo 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 the control Wistar rats and according to Wijsman et al. (1993) the apoptotic index in the ventral prostate of rats is less than 0.1%. The ventral prostate apoptotic index observed in the control and treated group of the present study were similar to the aforementioned findings, which indicated that *H. tomentosa* infusion did not cause any significant effect that altered the normal programmed cell death rate of the ventral prostate.

In the present study, there was no alteration in the weight of male reproductive accessory organs, with maintenance of stereological and morphometrical parameters of the ventral prostate. Also, there was an identical pattern of androgen receptor expression and apoptotic index in the ventral prostate of animals treated with *H. tomentosa*. Despite the observations of Gomes et al. (2011), that *H. tomentosa* infusion caused an increase of testosterone plasmic levels, the results of the present study showed no alteration caused by *H. tomentosa* infusion. Moreover, the normal apoptotic index and absence of histopathological alterations in the ventral prostate of Wistar rats treated with *H. tomentosa* also indicated that this treatment did not cause any injury to

the ventral prostate tissue. The route, period and dose of *H. tomentosa* root infusion administration to adult Wistar rats did not result in benefit or damage to their ventral prostate. Based on this demonstration that the infusion did not damage the prostate, future studies would be important in order to elucidate the apparent antioxidant potential of this plant on prostate tissue.

ACKNOWLEDGEMENTS

We thank FAPESP (process number 2009/07824-3), FAEPEX (process number 92910) and CAPES/PROEX for financially supporting this work. We thank the students of the Laboratory of Microscopy and Microanalyses (UNESP – Rio Preto) for technical support. We also thank Felipe Boschiero from “Espaço da Escrita” for correct the formatting of this work.

REFERENCES

- Chierogatto LC (2005). Effect of chronic treatment with extracts of *O. Heteropterys aphrodisiaca* Mach. e *Anemopaegma arvense* (Vell.) Stellf not Wistar adult testis of rats. Ph. D. Thesis, Federal University of Viçosa.
- Cid TP, García JRC, Alvarez FC, de Arriba G (2003). Antioxidant nutrients protect against cyclosporine A nephrotoxicity. *Toxicology* 189(1-2):99-111.
- Cordeiro RS, Scarano WR, Campos SGP, Santos FCA, Vilamaior PSL, Góes RM, Taboga SR (2008). Androgen receptor in the Mongolian gerbil ventral prostate: Evaluation during different phases of postnatal development and following androgen blockage. *Micron* 39(8):1312-1324.
- Galvão SMP, Marques LC, Oliveira MGM, Carlini EA (2002). *Heteropterys aphrodisiaca* (extract BST0298): a Brazilian plant that improves memory in aged rats. *J. Ethnopharmacol.* 79(3):305-311.
- García-Flórez M, Oliveira CA, Carvalho HF (2005). Early effects of estrogen on the rat ventral prostate. *Braz. J. Med. Biol. Res.* 38(4):487-497.
- Gomes MLM, Monteiro JC, Freitas KM, Sbervelheri MM, Dolder H (2011). Association of the infusion of *Heteropterys aphrodisiaca* and endurance training brings spermatogenetic advantages. *Biol. Res.* 44(3):235-241.
- Hayashi N, Sugimura Y, Kawamura J, Donjacour AA, Cunha GR (1991). Morphological and functional heterogeneity in the rat prostatic gland. *Biol. Reprod.* 45(2):308-321.
- Hoehne FC (1920). The herbalists who sell the city of Sao Paulo. Sao Paulo-SP, House Duprat.
- Marques LC, Pieri C, Roman-Júnior WA, Cardoso MLC, Milaneze-Gutierrez MA, Mello JCP (2007). Control pharmacognostic the roots of *Heteropterys aphrodisiaca* O. Mach. (Malpighiaceae). *Rev. Bras. Farmacogn.* 17(4):604-614.
- Mattei R, Barros MP, Galvão SMP, Bechara EJH, Carlini ELA (2001). *Heteropterys aphrodisiaca* O. Machado: Effects of Extract BST 0298 on the Oxidative Stress of Young and Old Rat Brains. *Phytother. Res.* 15(7):604-607.
- Monteiro JC (2007). Associação de Ciclosporina e *Heteropterys aphrodisiaca* (nó-de-cachorro) administrados a ratos Wistar: estrutura, ultra-estrutura e morfometria testicular. Master's Thesis. State University of Campinas.
- Monteiro JC, Gomes MLM, Nakagaki WR, Tomiosso TC, Sbervelheri MM, Dolder H (2010). Does *Heteropterys aphrodisiaca* administration and endurance training alter bones of mature rats? *J. Morphol. Sci.* 27(2):17-23.
- Monteiro JC, Predes FS, Matta SLP, Dolder H (2008). *Heteropterys aphrodisiaca* Infusion Reduces the Collateral Effects of

- Cyclosporine A on the Testis. *Anat. Rec.* 291(7):809-817.
- Pio Corrêa M (1984). Dictionary of Useful Plants of Brazil Exotic and Cultured. Rio de Janeiro-RJ, Ministry Agric.Braz. Institute for Forestry Development.
- Pott A, Pott VJ (1994). Plantas do Pantanal. Empresa Brasileira de Pesquisa Agropecuária, Brasília, Brazil.
- Rezzani R (2004). Cyclosporine A and adverse effects on organs: histochemical studies. *Prog. Histochem. Cytoc.*, 39(2): 85-128.
- Rizzini CT (1983). Efeitos psicotrôpicos de plantas Brasileiras parte II: aspectos botânicos. *Cienc. Cult.* 35:434-438.
- Sbervelheri MM, Gomes MLM, Monteiro JC, Dolder H (2009). Toxicological analysis of liver, kidney and blood plasma parameters of male Wistar rats treated with nó-de-cachorro infusion (*Heteropterys aphrodisiaca*, O.Mach). *Acta Microscop.* p.18B.
- Scarano WR, Toledo FC, Guerra MT, Campos SGP, Júnior LAJ, Felisbino SL, Anselmo-Franci JA, Taboga SR, Kempinas WG (2009). Long-term effects of developmental exposure to di-n-butyl-phthalate (DBP) on rat prostate: Proliferative and inflammatory disorders and a possible role of androgens. *Toxicology* 262(3):215-223.
- Shabisgh A, Tanji N, D'Agati V, Burchardt M, Rubin M, Goluboff ET, Heitjan D, Kiss A, Buttyan R (1999). Early effects of castration on the vascular system of the rat ventral prostate gland. *Endocrinology* 140(4):1920-1926.
- Spaziani E (1975). Accessory reproductive organs in mammals: control of cell and tissue transport by sex hormones. *Pharmacol. Rev.* 27(2):207-286.
- Wijsman JH, Jonker RR, Keijer R, van de Velde CJ, Cornelisse CJ, van Dierendonck JH (1993). A new method to detect apoptosis in paraffin sections: *In situ* end-labeling of fragmented DNA. *J. Histochem. Cytochem.* 41(1):7-12.