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SÉRGIO MARCELINO DE OLIVEIRA

CONTROLE HORMONAL DA PRÓSTATA FEMININA DO GERBILO SOB INFLUÊNCIA DE MÚLTIPLAS PRENHEZES E REPOSIÇÃO HORMONAL

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

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DEDICATÓRIA

A Deus, que sempre me acompanha pelos caminhos.

A minha querida mãe, guerreira incansável, que sempre entendeu e apoiou-me em minhas escolhas.

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RESUMO

A próstata feminina (glândula parauretral de Skene) está localizada na parede da uretra feminina e histologicamente possui as mesmas partes da próstata masculina, apresentando um epitélio colunar pseudoestratificado e componentes celulares, enzimáticos e estromais necessários para sua função exócrina e neuroendócrina. O tecido prostático possui uma alta dependência em relação aos andrógenos, e a ação destes hormônios sobre as células prostáticas é mediada via receptor nuclear. Nem todos os tipos celulares prostáticos possuem receptor para andrógenos, sugerindo que somente certos tipos celulares podem ser considerados alvos diretos da ação de andrógenos. A próstata feminina também é alvo da testosterona, pois estudos recentemente publicados demonstram que a reposição hormonal com este hormônio promove o desenvolvimento morfofuncional da próstata feminina em gerbilos. Mesmo sendo a próstata um tecido andrógeno dependente, os estrógenos são necessários para manutenção do seu funcionamento, o que se explica devido a presença dos dois tipos de receptores de estrógeno (ER-α e ER-β). Dados recentes da literatura propõem que os hormônios relacionados com a gravidez e ciclo ovariano promovem certo grau de proteção para o tecido da mama contra o câncer e que a aplicação de estradiol e progesterona previne a carcinogênese de mama em ratas nulíparas, mimetizando o efeito da gravidez, e ainda, que tanto o câncer de próstata quanto o câncer de mama, além de serem os principais tumores que incidem em homens e mulheres, respectivamente, e possuírem associações epidemiológicas e etiológicas, apresentam várias anormalidades genéticas em comum. O presente trabalho teve como objetivo avaliar as modificações morfo-funcionais na próstata feminina do gerbilo da Mongólia sob a influência da prenhez e avaliar a influencia desse estágio do ciclo reprodutivo na homeostase dos compartimentos prostáticos das fêmeas velhas. Para tanto, foram utilizados 50 animais, sendo: 5 fêmeas adultas nulíparas (AN); 5 fêmeas adultas multíparas (AM); 5 fêmeas velhas nulíparas (VN); 5 fêmeas velhas multíparas (VM); 5 fêmeas velhas nulíparas (VNT) e 5 velhas multíparas (VMT), as quais receberam suplementação de 0,25ml de testosterona em dias alternados (1mg/kg/dia de cipionato de testosterona em óleo vegetal) por 21 dias; 5 fêmeas velhas nulíparas (VND) e 5 velhas multíparas (VMD) as quais receberam suplementação diária de 0,1ml DHEA (5mg/kg diluída em solução salina) por 21 dias; 5 fêmeas velhas nulíparas (VNE_2) e 5 velhas multíparas (VME_2), as quais receberam suplementação de 0,1ml de estradiol (10mg/ml de óleo

vegetal), em dias alternados, por 21 dias. O grupo VM apresentou uma maior incidência de lesões proliferativas, o que nos leva a concluir que o tecido prostático de fêmeas que passam por sucessivas gravidezes possuem maior probabilidade de serem acometidas por tais lesões. Além disso, a reposição hormonal seja com testosterona, estrógeno ou dihidrotestosterona se mostrou mais agressivo no caso de fêmeas multíparas, pois a incidência de lesões proliferativas foi maior neste grupo.

ABSTRACT

The gerbil female prostate is located paraurethrally and has all the main histological components of the male prostate, like secretor epithelium and fibromuscular stroma. This gland, like the prostate in males, is targeted by testosterone action, which promotes morphofunctional development. Furthermore, estrogens are required to maintain the male and female prostate and this gland presents both estrogen receptors (ER-α and ER-β). In the present work the structural and morphometric-stereological and serological aspects, as well as the quantification of the incidence, multiplicity and percentage of acini affected by different lesions were analyzed. Fifty senile female gerbils were divided into ten groups (five animals each): Adult nulliparous gerbils (AN) and adult multiparous gerbils (AM); senile nulliparous (SN) and senile multiparous (SM) female gerbils; senile nulliparous gerbils (SND) and senile multiparous gerbils (SMD) that received daily 0,1 ml of DHEA during 21 days; senile nulliparous gerbils (SNE) and senile multiparous gerbils (SME) that received 0,1 ml of β-estradiol on alternate days during 21 days; senile nulliparous gerbils (SNT) and senile multiparous gerbils (SMT) that received 0,2ml of T (1mg/kg/day) for 21 days. This work was performed to quantify the incidence, multiplicity and percentage of acini affected by different lesions in prostate of nulliparous and multiparous senile female gerbils under different types of hormonal replacement using morphometric, stereological and immunohistochemical techniques and to try to establish a relationship between hormonal serum levels in these groups. The proliferative ratio (PCNA/TUNEL) were higher in al multiparous groups when compared with nulliparous groups, mainly in SMT, the same group where were found higher multiplicity of proliferative lesions.

1. Morfologia da próstata feminina

A próstata feminina (glândula parauretral de Skene) é uma glândula anexa do sistema genital feminino e possui, segundo Reinier De Graaf (1672), homologia com a próstata masculina. Está localizada na parede anterior da uretra feminina e histologicamente possui as mesmas partes da próstata masculina, apresentando um epitélio colunar pseudoestratificado e componentes celulares, enzimáticos e estromais necessários para sua função exócrina e neuroendócrina. (ZAVIAČIČ *et al.*, 2000). Zaviačič e colaboradores (1997) demonstraram a presença de células neuroendócrinas na parede dos ductos prostáticos. Além disso, atividade positiva da enzima fosfatase ácida prostática (PAP) foi evidenciada por Zaviačič (1984) e por Custódio e colaboradores. (2004).

Em mulheres, no epitélio glandular prostático tem sido evidenciada a presença de células epiteliais secretoras e também de células epiteliais basais (ZAVIAČIČ *et al*., 2000). Estas células epiteliais secretoras apresentam abundância em vesículas secretoras e grânulos, assim como retículo endoplasmático rugoso (RER), complexo de Golgi desenvolvido e numerosas mitocôndrias, caracterizando sua alta atividade secretora (ZAVIAČIČ *et al*., 2000). Em fêmeas de Gerbilo da Mongólia (*Meriones unguiculatus*), foi demonstrado que a próstata feminina possui alvéolos e ductos circundados por um estroma fibromuscular. O epitélio é cúbico simples, possuindo dois tipos celulares distintos: células secretoras cilíndricas e as células basais, restritas ao compartimento basal do epitélio. Já o estroma apresenta vasos sanguíneos, células musculares lisas circundando os alvéolos e ductos prostáticos, eventuais fibras musculares esqueléticas, dentre outros tipos celulares. No estroma encontram-se ainda a maioria dos

componentes fibrosos da matriz extracelular, sendo as fibras de colágeno as mais abundantes (dos SANTOS *et al*., 2003).

O câncer de próstata é o principal tipo de câncer em homens nos países ocidentais e a segunda causa de morte por câncer no gênero masculino (PARKER *et al.*, 1997, JEMAL *et al.,* 2007). Evidências de adenocarcinoma prostático em biópsias de tecido prostático feminino têm sido relatadas em alguns trabalhos científicos (SLOBODA *et al*., 1998; ZAVIAČIČ *et al.*, 1993; SVANHOLM *et al.*, 1987). Além disso, pesquisas recentes demonstraram que o envelhecimento pode promover um aumento na incidência de lesões proliferativas na próstata feminina do gerbilo (CUSTODIO *et al*., 2010) e estudos tem alertado que alguns adenocarcinomas uretrais femininos tem sido diagnosticados de forma equivocada, pois apresentam evidências de origem prostática (Reis *et.al*., 2009).

O gerbilo tem se prestado como um excelente modelo experimental para estudo com a próstata devido à facilidade com que pode ser manuseado no biotério, além de ser um animal extremamente dócil e de fácil adaptação ao cativeiro. O interesse biológico em relação à próstata de *Meriones unguiculatus* se justifica devido a algumas semelhanças morfofuncionais existentes em relação à próstata humana.

2. Regulação hormonal da próstata feminina

Da mesma maneira que a masculina, a próstata feminina está sob influência da testosterona (T) tanto para sua diferenciação e desenvolvimento peri-natal, quanto para sua manutenção (SANTOS *et al.,* 2006). Além disso, a manutenção da próstata feminina está sob

influência de estrógenos (E) sendo que esta glândula apresenta atividade tanto do receptor de estrógeno alfa (ER- α) quanto beta (ER-β) (WEIHUA *et al.*, 2001). Alguns pesquisadores (MCPHERSON *et al.,* 2008) evidenciaram que o estrógeno tem um papel crucial na diferenciação e desenvolvimento prostático. Além disso, estudos desenvolvidos em nosso laboratório sugerem que o surgimento e proliferação de lesões prostáticas podem ser promovidos pela administração de E (SCARANO *et al.,* 2005; SCARANO *et al*., 2004). Biancardi e colaboradores (2010) demonstraram que a administração de T promove intenso crescimento da próstata feminina de rato, a qual se torna ativa nas funções sintética e secretora.

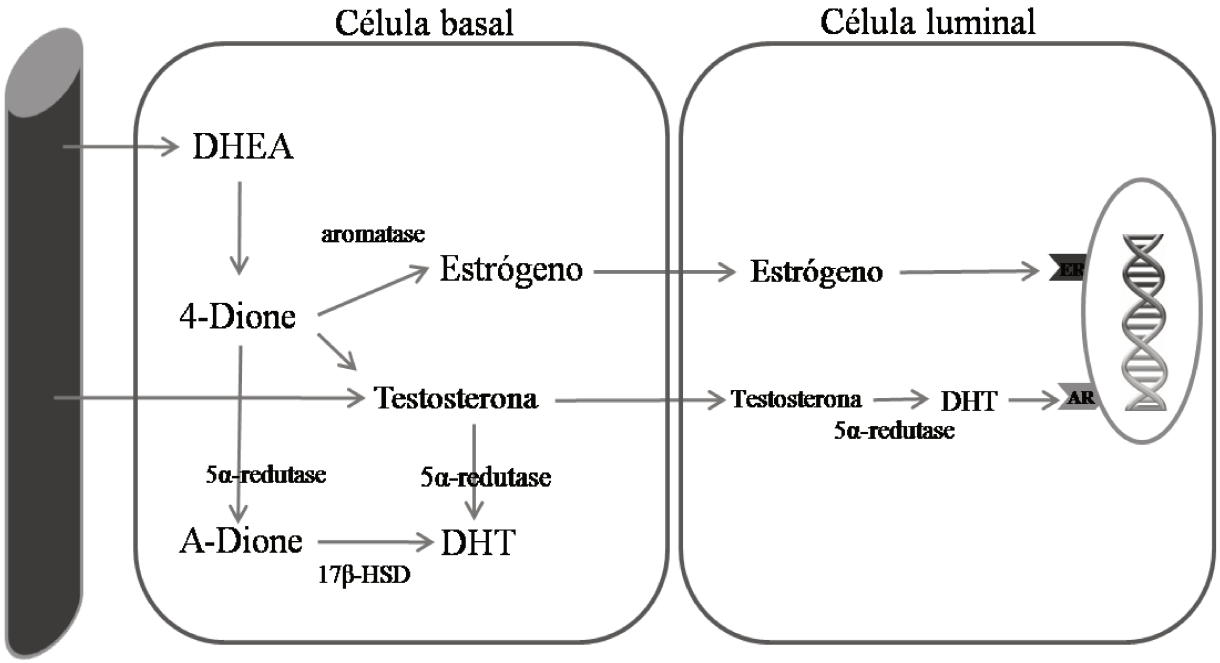

Fig. 01: *Vias metabólicas da ação de hormônios esteróides na próstata: DHT, dighidrotestosterona; ER, receptor de estrógeno; AR, receptor de andrógeno (adaptado de LABRIE et al., 1998).*

A progesterona (P) que é um hormônio essencial para a função secretora do endométrio, permitindo a implantação do embrião e mantendo a viabilidade fetal, também tem ação sobre o tecido prostático. O receptor de progesterona (PgR) é usado como marcador molecular no tratamento de câncer de mama (CHOID *et al*., 2005). Estudos têm mostrado sua associação com,

por exemplo, a proteína Par-4, que tem a capacidade de induzir apoptose. Esta associação promove a inibição da ação da Par-4, causando uma diminuição da atividade apoptótica o que leva à progressão do câncer (ZAPATA-BENAVIDES *et al*., 2009). Se a importância do receptor de andrógeno (AR) na evolução do câncer de próstata está bem estabelecida, muitos trabalhos apontam também para a potencial implicação do PgR na carcinogênese prostática (HIRAMATSU *et al.,* 1996; WERNERT *et al*., 1988).

A dehidroepiandrosterona (DHEA), o sulfato de dehidroepiandrosterona (DHEA-S) e a androstenediona são precursores de esteróides secretados pela glândula adrenal, que podem ser convertidos em potentes estrógenos ou andrógenos em diferentes tecidos. A formação do mais potente andrógeno natural, a dihidrotestosterona (DHT) envolve inúmeras enzimas, sendo a mais importante a 5α-redutase. Já a formação do mais importante estrógeno natural, o 17β-estradiol depende, principalmente, da enzima aromatase (Fig. 01) (LABRIE *et al*., 1998). A produção de DHEA pelas adrenais sofre um intenso declínio em fêmeas durante a menopausa, acarretando no aparecimento de várias patologias, dentre elas a osteoporose (JOHNSTON & EPSTEIN, 1981). Já que a reposição hormonal usando estrógeno pode aumentar o risco de câncer de mama (COLDITZ *et al*., 1995), Labrie e colaboradores (1998) sugerem a administração de DHEA para prevenir a osteoporose em mulheres após a menopausa. Vale ressaltar que vários cânceres (mama, próstata, ovário e útero) são dependentes de hormônios esteróides, o que leva a uma incessante procura por substâncias inibidoras da atividade enzimática que produz esses hormônios, que podem ser combinados com potentes e específicos antiestrógenos e antiandrógenos (LUO et al., 1996; OLIVEIRA et al., 2006).

OBJETIVOS

Este trabalho teve como objetivo a análise e quantificação da incidência de lesões proliferativas na próstata de gerbilos fêmeas nulíparas e multíparas procurando estabelecer relações entre a quantidade de lesões com a taxa proliferativa do epitélio e a dosagem hormonal de cada grupo. Além disso, procurou-se determinar como as reposições hormonais utilizando E, T e DHEA atuam na remodelação tecidual e manutenção da arquitetura prostática bem como na promoção/prevenção de lesões prostáticas, tanto em fêmeas nulíparas, quanto em fêmeas multíparas.

Artigo 01.

Microscopic evaluation of proliferative disorders in the gerbil female prostate: evidence of aging and multiple pregnancies influences

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Microscopic evaluation of proliferative disorders in the gerbil female prostate: evidence of aging and the influence of multiple pregnancies

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Running Title: Pregnancy, aging and female prostate disorders

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Abstract

The gerbil female prostate is located paraurethrally and has all the histological components of the male prostate, like secretor epithelium and fibromuscular stroma. This gland, like the prostate in males, is targeted by testosterone action, which promotes morphofunctional development. Furthermore, estrogens are required to maintain the male and female prostate and this gland presents both estrogen receptors (ER-α and ER-β). In the present work the structural and morphometric-stereological and serological aspects, as well as the quantification of the incidence, multiplicity and percentage of acini affected by different lesions were analyzed. Animals were divided into four groups: Five adult nuliparous gerbils (AN); five adult multiparous gerbils (AM); five senescent nulliparous gerbils (SN); five senescent multiparous gerbils (SM), and were weighed and sacrificed by CO2 inhalation. The ventral prostate was dissected out, weighed and fixed to perform histological and morphometric-stereological analysis and quantification of prostate disorders. A high rate of lesions, mainly dysplasia, was identified in tissue from senescent multiparous and adult multiparous animals. Prostatitis was found mainly in SN animals, while dysplasia, hyperplasia, neoplasia, PIA and adenocarcinoma were common in SM ones. Although the proliferative lesion incidence was high in AN group, it was highest in the SM group. The hormonal events which occur due to the estrous cycle in female gerbils (after and before each pregnancy) may be responsible for the high number of lesions observed in our study and all the data presented herein lead us to assume that pregnancy promotes augmentations in both the incidence and multiplicity of proliferative disorders in the gerbil female prostate since progesterone levels remain high during pregnancy.

Key words: female prostate, aging, pregnancy, steroid hormone, proliferative lesion, progesterone

1. Introduction

The human female prostate was firstly described by Reinier de Graaf in the Century 18th (Zaviačič, 1999), demonstrated its homology with the male prostate. In females this gland is located in the urethral wall and exhibits the same structures as the male version: pseudostratified columnar epithelium and the cellular, enzymatic and stromal compounds that are necessary to its functionality (Custodio et al., 2004; Zaviačič et al., 2000). In females, little is known about the prostate gland, especially regarding to its physiology and contribution to the genial tract (Zaviačič et al., 2000). However, some works indicate that testosterone have stimulatory effects on the female prostate, inducing epithelial cell proliferation, differentiation, secretory activity, and dysplasia (Santos et al., 2006). Furthermore, foci of prostatic adenocarcinoma were found in female prostate samples (Sloboda et al., 1998; Svanholm et al., 1987; Zaviačič et al., 1993) and evidences in the establishment that female urethral carcinoma is originated from Skene´s gland, may have influence on diagnostic and treatment strategies to this pathology in the future (Reis et al., 2011).

The female prostate, like in male, is target of testosterone (T) hormone action, which promotes morphofunctional development during embryogenesis and functional maintenance in the adult gland (Santos et al., 2006). Mongolian gerbil (Meriones unguiculatus) have been a good experimental model, since the previous data found by our group been demonstrated that histological, histochemical, and ultrastructural features of the adult gerbil's prostate are comparable to the human prostate (Custódio et al., 2004; Scarano et al., 2005; Santos et al., 2006).

Estrogens (E) are required for maintenance of the male and female prostate, a gland that presents both estrogen receptors, ER-α and ER-β (Weihua et al., 2001). Besides this hormone can

play a key role in prostate development and differentiation (McPherson et al., 2008). Studies performed by our research group suggest that the enlargement and proliferation of prostatic lesions can be promoted by E administration (Scarano et al., 2004; Scarano et al., 2005).

Some studies (Guzman et al., 1999; Medina, 2004; Sivaraman and Medina, 2002) have shown that pregnancy and ovarian-cycle hormones like E and progesterone (P) provide a type of protection against cancer in breast tissue. Furthermore, Guzman et al. (1999) demonstrated that E and P administration prevents mammalian carcinogenesis in nuliparous rats, (for example) like after pregnancy. In contrast, Liang and colleagues (2010) shows that progestins stimulated the growth of breast tumors independent of exposure timing and protocol, and stimulated vascular endothelial growth factor elaboration and increased tumor vascularity and also increased lymph node metastasis of BT-474 cells. According to López-Otín and Diamandis (1998) pregnancy increases the risk of tumoral proliferation in breast tissue, a tissue that has some similarities with prostate including alterations in androgen receptor (AR) gene while others involved in hereditary breast cancer (BRCA1,BRCA2, p53 and RB1) are genetic abnormalities common to breast and prostate cancer.

Based on these considerations, the aim of this work was to evaluate in nulliparous and multiparous female gerbils the microscopic disorders presented by prostate gland, besides to quantify the incidence, multiplicity and percentage of acini affected by different lesions.

2. Material and methods

2.1. Animals and Experimental Design

Ten female adult gerbils (Meriones unguiculatus, Gerbilinae, Criscetidae) and ten female senescent (± 15 months old) gerbils were housed under standard conventional conditions (25ºC , 40% – 70% relative humidity, 12 hr light/12 hr dark) and allowed access to chow and water ad libitum. The vaginal smears were collected and used as an estrous cycle indicator; only animals in proestrus were used in these experiments (Nishino and Totsukawa, 1996). Animals were divided into four groups: Five adult nuliparous gerbils (AN); five adult multiparous gerbils (AM); five senescent nulliparous gerbils (SN); five senescent multiparous gerbils (SM). Experiments were performed according to the Guide for Care and Use of Laboratory Animals. Animals were weighed and sacrificed by compressed CO2 inhalation. The ventral prostate was carefully dissected out, weighed and fixed. Animal handling and experimental design were done in accordance to ethical guidelines from São Paulo State University, which follows the Guide for Care and Use of Laboratory Animals (Protocol CEEA - 015/07). The quantity of animals needed in this study is justified by the large number of analytical procedures employed.

2.2. Histology

Prostatic samples were fixed by immersion in Karnovsky's solution (5% parformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) for 24 h. After fixation, the material was washed with running tap water, dehydrated in an ethanol series, and embedded in glycol methacrylate resin (Leica historesin embedding kit, Leica, Nussloch, Germany) and sectioned at 3 μm on a Leica automatic rotatory microtome (Leica RM2155, Nussloch, Germany). Histological sections were subjected to Hematoxylin-Eosin (H&E) staining for general studies.

Microscopic analyses were performed on Zeiss- Jenaval (Zeiss-Jenaval, Jena, Germany) or an Olympus BX60 light photomicroscope (Olympus, Hamburg, Germany). Microscopic fields were digitized using the software Image-Pro Plus, Version 4.5 for Windows.

2.3. Morphometric-stereological analysis

Random fields of Hematoxylin-Eosin (H&E) histological sections of female prostate from each experimental group were analyzed by Image -Pro® Plus version 4.5 for Windows.

Stereological measurements were obtained by the M120 multipoint test system and 60-line test proposed by Weibel (1963) to compare the relative proportion (relative volume in %) of each component of prostatic tissue (epithelium, lumen, muscular stroma and nonmuscular stroma and smooth muscle cells - SMC). 30 random microscopic fields were captured from each experimental group. The relative values were determined by counting the points coincident with the test grade divided by the total number of points.

2.4. Statistical analysis

Data were performed with Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA). The ANOVA and Tukey HSDs test were employed, and $p \leq 0.05$ was considered statistically significant. Differences in prostatic complex absolute weight were analyzed by the Student's t test, and $p \leq 0.05$ was considered statistically significant.

2.5. Hormonal Dosage

Blood samples were collected from all animals by decapitation. Serum quantification of T, E and P was done using the Modular Analyzer for Immunoassay of Chemiluminescence ECi (Johnson & Johnson) according to the procedure of Weeks and Woodhead (1984). For each group sample, at least 3 animals were utilized. All sample tests were done in triplicate.

2.6. Quantification of prostate disorders

H&E histological sections from adult and senescent nuliparous/multiparous animals were chosen randomly (7 sections per animal). The lesions were classified according to Shappell and colleagues (2004) in addition to the Classification of Urinary System Tumors and Male Genital Organs from the World Health Organization – WHO (2004): Prostatic hyperplasia, prostate microcalculi and/or calculi (small structures, with crystalloid aspect, scattered in the luminal acini were identified as microcalculi, while the real prostatic calculi had larger dimensions and occupied most acini) and prostatitis were classified as benign lesions; high-grade prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) were classified as pre-malignant ones, and adenocarcinomas as malignancies.

The glandular lesion incidence (injury diagnosis/ sample) was determined by identifying the different lesions in relation to the total sample number. The multiplicity (specific number of lesions/ sample) was calculated by the relative frequency of each lesion identified in the histological section to the total number of examined animals.

2.7. Immunohistochemical Methods

Pretreatment of sections by heating in citrate buffer, pH 6.0 (using a pressure cooker), was performed to enhance Bcl-2, p53, and caspase-3 immunostaining and treated for 20 min in 3% H2O2 in methanol to block endogenous peroxidases. They were subsequently treated with a background sniper blocker to eliminate unspecific bindings (Biocare Medical, Concord, CA, USA), during 15 min. Sequentially, slides were incubated with the following primary antibodies (Santa Cruz Biotechnology, Palo Alto, CA, USA) diluted 1:100 in 1% BSA: mouse anti-human PCNA (sc-56, 1 h, at 37o C). Primary antibodies anti-PCNA were detected by Polymer conjugated to peroxidase (Novolink Polymer; Novocastra, Norwell, MA, USA), for 40 min. The reactions were revealed with diaminobenzidine (0.03% in TBS) and sections were stained with haematoxylin. Negative controls were obtained by omission of the primary antibody.

3. Results

3.1. Morphological and morphometric analysis

Adult nulliparous animals showed a characteristic architecture of prostatic tissue, with thin and simple cubic/columnar epithelium (Fig. 1a). AN presented a large number of clear cells distributed in the epithelium, and various ciliated cells were identified (Fig. 1b). However, AM presented proliferative characteristics and higher epithelial cells, in which some lesions were found (Fig. 1c-d).

The SN animals displayed relatively preserved architecture of prostatic tissue (Fig. 2a), and cuboidal epithelial cells with no proliferative characteristics (Fig. 2i). The stroma of these animals presented few collagen fibers and highly organized muscular cells (Fig. 2b).

SM tissues possessed a proliferative epithelium (Fig. 2j), with columnar epithelial cells, numerous microacini, atypical nuclei (2c-h) and presence of calculi (Fig. 2g). Furthermore, stromal alterations can be found in SM samples, such as a marked modification in the arrangement and distribution of collagen fibers, including the fibrillar molecular aggregation. In addition the blood vessels associated with proliferative epithelium were more conspicuously observed (Fig. 2c,d and h).

Adult gerbils presented mean epithelial height of 11.22 ± 3.59 µm ($p \leq 0.05$) in nulliparous animals and 20.98 ± 6.48 µm ($p \leq 0.05$) in multiparous ones. In old animals the epithelial height in senescent animals was 17.38 ± 2.45 µm ($p \leq 0.05$) in nulliparous animals and 22.93 ± 5.35 µm ($p \leq 0.05$) among the multiparous sample.

3.2. Hormonal serum levels

The most evident difference were observed in progesterone levels, once that this hormone concentration was more than two times higher in the multiparous than in the nuliparous animals. AM presented progesterone levels around 3.5 ng/ml ±0.005, while in SM was 2.03 ng/ml ±0.569. In nuliparous animals, adult had 0.70 ng/ml ±0.23 and senescent 0.94 ng/ml ±0,84. The hormonal ratio between progesterone and testosterone (P/T) and between progesterone and estrogen (P/E) revealed also elevated values in multiparous animals. All the intergroup differences revealed by hormonal concentration in serum testosterone, progesterone and estrogen levels can be observed in Table 01.

3.3. Quantification of prostate disorders

A high rate of lesions, mainly dysplasia, was identified in tissue from SM and AM animals in contrast with AN and SN groups (Table 1). Prostatitis was found mainly in SN animals, while dysplasia, hyperplasia, neoplasia, PIA and adenocarcinoma were observed among SM.

Among the adult gerbils, the proliferative lesion incidence was higher in AN than in AM, but in senescent females, this incidence was highest among SM (Table 1).

4. Discussion

Since century 18th, when the female prostate anatomical studies of de Graaf were first published, many papers have focused on the origins and function of this gland (Zaviačič, 1999). The proliferative lesion incidence in the female prostate and its relation to age has been reported in the literature (Custodio et al., 2008). In males, these lesions can be promoted by administration of steroid hormones (Cunha et al., 2001; Risbridger et al., 2003; Scarano et al., 2005). In females, some experiments showed that hormonal administration induced an imbalance of prostate homeostasis leading to an increase in proliferative lesion incidence (Santos and Taboga, 2006). However, many times, these prostatic lesions were diagnosed incorrectly as urinary tract disorders (Zaviačič 1999).

All the main known proliferative disorders were found in our investigation and the most commonly identified lesion was dysplasia, which promotes changes in acinar morphology such as epithelial infolding and irregular acini. In addition, all the other lesions presented high multiplicity and incidence in SM animals, except prostatitis microcalculi and calculi.

Hyperplasia, a disorder that causes urethral obstruction and urinary retention (Zaviačič, 1999), was frequent among SM in the present study. This lesion, that promotes increase in glandular volume as evidenced by acinar expansion and regions of epithelial metaplasia, has been seen in women but frequently not recognized and treated (Folson and O'Brien, 1945) and, in men, causes urethral obstructions (Untergasser et al., 2005). Marcelli and Cunningham (1999) showed that an increased number of cells are related to high proliferation and low apoptosis, which is one of the consequences of lumen enlargement in the prostate. The role of the AR/androgen signaling in the precancerous prostatic hyperplasia and dysplasia that progress to adenocarcinomas is amply evident based on several lines of studies (Culig et al., 2002) whereas the multiparous prostate can be influenced by T from natural production (Labrie et al., 1998) and from male fetuses (Kensinger et al., 1986).

rostatic intraepithelial neoplasia (PIN) was highly present in the SM group. This PIN is defined as a pre-malignant lesion (O'Shaughnessy et al., 2002) that shows not only morphological cell abnormality including enlarged cells with atypical nucleoli, but also the presence of microacini and vascularized stroma, as described by Brawer (1992).

A brief exposure to estrogen in the neonatal period permanently programs the prostate in terms of its growth, morphology, responsiveness to androgens, and susceptibility to diseases. In male mice, neonatal estrogenization leads to squamous metaplasia in the adult prostate (Strauss et al., 1998) while menstrual/reproductive events are the most important breast cancer risk factors (Henderson et al., 1988). Androgens (androstenedione and T) are necessary precursors for the occurrence of E synthesis in the ovary. Androgens, secreted by both the ovaries and adrenals, circulate at a concentration similar to E during the preovulatory peak and at a higher concentration during the rest of the menstrual cycle. In humans, after menopause, the secretion of

P and E falls dramatically, but androgens continue being secreted by adrenals in a slightly higher proportion than during the fertile period of life (Honma et al., 2006).

In the Mongolian gerbil, the length of gestation is 24-26 days (Nakai et al., 1960) and parental care by female and male immediately after the birth had been noticed (Prates and Guerra, 2005). Prolactin, released by the pituitary after parturition, is involved in rodent maternal behavior, although species differences exist during both the onset and maintenance of this behavior (Bridges et al., 1997; McCarthy et al., 1994). Furthermore, it has been observed that prolactin enhances the effects of androgens in the prostate gland and/or the seminal vesicles of rats, mice, and guinea pigs as well as in the accessory sex organs of other species (Thomas and Keenan, 1976).

The age-related increase in prevalence of chronic prostatitis is associated to a T concentration besides a T/E ratio declines (Kaufman and Vermeulen, 2005; Vermeulen et al., 2002), however this pathology presents uncertain etiology (Piovesan et al., 2009). In addition, the levels of hormones as estrogen and progesterone cannot be neglected in this process, once that the interaction of them may be one key point to understanding this disorder. The high incidence and multiplicity of proliferative lesions in the SM prostate may be related to the high P/T and P/E ratios in this group. These data might be causal and can be explained by the weakening anti-inflammatory action of T and the intensifying pro-inflammatory influence of E (Straub, 2007).

All these hormonal events which occur due to the estrous cycle in female gerbils (after and before each pregnancy period) may be responsible for the elevate number of lesions observed in the present study. All the collected data allow us to assume that pregnancy promotes increase in the incidence and in the multiplicity of microscopically proliferative disorders in the gerbil female prostate. This fact is probably due to the P high levels which remain high during the all period of

pregnancy. Thus, these analyses justify the employing of this experimental model for the study of morphofunctional aspects of gerbil with especial attention to aging, mainly under hormonal replacement, like treatments used in human females after menopause.

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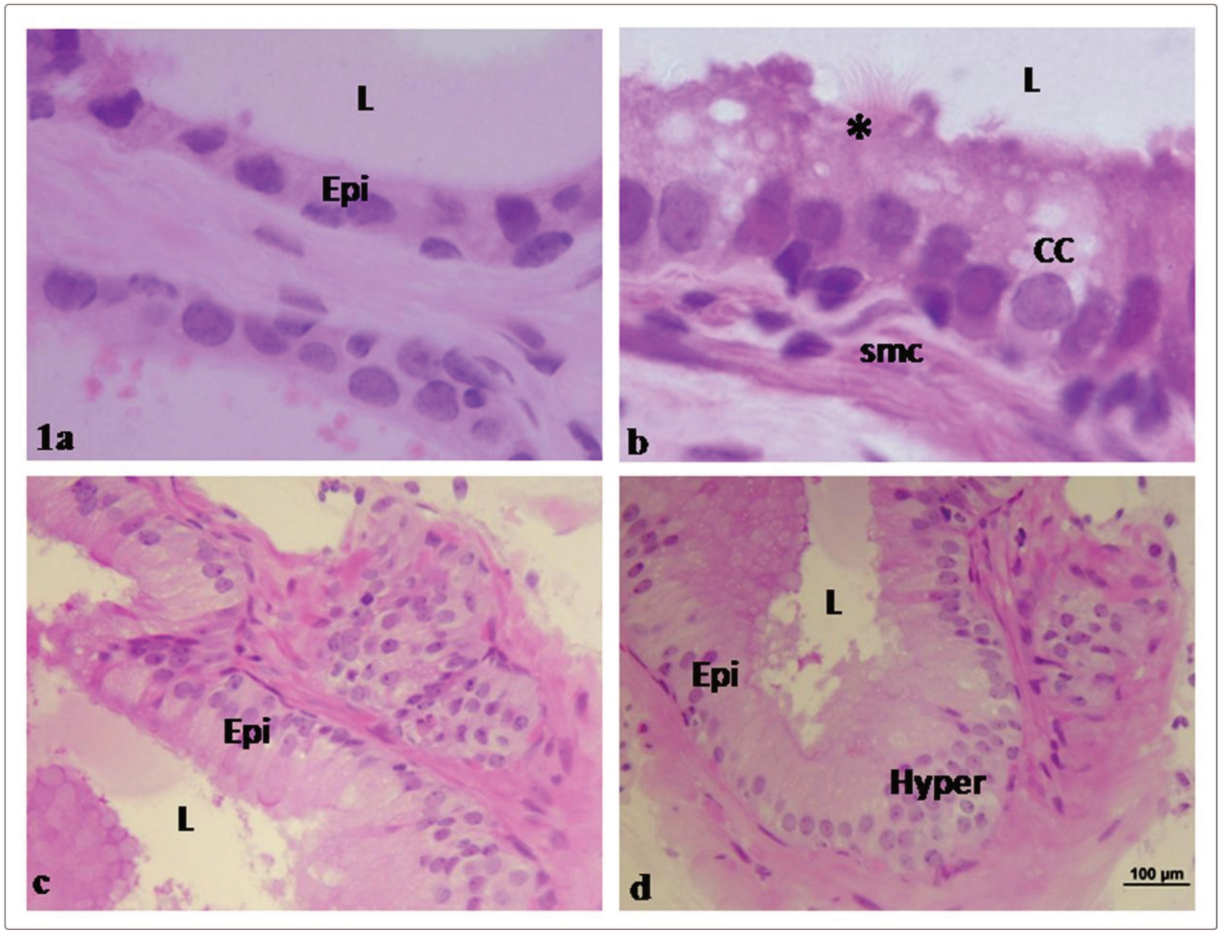
FIGURES CAPTIONS

Figure 1: Prostate samples from adult nulliparous (a-b) and multiparous animals (c-d). Hematoxylin-eosin. L, lumen; *, ciliated cell; Epi, epithelium; smc, smooth muscle cell; CC, clear cell; Hyper, hyperplasia (magnification:1a, 1b and 1d- 100X; 1c- 40X).

Figure 2: Prostate samples from senescent nulliparous (a-b) and multiparous animals (c-h). Hematoxylin-eosin. L, lumen; Epi, epithelium; smc, smooth muscle cell; *, microacinous; bv, blood vessel; Co, collagen; Dysp, dysplasia; Ca, calculi; PIN, prostatic intraepithelial neoplasia . (magnification: 2a, 2c and 2h-40X; 2f-20X; 2b, 2d, 2e and 2g- 100X). Immunohistochemical staining to PCNA from senescent nulliparous (2i) and multiparous animals (2j). Immunoreactive nuclei are observed in the epithelium (arrowheads).

Table 1: Absolute and relative quantitative data of the gerbil female prostate proliferative lesions: Incidence (injury diagnosis/sample)-different lesions in relation to the total sample number; Multiplicity (specific number of lesions/sample)-frequency of each lesion identified in the histological section relative to the total number of examined animals; Percentage of lesions per acini. Different indices (a, b, c, d) indicate statistically significant differences at $p \leq 0.05$.

Table 2: Hormone serum levels. Dosage of progesterone, testosterone and estrogen. Hormonal ration between different hormones. Different indices (a, b, c, d) indicate statistically significant differences at $p \leq 0.05$.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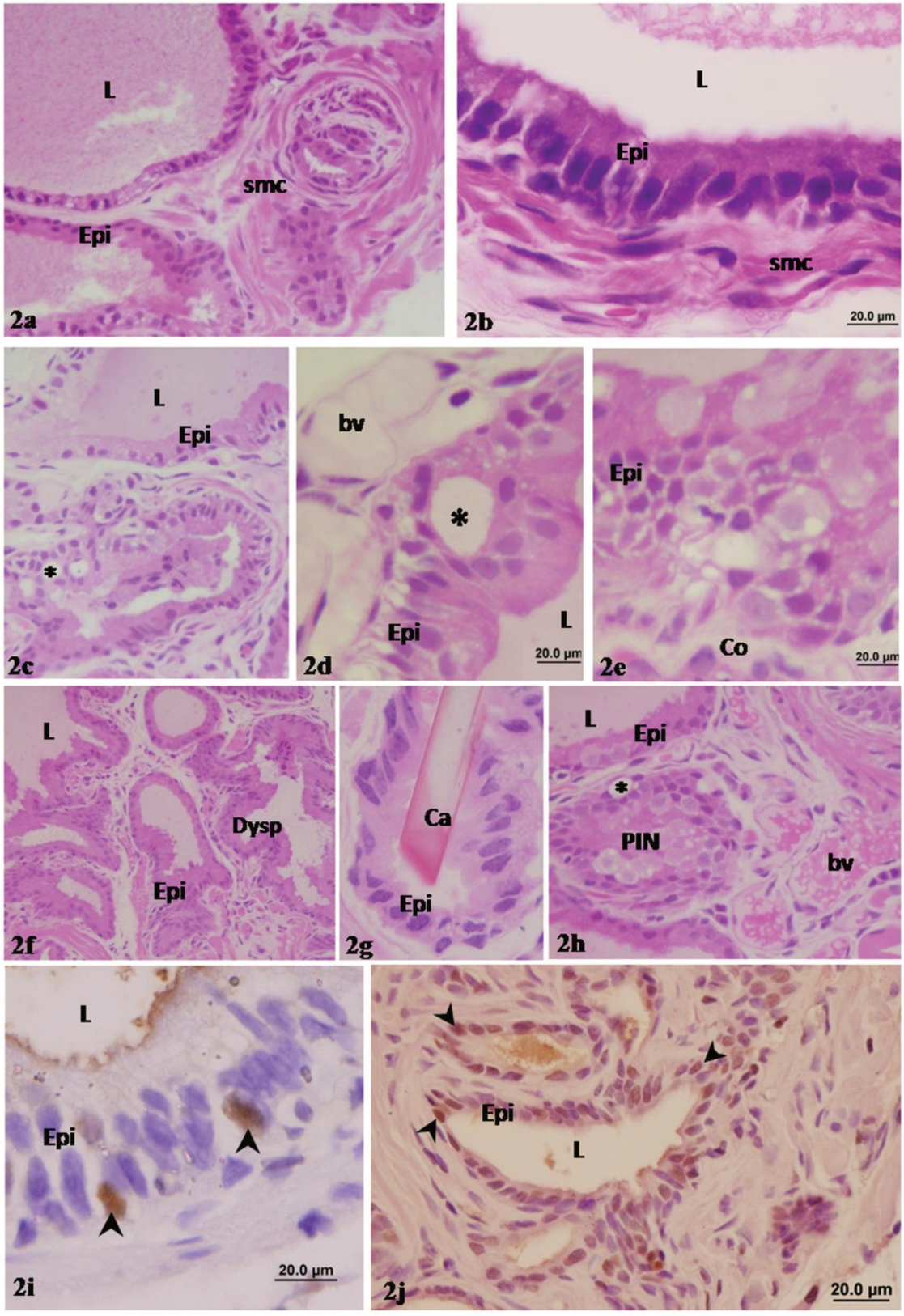
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Table 01	Indices of proliferative lesions							
	Percentage of lesions per acinus (%)							
	Dysplasia	Hyperplasia	Prostatitis	Microcalculi	Calculi	PIN	PIA	Adenocarcinoma
AN	21	16	0	21	0	5	0	0
AM	31	15	0	8	8	0	0	0
SN	23	9	23	5	14	0	0	0
SM	29	23	10	13	10	26	3	3
	Incidence (%)							
	Dysplasia	Hyperplasia	Prostatitis	Microcalculi	Calculi	PIN	PIA	Adenocarcinoma
AN	67	67	0	33	0	33	0	0
AM	67	67	0	33	33	0	0	0
SN	67	67	67	33	67	0	0	0
SM	67	67	67	67	67	100	33	33
	Multiplicity - Mean ±St Err							
	Dysplasia	Hyperplasia	Prostatitis	Microcalculi	Calculi	PIN	PIA	Adenocarcinoma
AN	1.3 ±0.6^{abc}	1.0 ±0.5^{abc}	0.0 ±0.0^{ab}	1.3 ±1.3^{ad}	0.0 ±0.0^{ab}	0.3 ±0.3^{abc}	0.0 ±0.0	0.0 ±0.0
AM	2.3 ±0.3^{bac}	0.6 ±0.3^{bac}	0.0 ±0.0^{ba}	0.3 ±0.3^{bc}	0.3 ±0.3^{ba}	0.0 ±0.0^{bac}	0.0 ±0.0	0.0 ±0.0
SN	1.6 ±0.8^{cab}	0.6 ±0.3^{cab}	1.6 ±1.2^{cd}	0.3 ±0.3^{cb}	1.0 ±0.5^{cd}	0.0 ±0.0^{cab}	0.0 ±0.0	0.0 ±0.0
SM	4.7 ±1.4^{d}	5.0 ±0.5^{d}	1.6±0.3^{dc}	1.6 ±0.3^{da}	1.6 ±0.3^{dc}	4.3 ±0.8^{d}	0.3 ±0.3	0.3 ±0.3

Table 02	Hormonal serum levels			
Hormonal dosage ±St Err	***AN***	***AM***	***SN***	***SM***
PRG (ng/ml)	0.70 ±0,23 ^{acd}	3.50 ±0,005 ^{bcd}	0.94 ±0,84 ^{cabd}	2.03 ±0,569 ^{dabc}
TESTO (ng/ml)	0.13 ±0,01 ^{abd}	0.16 ±0,003 ^{bad}	0.37 ±0,09 ^{c}	0.06 ±0,016 ^{dab}
EST (ng/ml)	0.03 ±0,005 ^{abcd}	0.02 ±0,006 ^{bacd}	0.02 ±0,001^{cabd}	0.02 ±0,001^{dabc}
Hormonal Ratio	***AN***	***AM***	***SN***	***SM***
T/E	4	6	18	3
T/P	0.18	0.1	0.40	0.03
E/P	0.047	0.0013	0.02	0.01
P/T	5.53	104	2.51	32
P/E	21	763	45	92

Artigo 02.

Multiparous old female prostate shows an increased rate of proliferative disorders after hormonal replacement.

A ser submetido para o periódico *General and Comparative Endocrinology*

MULTIPAROUS OLD FEMALE PROSTATE SHOWS AN INCREASED RATE OF PROLIFERATIVE DISORDERS AFTER HORMONAL REPLACEMENT.

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Running Title: Pregnancy plus hormonal replacement induces proliferative disorders in female prostate.

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ABSTRACT

The human female prostate was first described by Reinier DeGraaf in 1672, which showed its homology with male prostate. Besides was found focuses of prostatic adenocarcinoma in female prostatic samples by some researches. Maintenance of the female and male prostate is under influence of steroid hormones and these that can promote disruption of prostatic tissue initiating proliferative disorders. Forty senile female gerbils were divided into eight groups (five animals each): senile nulliparous (SN) and senile multiparous (SM) female gerbils; senile nulliparous gerbils (SND) and senile multiparous gerbils (SMD) that received daily 0,1 ml of DHEA during 21 days; senile nulliparous gerbils (SNE) and senile multiparous gerbils (SME) that received 0,1 ml of β-estradiol on alternate days during 21 days; senile nulliparous gerbils (SNT) and senile multiparous gerbils (SMT) that received 0,25ml of T (1mg/kg/day) for 21 days. This work was performed to quantify the incidence, multiplicity and percentage of acini affected by different lesions in prostate of nulliparous and multiparous senile female gerbils under different types of hormonal replacement using morphometric, stereological and immunohistochemical techniques and to try to establish a relationship between hormonal serum levels in these groups. The proliferative ratio (PCNE/TUNEL) were higher in al multiparous groups when compared with nulliparous groups, mainly in SMT, and a high quantity of proliferative lesions were found in SM and SMT when compared with SN and SNT respectively.

Key words: hormonal replacement, female prostate, pregnancy, aging, histopathology

INTRODUCTION

The female prostate gland is located in female urethral wall and, like in males, show an pseudostratified columnar with all the cellular, enzymatic and stromals compounds that are necessary to its functionality (Custodio et al., 2004; Zaviačič et al., 2000). The human female prostate was first described by Reinier DeGraaf (1672), which showed its homology with male prostate. Besides was found focuses of prostatic adenocarcinoma in female prostatic samples by some researches (Sloboda et al., 1998; Svanholm et al., 1987; Zaviacic et al., 1993). Like in males, testosterone (T) is the main hormone that promotes morfofunctional development of gerbil female prostate (Santos et al., 2006).

Maintenance of the female and male prostate is under influence of Estrogens (E) and this gland presents activity of both estrogen receptors, ER-α and ER-β (Weihua et al., 2001). Some researchers have shown that this hormone can play a key role in prostate development and differentiation (McPherson et al., 2008). Studies performed by our research group suggest that the enlargement and proliferation of prostatic lesions can be promoted by E administration (Scarano et al., 2005; Scarano et al., 2004).

Dehydroepiandrosterone (DHEA) is the mainly steroid hormone produced by human adrenal cortex. Serum levels of DHEA and its sulfated conjugation product, DHEAsulfate (DHEAS), peak in men and women at 30 years old and decrease progressively and profoundly after this age (Orentreich et al., 1992). This hormone is widely available as an dietary supplement and is increasingly self prescribed for its alleged anabolic and anti-aging effects (Labrie et al., 1998).

Nuclear or membrane receptor for DHEA has not yet identified, and its mechanisms of action remain a subject of active investigation. DHEA has been shown to promote many of its effects via the androgen receptor (AR) and/or ER after its enzymatic conversion to androgen or estrogen (Labrie et al., 1998), although direct effects of DHEA on the AR and ER have also been demonstrated (Martin et al., 2004; Tan et al., 1997). Furthermore Liang et al. (2010) shows that progestins stimulated the growth of breast tumors independent of exposure timing and protocol, and stimulated vascular endothelial growth factor elaboration and increased tumor vascularity and also increased lymph node metastasis of BT-474 cells. And according to López-Otín and Diamandis (1998) pregnancy increases the risk of tumoral proliferation in breast tissue, a tissue that has some similarities with prostate including alterations in AR gene while others involved in hereditary breast cancer (BRCA1,BRCA2, p53 and RB1) are genetic abnormalities common to breast and prostate cancer.

This work was performed to quantify the incidence, multiplicity and percentage of acini affected by different lesions in prostate of nulliparous and multiparous senile female gerbils under different types of hormonal replacement using morphometric, stereological and immunohistochemical techniques and to try to establish a relationship between hormonal serum levels in these groups.

MATERIAL E METHODS

Animals and Experimental Design

Forty senile female gerbils (*Meriones unguiculatus*, Gerbilinae, Criscetidae) were housed under standard conventional conditions (25ºC , 40% – 70% relative humidity, 12 h light/12 h

dark) and allowed access to chow and water *ad libitum*. The vaginal smears was collected and used as an indicator of estrous cycle and animals in proestrus phase were used in these experiments (Nishino and Totsukawa, 1996). Animals were divided into eight groups (five animals each): senile nulliparous (SN) and senile multiparous (SM) female gerbils; senile nulliparous gerbils (SND) and senile multiparous gerbils (SMD) that received daily 0,1 ml of DHEA during 21 daysm(5mg/kg/day); senile nulliparous gerbils (SNE) and senile multiparous gerbils (SME) that received 0,1 ml of β-estradiol on alternate days during 21 days (2mg/kg/day); senile nulliparous gerbils (SNT) and senile multiparous gerbils (SMT) that received 0,25ml of T (1mg/kg/day) for 21 days.(adapted of Pollard and Luckert, 1987; Santos et al., 2006)

Experiments were performed according to the *Guide for Care and Use of Laboratory Animals.* Animals were weighed and sacrificed by compressed CO_2 inhalation. The ventral prostate was carefully dissected out and fixed.

Histology

Prostatic samples were fixed by immersion in Karnovsky's solution (5% parformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) for 24 h. After fixation, the material was washed with running tap water, dehydrated in an ethanol series, and embedded in glycol methacrylate resin (Leica historesin embedding kit, Leica, Nussloch, Germany) and sectioned at 3 μm on a Leica automatic rotatory microtome (Leica RM2155, Nussloch, Germany). Histological sections were subjected to Hematoxylin-Eosin (H&E)(Weeks and Woodhead, 1984) staining for general studies. Microscopic analyses were performed on Zeiss- Jenaval (Zeiss-Jenaval, Jena, Germany) or an Olympus BX60 light photomicroscope

(Olympus, Hamburg, Germany). Microscopic fields were digitized using the Image-Pro Plus, Version 4.5 for Windows software.

Immunohistochemical Analysis of Proliferating Cell Nuclear Antigen (PCNA)

Pretreatment of sections by heating in citrate buffer, pH 6.0 (using a pressure cooker), was performed and treated for 20 min in 3% H_2O_2 in methanol to block endogenous peroxidases. They were subsequently treated with a background sniper blocker to eliminate unspecific bindings (Biocare Medical, Concord, CA, USA), during 15 min. Sequentially, slides were incubated with the following primary antibodies (Santa Cruz Biotechnology, Palo Alto, CA, USA) diluted 1:100 in 1% BSA: mouse anti-human PCNA (sc-56, 1 h, at 37ºC). Primary antibodies anti-PCNA were detected by Polymer conjugated to peroxidase (Novolink Polymer; Novocastra, Norwell, MA, USA), for 40 min. The reactions were revealed with diaminobenzidine (0.03% in TBS) and sections were stained with haematoxylin. Negative controls were obtained by omission of the primary antibody.

Detection of apoptotic cells

Apoptotic cells were detected *in situ* using the DNA fragmentation assay associated to cell death based in TUNEL reaction (TdT-Fragel-Calbiochem; CN Biosciences, La Jolla, CA, USA), following the manufacturer's instructions. Briefly, after digestion with proteinase k (1:100 in 10 mM Tris pH 8.0) at room temperature for 23 min, slides were immersed in a solution of 3% H2O2 in methanol for 5 min to block endogenous peroxidases. In the next step, they were incubated with biotinilated TdT followed by enzyme deoxinucleotidyl terminal transferase (TdT), for 1 h at 37ºC. At the end of the reaction, the biotinilated nucleotides were detected by streptoavidin conjugated to peroxidase, and the reaction was revealed using diaminobenzidine

(0.07% in distillate water). Slides were finally stained with haematoxylin. Negative controls were obtained by omitting the incubation with TdT enzyme.

Morphometric-stereological analysis

Random prostatic areas from H&E sections were analyzed by Image-Pro Plus version 4.5 for Windows software. The morphometric-stereological analyses were carried out using Weibel's multipurpose graticulate with 120 points and 60 test lines (Weibel, 1978) to compare the relative proportion (%) of epithelium, lumen, non-muscular stroma, and smooth muscular cell. The obtained data were evaluated by analyses of mean ± standard error (SE).

Statistical analysis

Data were performed with Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA). The ANOVA and Tukey HSDs test were employed, and $p \leq 0.05$ was considered statistically significant.

Hormonal Dosage

Blood samples were collected from all animals by decapitation. Serum quantification of T, E and P was done using the Modular Analyzer for Immunoassay of Chemiluminescence ECi (Johnson & Johnson) in accord with Weeks and Woodhead (1984). For each treatment interval samples with a minimum of 3 animals were utilized. In each of these samples the test was done in triplicate.

Prostatic disorders quantification

Histological sections stained with H&E from animals of all experimental groups were randomly chosen. The lesions were classified according to Shappell and co-workers (2004) in addition to the Classification of Urinary System Tumors and Male Genital Organs from the World Health Organization - WHO (2004) Prostatic hyperplasia, microcalculi, prostate calculi and prostatitis were classified as benign lesions; high-grade prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) were classified as pre-malignant ones, and adenocarcinoma as malignancies.

The gland incidence (injury diagnosis/ sample) of lesion was achieved by identifying the different lesions in relation to the total sample number. The multiplicity (specific number of lesions/ sample) was calculated by the frequency of each lesion was identified in the histological section in relation to the total number of examined animals.

RESULTS

Morphological and morphometrical analysis

Senile nulliparous samples showed a characteristic architecture of prostatic tissue, with a simple columnar epithelium (Fig. 1a). In contrast, SM presented a taller epithelium in which an increased amount of proliferative lesions were identified (Fig. 1b) and these lesions almost always were associated with numerous blood vessels (Fig. 1b, inset).

Hormonal replacement leads, in all groups, to a taller epithelium, when compared with their control groups, with proliferative characteristics, like atypical nuclei (Fig. 1g, inset),

epithelial buds (Fig. 1c, inset) and increased number of clear cells (Fig. 1f, inset). In the treated groups the epithelium became height when compared with SN/SM, and besides secretor cells showed numerous apical vesicles (Fig. 1c and1e). These groups presented, in all almost cases, numerous crystalloids inclusions into the luminal compartment, called calculi (Fig. 1f).

SN presented mean epithelial height of 17.38 ± 2.45 µm ($p \leq 0.05$), in contrast with 22.93 ± 5.35 µm ($p \leq 0.05$) in SM. Furthermore, SND showed mean epithelial height of 22.86 ± 7.92 µm ($p \leq 0.05$), SNE of 20.29 ± 6,78 µm ($p \leq 0.05$) and SNT of 23.66 ± 8.89 µm ($p \leq 0.05$) in contrast with SMD that measured 26.85 ± 8.11 µm ($p \leq 0.05$), SME 22.33 ± 4.36 µm ($p \leq 0.05$) and SMT 27.48 ± 7.71 µm ($p \leq 0.05$).

Hormonal serum levels

The intergroup differences revealed by hormonal concentration in serum testosterone, progesterone and estrogen levels can be observed in Table 01.

Prostatic disorders quantification

In treated groups were found a large number of proliferative lesions (Fig. 1c-h). SMT showed the higher multiplicity of almost all types of lesions (Tab. 01). Dysplasia, hyperplasia, prostatitis and PIN was the mainly type of lesion found in SM and SMT groups and in both cases the ratio between PCNA positive cells and TUNEL positive cells were higher when compared with SN and SNT, respectively (Tab. 01).

Proliferative ratio

The proliferative ratio (PCNA/TUNEL) were higher in al multiparous groups when compared with nulliparous groups, mainly in SMT, the same group where were found higher multiplicity of proliferative lesions (Tab.01). The analyze of proliferate and apoptotic cells in the prostate epithelium revealed an increase in positive PCNA nuclei in treated groups (Fig. 2c-2h) when compared with not treated groups (Fig. 2a and 2b) and a decreased in positive TUNEL nuclei in treated animals (Fig. 2c-2h, inset; table 01) in relation with control groups (Fig. 2a and 2b, inset).

DISCUSSION

The high incidence of PCNA positive cells in SM in contrast with SN group can be relationship to expressive ratio between P to T and P to E levels. The serum levels of P remains higher in multiparous animals, while T and E remains in low level, when compared with nulliparous animals. Zapata-Benavides et al.(2009) showed that overexpression of PGr inhibit the action of PAR-4 protein, that is responsible to promote apoptosis process.

Prostate tissue is under steroids hormones influence to maintain its normal architecture and secretor function (Prins et al., 1991; Taplin and Ho, 2001; Weihua et al., 2001). The hormonal imbalance leads to several types of disruption in prostatic homeostasis and can promote the onset of epithelial proliferative lesions in female prostate (Kurita et al., 2001; Santos et al., 2008). Hormonal replacements are widely used in women after menopause, including T, E and DHEA supplementation (Barnhart et al., 1999; Burger et al., 1987; Buvat, 2003). The adrenal precursor steroids DHEA-S and DHEA can be transformed into androgens and/or estrogens in

peripheral target tissues depends upon the level of expression of the various steroidogenic and metabolizing enzymes in each cell of these tissues (Labrie et al., 2005), and this conversion can be responsible for the high serum levels of E in DHEA treated animals.

Epidemiological research demonstrates that higher endogenous androgen levels increase breast cancer risk in postmenopausal women (Schover, 2008), a tissue that has some similarities with prostate including alterations in androgen receptor (AR) gene while others involved in hereditary breast cancer (*BRCA1,BRCA2, p53 and RB1*) are genetic abnormalities common to breast and prostate cancer (Lopez-Otin and Diamandis, 1998). Androgens and estrogens hormones have crucial roles in certain disease states, particularly in mammary and prostate carcinomas (Aumuller et al., 1982; Brueggemeier et al., 2005; Cunha et al., 2001).

These hormones commonly used in hormonal replacement can be responsible to increase the risk of incidence of proliferative lesions in female prostate, which was seen in the multiparous female gerbils treated with hormones, that showed a high incidence of epithelial lesions like HGPIN that has a strong association with acinar-type prostatic adenocarcinoma. HGPIN and acinar-type prostatic adenocarcinoma both show similar molecular alterations, providing further evidence of their association (Dickinson, 2010).

The high incidence of proliferative lesions in senile multiparous groups seems to be related not only with high percentage of positive PCNA cell, but to with decreased of number of TUNEL positive cells that was higher in SM, which corroborates with Niu et al., (2008) that showed that mice lacking the prostate epithelial AR have increased apoptosis in epithelial luminal cells and increased proliferation in epithelial basal cells and with Un-no et al., (2007) that showed

that estrogen exposure in the neonatal period to Wistar rats decreases the number of ERb in the mature adult and accelerates cell proliferation.

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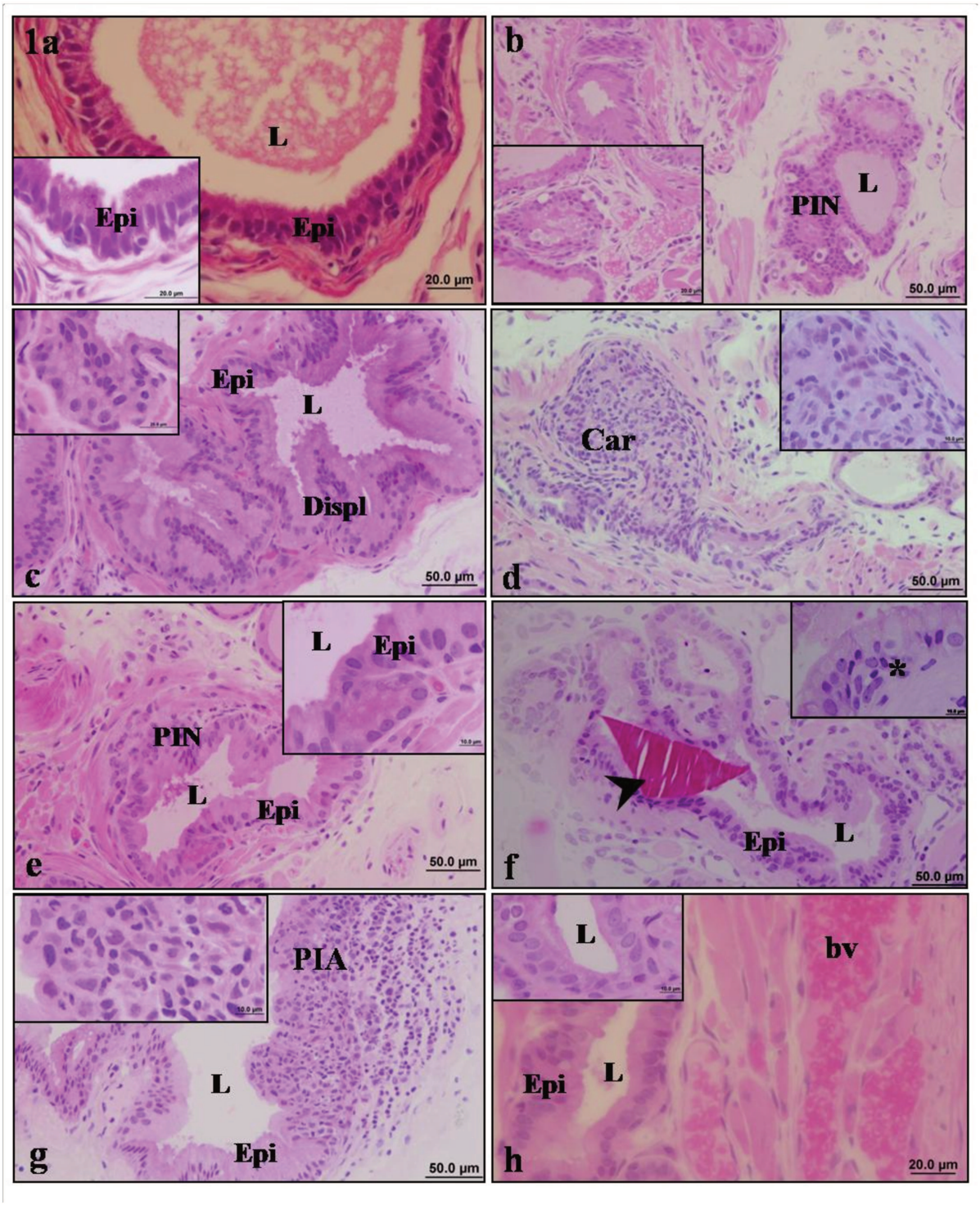
FIGURES CAPTIONS

Fig. 01: Hematoxilyn-eosin staining. a- SN; b- SM; c- SND; d- SMD; e- SNE; f- SME; g- SNT; h- SMT. Epi- epithelium; L- lumem; arrowhead- calculi; *- clear cell; PIN- prostatic intraepithelial neoplasia; Displ- displasia; Car- carninoma; PIA- proliferative inflammatory atrophy.

Fig. 02: Immunohistochemical staining to PCNA (insets- TUNEL). a- SN; b- SM; c- SND; d- SMD; e- SNE; f- SME; g- SNT; h- SMT. Epi- epithelium; L- lumem; arrowhead- positive stain nuclei.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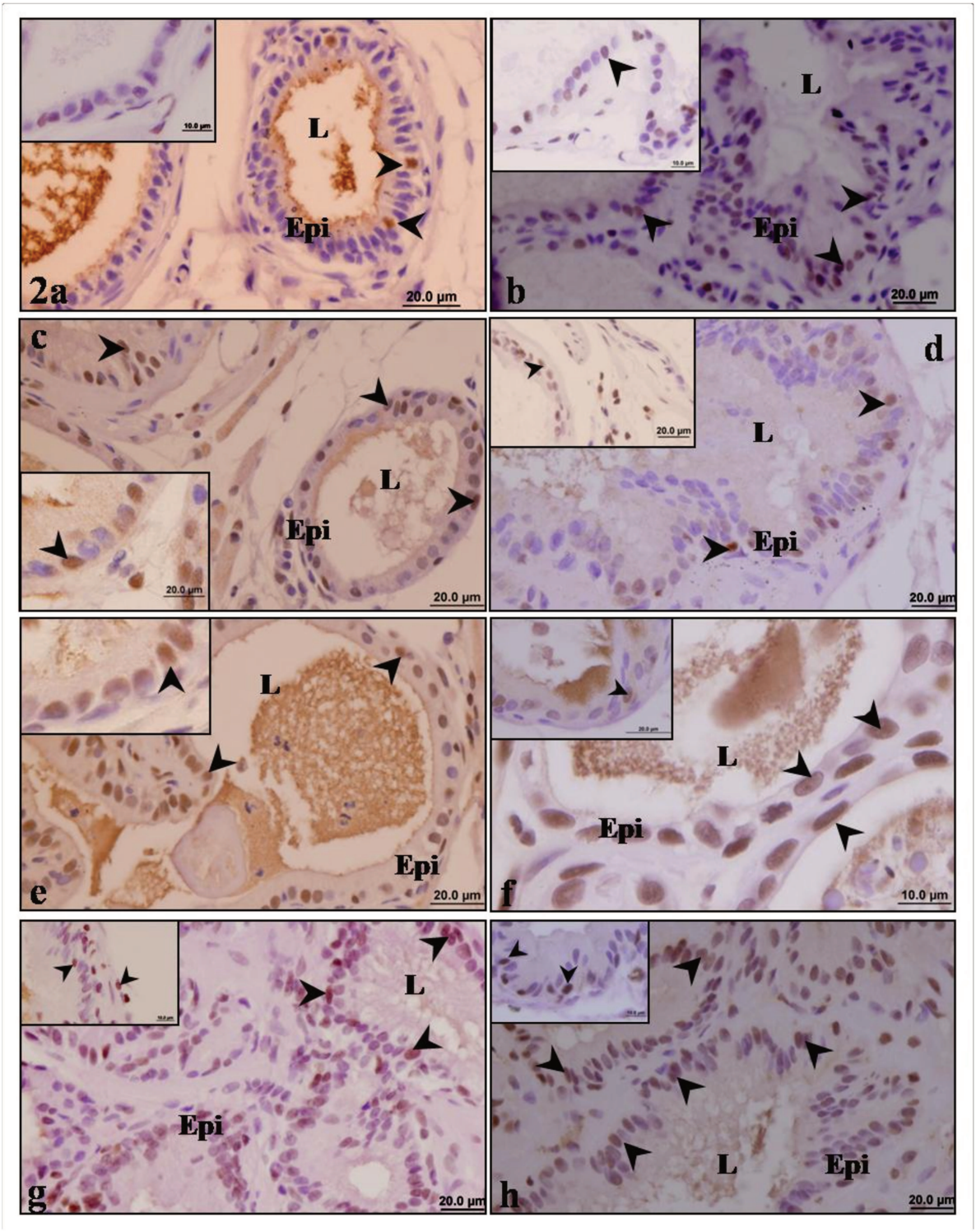
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Table 01: **Multiplicity - Mean ±St Err**

	Dysplasia	Hyperplasia	Prostatitis	Microcalculi	Calculi	PIN	PIA	Adenocarcinoma
SN	1.6 ±0.8^{abcdcfg}	0.6 ±0.3^{abcfg}	1.6 ±1.2^{abcdcfg}	0.3 ±0.3^{abcdeg}	1 ±0.6*	0^{abcg}	0^{abcdcfg}	0*
SND	3.3 ±1.8^{bacdcfgh}	2.7 ±0.8^{bacdcfg}	2.4 ± 0.8^{bacdcfgh}	0.3 ±0.3^{bacdcg}	1.3 ±0.3*	3 ±1^{bacdcfgh}	0.3 ±0.3^{bacdcfg}	0*
SNE	2 ±0.6^{cabdcfg}	4.3 ±0.6^{cabdfg}	3.6 ±1.7^{cabdcfgh}	0.3 ±0.3^{cabdeg}	1 ±0.6*	1.3 ±0.8^{cabdcfg}	0.3 ±0.3^{cabdcfg}	0*
SNT	3 ±0.6^{dabccfgh}	2.7 ±0.6^{dbccfgh}	3 ±1.8^{dabccfgh}	0^{dabcdcg}	0*	3.3 ±0.7^{dbcdcfg}	0^{dabccfg}	07 ±0.3*
SM	4.7 ±1.5^{ebcdfgh}	5 ±0.6^{ebdfgh}	1.7 ±0.3^{eabcdcfg}	1.3 ±0.3^{eabcdfg}	1.6 ±0.3*	4.3 ±0.8^{ebcdcfh}	0.3 ±0.3^{eabcdfg}	0.3 ±0.3*
SMD	2.3 ±1.4^{fabcdegh}	2.3 ±0.3^{fabcdeg}	1,3 ±0.6^{fabcdeg}	3.3 ±0.3^{feg}	0.7 ±0.3*	3.7 ±0.3^{fbcdegh}	0.3 ±0.3^{fabcdeg}	0.3 ±0.3*
SME	4 ±0.6^{gabcdcfh}	2.7 ±0.7^{gabcdcf}	4 ±1^{gabcdegh}	1.7 ±0.8^{gabcdcfg}	1 ±0.6*	1 ±0.6^{gabcdfg}	0^{gabcdcf}	0*
SMT	6 ±0.6^{hbdcfg}	6 ±0.6^{hdc}	5 ±0.6^{hbcdh}	4.7 ±0.8^{hf}	1.7 ±0.8*	5.7 ±0.3^{hbdef}	2.7 ±0.3^{h}	0.3 ±0.3*

Hormonal dosage after hormonal replacement

	SN	*SND*	*SNE*	*SNT*	*SM*	*SMD*	*SME*	*SMT*
PRG (Ng/ml)	0.94 (0.3)^{adch}	12 (1.8)^{bg}	24 (3)^{c}	2 (0.08)^{dach}	2 (0,5)^{eadh}	34 (16)^{f}	9 (0.5)^{gb}	1.53 (0.1)^{hadc}
TESTO (Ng/ml)	0.37 (0.02)^{afg}	0.59 (0.08)^{bc}	0.66 (0.05)^{cb}	11 (0.6)^{d}	0.06 (0.01)^{e}	0.32 (0.01)^{fag}	0.25 (0.01)^{gaf}	6.60 (0.05)^{h}
EST (Ng/ml)	0.02 (0)^{acdg}	0.11 (0.01)^{b}	- -	0.02 (0)^{eadg}	0.02 (0)^{dacg}	0.37 (0.2)^{e}	4.30 (0)^{f}	0.04 (0)^{gacd}
Hormonal Ratio	*SN*	*SND*	*SNE*	*SNT*	*SM*	*SMD*	*SME*	*SMT*
T/E	18	5.3	- -	582	3	0.88	0.1	182
T/P	0.4	0.05	0.03	4.66	0.03	0.01	0.02	6
E/P	0.022	0.01	- -	0.008	0.01	0.011	0.43	0.02
P/T	2.5	21	37	0.21	32	106	40	0.23
P/E	45	111	- -	125	92	93	2	42

Proliferative activity - % (SE)

	PCNA				***TUNEL***			
	SN	***SND***	***SNE***	***SNT***	***SN***	***SND***	***SNE***	***SNT***
Positive	4 (0.82)^{a}	30 (3.7)^{b}	68 (3.6)^{cd}	67 (1.8)^{dc}	19 (1.9)^{abc}	17 (1.5)^{bacd}	16 (1.5)^{cabd}	8 (1.7)^{dbc}
	SM	***SMD***	***SME***	***SMT***	***SM***	***SMD***	***SME***	***SMT***
Positive	63 (2)^{abcd}	72 (4.3)^{bacd}	78 (3.1)^{cabd}	77 (3.5)^{dabc}	36 (2.3)^{ab}	28 (3)^{ba}	15 (1.4)^{cd}	7 (2.9)^{d}

Proliferative Ratio	***SN***	***SND***	***SNE***	***SNT***	***SM***	***SMD***	***SME***	***SMT***
PCNA/TUNEL	0.21	1.76	4.25	8.37	1.75	2.57	5.2	11

- As fêmeas multíparas apresentaram uma maior incidência de lesões proliferativas, o que nos leva a concluir que o tecido prostático de fêmeas que passam por sucessivas gravidezes possuem maior propensão de serem acometidas por tais lesões.

- A reposição hormonal em fêmeas multíparas, seja com testosterona, com estrógeno ou com dehidroepiandrosterona é fator preponderante de aumento na incidência de lesões proliferativas quando comparado com as fêmeas nulíparas também submetidas às mesmas condições de reposição hormonal.

- O aumento na incidência de lesões proliferativas nas fêmeas multíparas se deve a um aumento do número de células em proliferação celular e diminuição do número de células em apoptose.

- A administração de DHEA promove um aumento na dosagem sérica de E devido à aromatização dos subprodutos esteroidais desse hormônio.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **"CONTROLE HORMONAL DA PRÓSTATA FEMININA DO GERBILO SOB INFLUÊNCIA DE MÚLTIPLAS PRENHEZES E REPOSIÇÃO HORMONAL"**:

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

(X) tem autorização da(s) seguinte(s) Comissão(ões) de Bioética ou Biossegurança*: CEEA-Comissão de Ética na Experimentação Animal – UNESP-BOTUCATU, sob Protocolo(s) nº 015/07.

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

Aluno: Sérgio Marcelino de Oliveira

Orientador: Prof. Dr. Sebastião Roberto Taboga

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

Nome:
Função:

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CERTIFICATE

We certify that the protocol nº 015/07 about *"Hormonal control of the gerbil's female prostate: Influence of multiple pregnancies and hormone replacement"* agree with ETHICAL PRINCIPLES IN ANIMAL RESEARCH adopted by Brazilian College of Animal Experimentation (COBEA) and was approved "Ad referendum" in that date by the BIOSCIENCE INSTITUTE/UNESP ETHICAL COMMITTEE FOR ANIMAL RESEARCH (CEEA).

Botucatu, march 30, 2007.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

NADIA JOVÊNCIO COTRIM
Secretária – CEEA

Disorders related with aging in the gerbil female prostate (Skene's paraurethral glands)

Alguns dados obtidos durante o desenvolvimento deste projeto foram utilizados na confecção de um artigo publicado em co-autoria no periódico *International Journal of Experimental Pathology.*

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ORIGINAL ARTICLE

Disorders related with ageing in the gerbil female prostate (Skene's paraurethral glands)

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Summary

The female organs, which are regulated by steroid hormones, are the targets of many studies and in particular those related to senescence. However, although the female prostate is an organ influenced by hormones and susceptible to pathological lesions, there is little information known about its histopathology. Thus, given the morpho-physiological similarity between the paraurethral glands (female prostate) in women and female gerbils, the present study aimed to identify the spontaneous histopathological changes in the rodent and thus to contribute to the understanding of lesions that also affect the human female prostate. The structural, ultrastructural, immunohistochemical, morphometric-stereological and serological aspects, were analyzed and the incidence, multiplicity and percentage of acini affected by different lesions were quantified. Benign prostate lesions including hyperplasia, prostatitis, microcalculi and calculi; preneoplastic lesions like dysplasias; premalignant lesions such as high grade prostatic intraepithelial neoplasia, as well as malignant ones (specifically adenocarcinoma), were identified in the adult gland. They were intensified during senescence, which is possibly due to the imbalance among steroid hormone levels. Although clinical attention focuses on other urogenital organs, the real possibility of histopathological lesions in the human female prostate should be considered. Preventive work with regard to the female prostate might be applied in a gynaecological context in order to monitor the gland and avoid possible disturbances to women's health, and thus improve quality of life.

Keywords
ageing, female prostate, histology, steroids

The ageing process is accompanied by a natural decrease in endocrine activity and a concomitant physiological decline that favours the development of histopathologies in the organism. According to Chahal and Drake (2007), glands suffer the effects of ageing. Since most of their functions are interrelated, a reduced function of a particular one could affect the others.

The functioning of the female genital apparatus during the climacteric period is a subject of tremendous concern

because of its hormonal dependence. However, these studies focus on changes in many organs, such as the breast, endometrium, ovaries and the uterus (Labrie 2006; Yeh 2007), whereas knowledge on the prostate during that period is scarce (Zaviačič 1999; Custodio *et al.* 2004, 2008).

The occurrence of the female prostate has been reported in several mammals, including humans (Zaviačič 1999) and rodents (Shehata 1980) while its morphophysiological (Gross and Didio 1987; Zaviačič 1999; Santos *et al.* 2003; Custodio *et al.* 2004, 2008) histochemical-enzymatic and immunohistochemical aspects (Tepper *et al.* 1984; Wernet *et al.* 1992) show a similarity to the male prostate.

Researches related to this gland in women are restricted to *postmortem* collection, which reduces relevant knowledge and hinders the study of spontaneous injuries. But the use of rodents as experimental models enables understanding of the biology in this gland, corroborated by quantitative and physiological studies, as well as experiments on hormonal manipulation already published (Santos *et al.* 2003, 2006, 2008; Custodio *et al.* 2004, 2008; Santos & Taboga 2006). The Mongolian gerbil (*Meriones unguiculatus*) has become an important biological model for studying the female prostate because of its similarity to the human gland. Thus, the present study on the gerbil female prostate fills a gap in the knowledge of the pathological processes that spontaneously develop in this gland and may provide possible contributions to the understanding of injuries that affect this gland in women.

Material and methods

Animal and sample preparations

Forty-five female gerbils (*Meriones unguiculatus*, Gerbilinae: Muridae) were used for this analysis, with 15 animals used for each phase of postnatal development: young (1 month), adult (4 months), and senile (18 months). The animals were maintained under conventional conditions of temperature and humidity (25 °C, 40–70% relative humidity, 12-h light/12-h dark), with free access to chow and water. After being anesthetized by CO_2 inhalation, the animals were decapitated. Blood samples from some of them were collected for serological analysis. The urethra plus adjacent tissues were dissected out using an Olympus SD-ILK stereoscopic microscope (Olympus Optical Co. LTD, Tokyo, Japan) to remove the adipose tissue and isolate the prostatic tissue plus the associated urethral segment. The separation of these components was performed by sectioning it at the base of bladder to obtain a block containing the entire Tokyo, urethra and prostate gland (UPG).

Animal care was performed according to the ethical guidelines of the Commission for Ethics in Animal Experimentation (CEEA) at the University of Campinas (UNICAMP), São Paulo, Brazil (process Nº 1213-1).

Serological analysis

After the animal's decapitation, blood was colleted and the serum was separated by centrifugation (300 ***g***) and stored at −20 °C for subsequent hormone assay. The determination of serum T levels was performed by luminescence immunoassay (mouse antitestosterone antibodies; Johnson & Johnson, Orthoclinical Diagnostics Division, Rochester, NY, USA) in an automatic analyzer: Vitros-ECi (Johnson & Johnson, Orthoclinical Diagnostics Division) for ultrasensitive chemiluminescence detection. The intraassay and interassay variations were 4.6 and 4.3% respectively. The tests are linear from 0 to 30 ng/ml (detection level). The sensitivity was 0.1–150 ng/ml for T and 0.1–3.814 pg/ml for E and for DHEA.

Structural analysis

The UPGs were fixed by immersion in Karnovsky's solution, or in 4% paraformaldehyde, during 24 h. After fixation, the tissues were dehydrated in ethanol gradient cleared in xylene, embedded in paraffin (Histosec, Merck, Darmstadt, Germany) or glycol methacrylate resin (Historesin embedding kit, Leica, Nussloch, Germany), and cut into 3 μm sections with a automatic rotatory microtome (Leica RM2155). Histological sections were stained with haematoxylin–eosin (H&E), Gömöri's reticulin, Feulgen reaction and AgNOR method. The specimens were analyzed with a Zeiss-Jenaval (Zeiss-Jenaval, Jena, Germany) or Olympus BX60 light microscope (Olympus, Hamburg, Germany), and the images were digitalized using the Image-Pro Plus version 4.5 for Windows software.

Immunocytochemistry analysis

Sections of 4% paraformaldehyde-fixed female prostates were subjected to immunocytochemistry for detection of Proliferating Cell Nuclear Antigen (PCNA). For immunohistochemical analysis, the sections were deparaffinized, rehydrated through graded alcohol, and antigen retrieval was performed in 10 mM citrate buffer pH 6.0, at 100 °C for 15 min. The blockade of endogenous peroxidases was obtained by covering the slides with H_2O_2 (3% in methanol) for 5 min. After pretreatment, the sections were incubated for 2 h at 37 °C with mouse anti-mouse PCNA antibody

(1:1000 Santa Cruz Biotech, Santa Cruz, CA, USA) diluted in 1% bovine serum albumin in Tris-buffered saline (TBS). After the slides were washed in TBS and incubated for 40 min at 37 °C with NovoLink Polymer (Novocastra Laboratories, New Castle, UK). After more washing in TBS, the sections were visualized by diaminobenzidine (DAB) solution and then counterstained with routine haematoxylin.

Ultrastructural analysis

The UPG fragments were fixed by immersion in 3% glutaraldehyde plus 0.25% tannic acid solution in Milloning's buffer, pH 7.3, containing 0.54% glucose for 24 h. After washing with the same buffer, they were postfixed with 1% osmium tetroxide for 2 h, washed again, dehydrated in graded acetone series, and embedded in Araldite resin. Ultrathin sections (50–75 nm) were contrasted with 2% uranyl acetate followed by 2% lead citrate in sodium hydroxide solution. The samples were evaluated with a LEO-Zeiss 906 (Zeiss, Cambridge, UK) transmission electron microscope operated in 80 kV.

Morphometric analyses

Nuclei of epithelial cells, stained by AgNOR method, with the following numbers of nucleoli: zero (not detectable), one, two, and more than two were counted in 25 random fields selected by age. The nucleolus number obtained was divided by totally nucleus analyzed in the respective fields. The absolute values found were converted into percentages.

Quantification of the prostatic disorders

To perform this analysis, histological sections stained with H&E from 10 adult and senescent animals were randomly chosen. Young animals were not subjected to such measurements since they did not exhibit any kind of lesion and were considered as control young group. The lesions were classified according to Shappell *et al.* (2004) in addition to the Classification of Urinary System Tumors and Male Genital Organs from the World Health Organization – WHO (2004). Thus, lesions classified as benign were prostatic hyperplasia, microcalculi, prostate calculi and prostatitis; the premalignant ones were high-grade prostatic intraepithelial neoplasia (PIN) and malignancies were adenocarcinomas. The more intense disorders of tissue architecture were identified as dysplasia. Although PIN can be classified by the WHO (2004) as low or high grade, and both have been identified in the gland, only the latter was quantified.

The incidence (injury diagnosis/sample) of lesion in the gland was obtained by identifying the different lesions in relation to the total sample number, while the multiplicity (specific number of lesions/ sample) was calculated by the frequency each lesion was identified in the histological section in relation to the total number of examined animals. The percentage of lesions per acinus was determined by the number of acini that developed lesions in relation to the total number of acini in the histological section. The acinar profile was indicated by the number of acini identified in each histological section in relation to the total number of acini in the whole group sample.

Statistical analysis

All the statistical tests were performed with Statistica 6.0 software (StarSoft, Inc., Tulsa, OK, USA). The quantitative results are expressed as mean ± standard deviation, and the analysis of variance and Tukey honest significance difference (HDS) tests were applied, with $P \leq 0.05$ was considered statistically significant.

Results

Evaluation of the hormonal serum levels

The adult age group presented higher serum levels of DHEA, T and E than the other ages, and during senescence the levels of DHEA and T showed significant reduction (Table 1). The rations between the levels of T and DHEA during all

Table 1 Serological dates of the young, adult and senile female Mongolian gerbil (n = 15/age)

Serological data			
	Ages		
Hormones	**Young**	**Adult**	**Senile**
Dehydroepiandrosterone (DHEA) (ng/ml)*	2.16 ± 0.1[a]	2.66 ± 0.3[a]	1.52 ± 0.1[b]
Testosterone (T) (ng/ml)*	0.60 ± 0.08[a]	1.48 ± 0.5[a]	0.18 ± 0.05[b]
Oestrogen (E) (pg/ml)	22.50 ± 1.6	25.02 ± 2.9	18.56 ± 2.0
T/E ratio	0.027 ± 0.005	0.06 ± 0.1	0.01 ± 0.028
T/DHEA ratio	0.28 ± 0.78	0.56 ± 1.6	0.12 ± 0.35

Values represent mean ± SD and asterisks represent statistically significant differences between the ages ($P \leq 0.05$). Superscript letters ([a,b]) represent statistically significant differences between the ages. Statistical analysis based on the ANOVA and Tukey Tests. Ratio between the serum levels of T/DHEA and T/E in relation to the ages of postnatal development is showed (Mean ± SD).

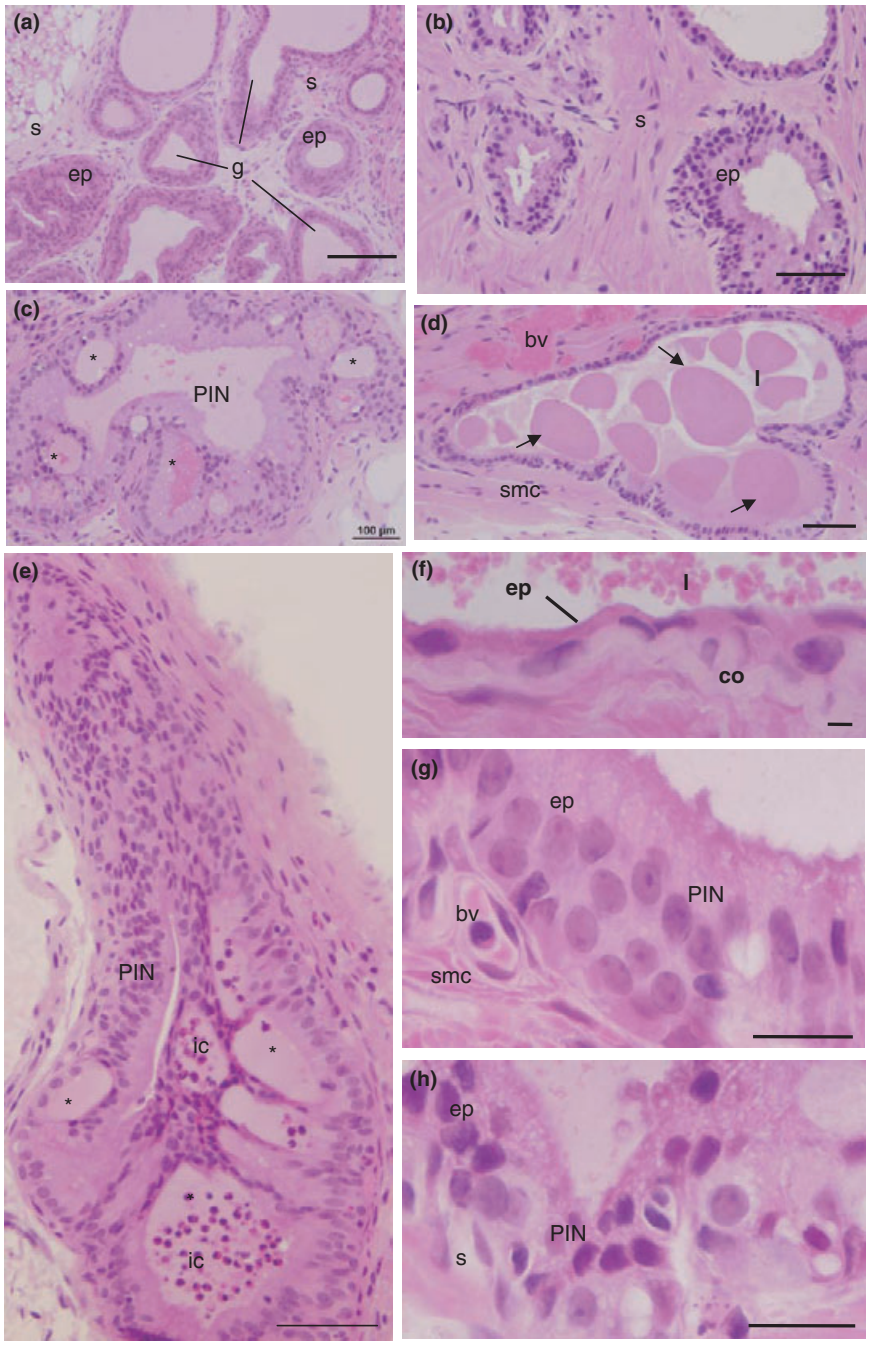

Figure 1 (a) Structural aspects of the control young female prostate. General view of the gland (*g*) with epithelium compartment (*ep*) inserted in the fibromuscular stroma (*s*) H&E. *Bar* 200 µm. (b–h) Histopathological aspects of the female prostate disorders in old gerbil stained by H&E. (b) Gland general aspect with epithelial hyperplasia. Stratified secretory epithelium (*ep*) inserted in dense stroma (*s*). *Bar* 100 µm. (c) General view of an acinus with intraepithelial neoplasia (*PIN*) containing several intraepithelial arcs (*). *Bars* 200 µm. (d) Alveolus with dysplasia and prostatic calculi (*arrows*) scattered although lumen (*l*) (*smc* smooth muscular cells, *bv* blood vessel). *Bars* 200 µm. (e) General view of an acinus with intraepithelial neoplasia (*PIN*) containing several intraepithelial arcs (*). To this lesion is associated inflammatory cells (*ic*). *Bars* 100 µm. (f) Detail of the gland showing the atrophic epithelium with extremely flat cells and microcalculi disperse throughout the lumen (*l*) (*co* collagen). *Bar* 50 µm. (g–h) Epithelial compartment (*ep*) proliferated showing prostatic intraepithelial neoplasia (*PIN*) and atypical cells. *Bar* 10 µm.

the ages of postnatal development were ten times higher that the ratios levels of T and E, however both hormonal rations were highlighted in adulthood (Table 1).

Structural and ultrastructural analysis

The control young group showed a developed epithelial compartment inserted in vascularized and innervated fibromuscular stroma (Figure 1a). All animals of this experimental group had no histopathological disorders. Continuous reticular fibre arrangements of the stroma were observed in intimate contact with epithelium (Figure 2c). Although some injuries were identified in the adult gland, the histological documentation was restricted to the senescent gland due to the higher lesion incidence found during this age. The structural analyses of senescent

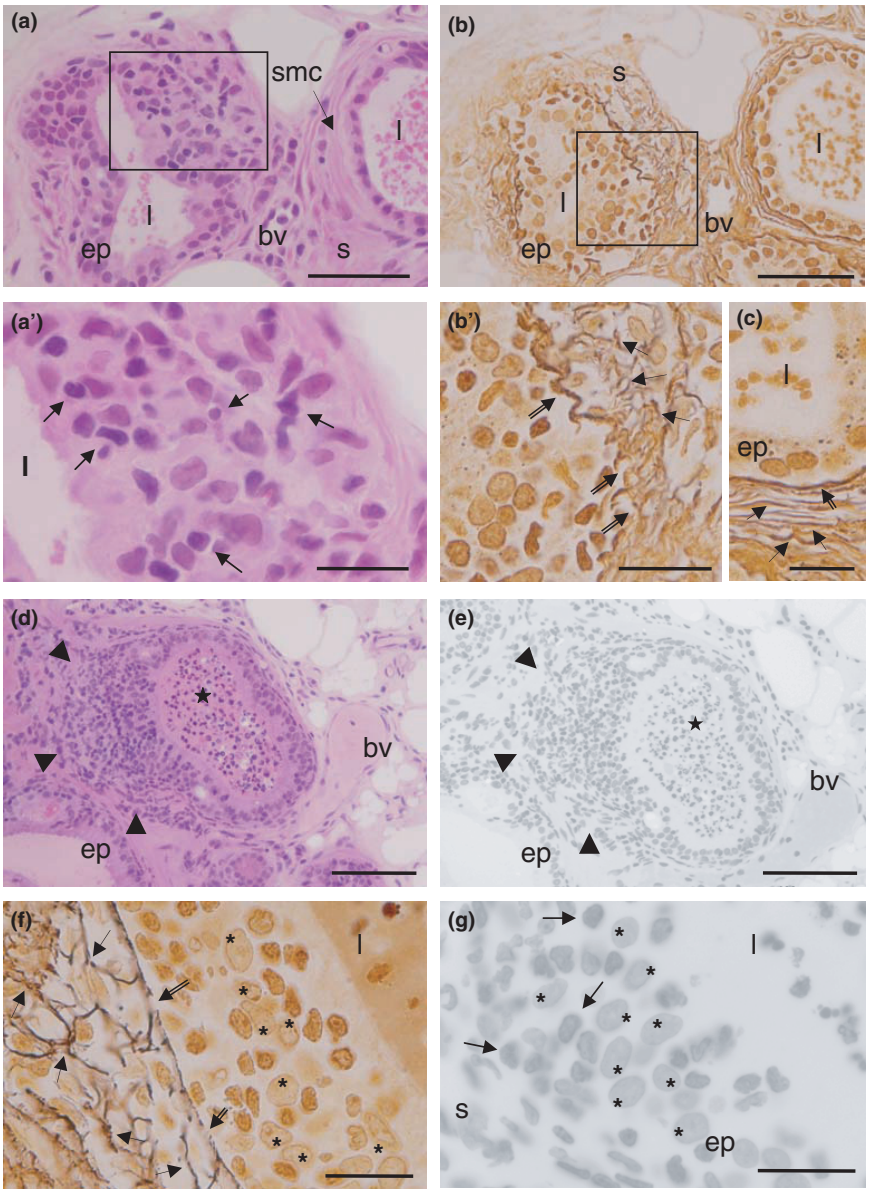

Figure 2 Histopathological aspects of the female prostate in old gerbil. (a) Gland alveolus showing morphologic disarrangement and cellular proliferation (*left*) distinguishing the adenocarcinoma contrasting with normal alveolus (*right*) (*ep* epithelium, *l* lumen, *s* stroma, *smc* smooth muscular cells, *bv* blood vessel). H&E. *Bar* 100 μm. (a') Detail of anomalous cell proliferation with inflammatory cells (*arrows*). *Bar* 20 μm. (b) Disarrangement of the reticular fibre (*left*) distinguishing the adenocarcinoma contrasting with normal one (*right*) (*ep* epithelium, *l* lumen, *s* stroma, *bv* blood vessel). Gömöri's stain. *Bar* 100 μm. (b') Detail of the fragmentation reticular fibres network in the basal lamina (*double arrows*) and stroma (*arrows*). *Bar* 20 μm. (c) In the normal acini, continuous basal lamina (*double arrows*) and organized reticular fibres network (*arrows*) is observed (*l* lumen, *ep* epithelium). Gömöri's stain. *Bar* 20 μm. (d) Anomalous epithelium cells proliferated with local invasion to stroma (*arrowheads*) associated with inflammatory cells distinguishing the adenocarcinoma of the gland *(asterisk* prostatitis, *ep* epithelium, *bv* blood vessel). H&E. *Bar*100 μm. (e) Adenocarcinoma of the gland stained by Feulgen reaction. (*arrowheads* epithelium cells proliferated, *asterisk* prostatitis). *Bar* 100 μm. (f) Detail of the base's epithelium, stained by Gömöri's stain, where can observe disrupted subepithelial (*double arrows*) reticular fibres and in the stroma (*arrows*) (*asterisk* epithelium cells). *Bar* 20 μm. (g) Detail of adenocarcinoma stained by Feulgen reaction showing different intensity of reaction in the chromatin. Note that dark inflammatory cells (*arrows*) and clear epithelial tumour cells (*asterisk*) (*ep* epithelium, *s* stroma). *Bar* 20 μm.

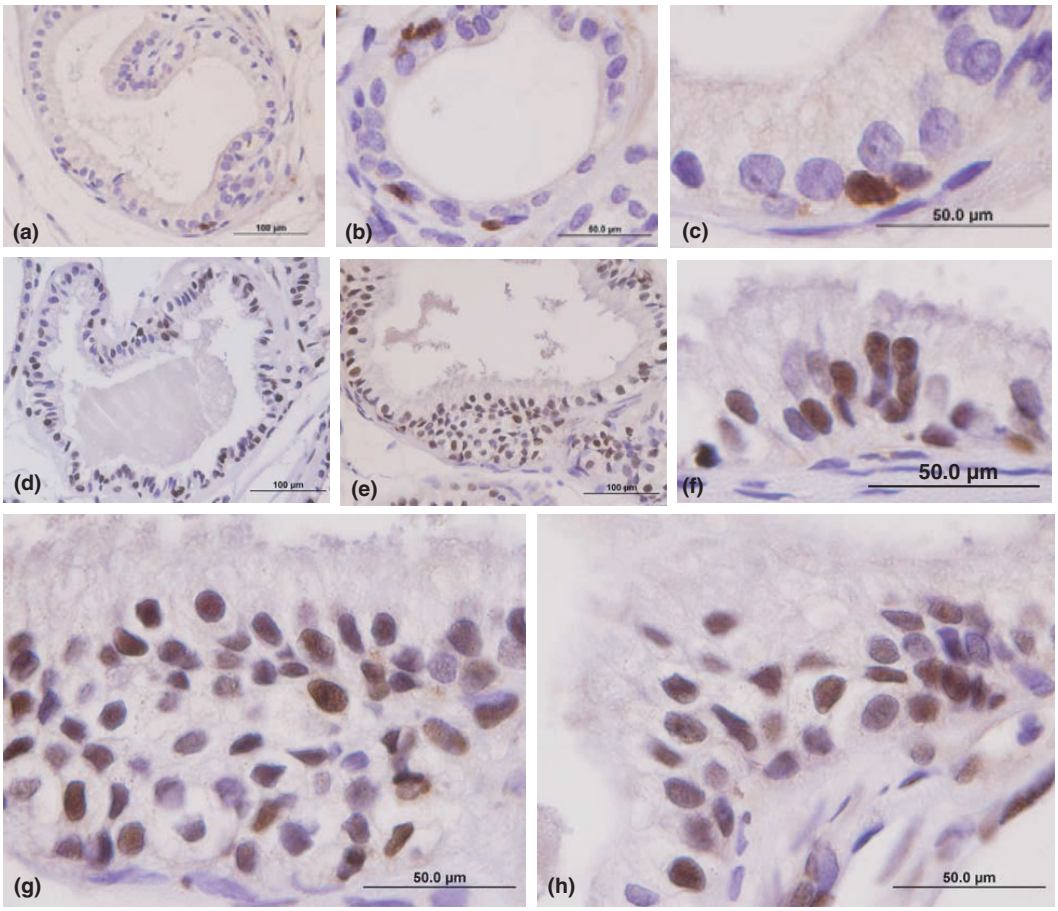

Figure 3 PCNA immunocytochemistry counterstained by haematoxylin. (a–c) Epithelial compartment of control female prostate free of the histopathology disorders. Demarcation of nuclear proliferation is very low in normal epithelium (immunoreactive nucleus is brown). *Bars:* (a) 200 µm, (b) 100 µm, (c) 50 µm. (d–h) Hyperplasic secretory epithelial compartment with large number of positive PCNA cells. *Bars:* (d,e) 200 µm, (f–h) 50 µm.

prostate exhibited important morphological alterations (Figures 1b–h and 2a–g). The epithelium and stromal compartment showed a cellular and stromal hyperplasia (Figure 1b). Prostatic calculi (Figure 1d) and multiples microcalculi (Figure 1f) were observed disperse throughout the lumen. Some alveoli had lost their structural organization showing the glandular dysplasia (undocumented data). Atypical cells observed in proliferating epithelium too showed nucleus with more that one nucleolus being identified premalignant lesions, like the high grade PIN (Figure 1c, e, g, h). Inflammatory cells were showed in the epithelium and lumen (Figures 1e and 2d,e), which in the latter a prostatitis was identified. The anomalous cell proliferations with disarrangement of the reticular fibres was an important tool for distinguishing an adenocarcinoma of the gland (Figure 2b,f).

The immunohistochemical analysis reveal that the normal epithelium of the female prostate presented few cells immunoreactive to PCNA reaction (Figure 3). While in glands that showed some disorders, such as hyperplasia, the number of cells positive PCNA was remarkable.

The ultrastructural analyses of the senile prostate (Figure 4a–d) showed a typical atrophic epithelial cells (Figure 4a) but its endomembrane system remained integrity with secretory organelles few developed. The formation of the intraepithelial arches (Figure 4b) and microlumens (Figure 4c) showed the disarrangement in the glandular ultrastructure. A dense stroma characterized by thick collagen layer was found in the subepithelial region (Figure 4a) and also between stromal cells such as fibroblasts and smooth muscle cells (Figure 4a). A continuous basement membrane limited the epithelial compartment in benign

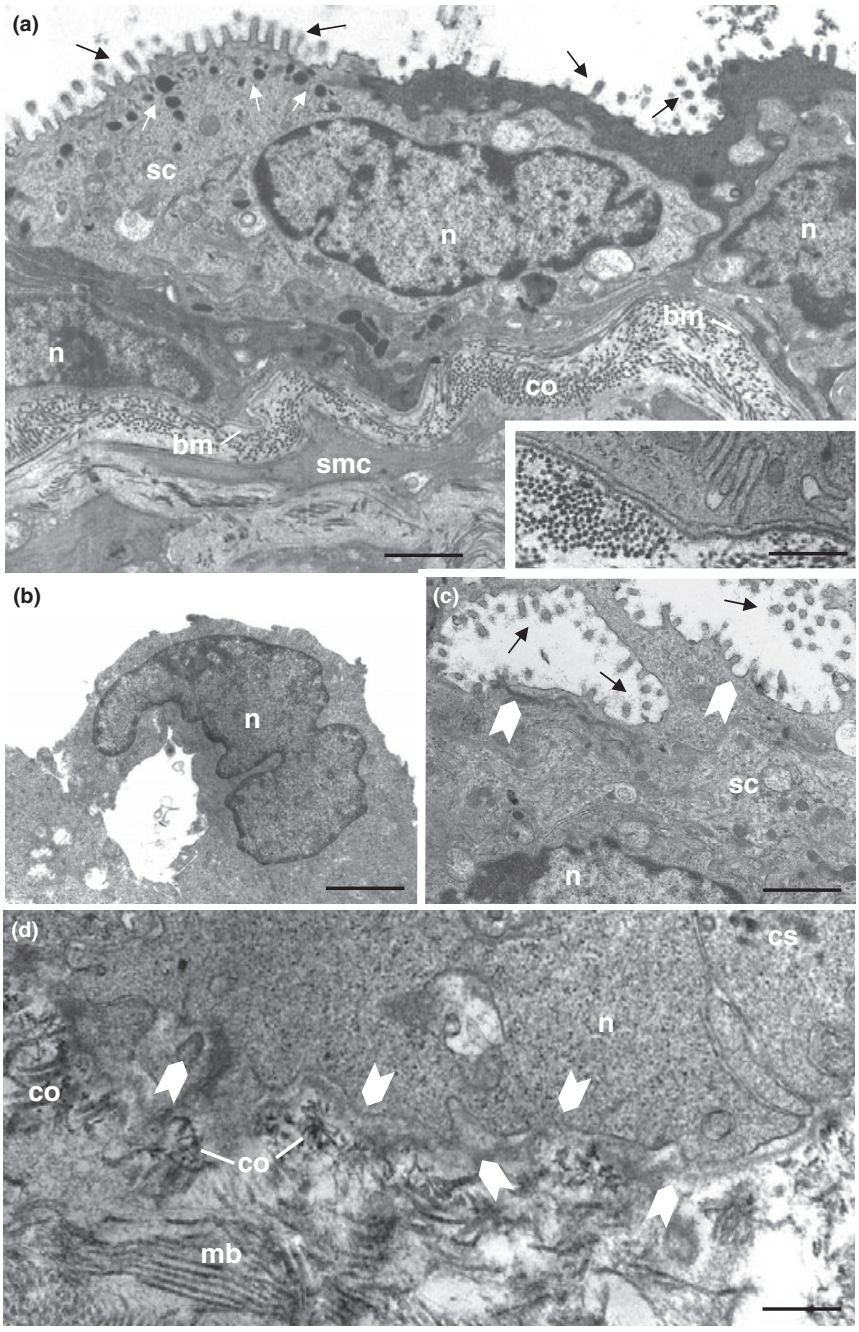

Figure 4 Ultrastructural aspects of the senile female prostate exhibiting disorders histopathology. (a) Squamous epithelium constitutive by secretory cells (*sc*) of heterogeneous phenotype and voluminous nucleus (*n*), with lipid droplets (*white arrows*) dispersed although cytoplasm and microvillous (*black arrows*) in its apical superficies. Dense stroma containing abundant collagen fibres (*co*) and smooth muscular cells (*smc*) which are below of the continuous basement membrane (*bm*). *Bar* 5.5 μm. *Inset:* Detail of the basal lamina showing the ultrastructural integrity. *Bar* 1.0 μm. (b) Detail of the intraepithelial arcs in early stage (*n* nucleus). *Bar* 1.86 μm. (c) Microlumens (*full arrows*) characteristics of the PIN high grade with cribriform pattern (*sc* secretory cell, *n* nucleus, *dark arrows* microvilous). *Bar* 0.7 μm. (d) Shown are fenestrations (*full arrows*) in the basement membrane (*bm*) allowing the contact between epithelial and stromal compartments designating a possible malignant disorder (*n* nucleus, *co* collagen). *Bar* 0.7 μm.

lesion, (inset of Figure 4a), while the disruption in this structure (Figure 4d) probably allowed epithelial cells to spread into the stromal region, which characterized malignant lesions of the senile prostates.

Analysis of nucleoli

The nucleoli of secretory epithelial cells in young prostates varied significantly, and in adulthood their frequency had dropped by almost 50%. The adult epithelial cells showed a significant rise in the number of nuclei without detectable nucleoli, which, moreover, were absent in senescence, the phase during which nuclei with a single nucleolus were significantly decreased. Those nuclei with two nucleoli were most abundant in adult cells, reducing significantly in senescent prostatic epithelium. The number of nuclei showing more than two nucleoli was reduced among adults, but rose significantly during senescence (Table 2).

Table 2 Percentage distribution of the nucleolus number by nuclei in secretory epithelial cells of the female prostate in young, adult and senescent ages

	% of the nuclei containing each number of nucleoli				
	Number of nucleoli in each nucleus				
Ages	0* (not detectable)	1*	2*	More than 2*	Average number of nucleoli in group*
Young	3.41 ± 1.45[a]	5.85 ± 1.52[a]	7.63 ± 1.82[a]	83.09 ± 3.68[a]	38.79 ± 3.35[a]
Adult	9.11 ± 1.98[b]	9.74 ± 2.67[a]	12.56 ± 2.75[a]	68.57 ± 5.34[b]	20.96 ± 1.32[b]
Senile	0.00 ± 0.00[a]	2.10 ± 1.27[b]	0.83 ± 0.58[b]	97.05 ± 1.69[c]	31.32 ± 2.23[a]

Values represent mean ± SD and asterisks represent statistically significant differences between the ages ($P \leq 0.05$). Superscript letters ([a,b,c]) represent statistically significant differences between the ages. Statistical analysis based on the ANOVA and Tukey Tests.

Table 3 Quantification of the prostatic disorders: lesion incidence and multiplicity, lesion percentage per acinus and acinar profile inter-ages

	Female prostate lesions							
	Benignant						Premalignant	Malignant
	Alveolus profile	Dysplasias*	Hyperplasias	Prostatitis	Prostatic micro calculi	Calculi	High-grade PIN	Adeno-carcinoma
Adult								
I (%)		20	70	10	30	30	30	10
M*	12.0 ± 1.81	0.2 ± 0.13[a]	2.1 ± 0.87	0.3 ± 0.3	0.5 ± 0.30	0.6 ± 0.33	0.6 ± 1.07	0.1 ± 1.0
% Lesions*		2.67 ± 1.79[a]	16.7 ± 4.63	1.30 ± 1.30	3.25 ± 1.81	4.16 ± 2.13	5.11 ± 3.10	1.0 ± 1.0
Senile								
I (%)		90	100	20	30	30	20	20
M*	17.5 ± 2.57	1.9 ± 0.54[b]	4.2 ± 0.98	0.4 ± 0.30	1.4 ± 0.85	0.5 ± 0.26	1.6 ± 1.07	0.3 ± 0.21
% Lesions*		10.28 ± 1.90[b]	23.38 ± 3.94	2.08 ± 1.42	9.27 ± 6.41	3.47 ± 2.02	6.51 ± 4.37	1.74 ± 1.16

Values represent mean ± SD and asterisks represent statistically significant differences between the ages ($P \leq 0.05$). Superscript letters ([a,b,c]) represent inter-ages significant differences. Statistical analysis based on the ANOVA and Tukey Tests.

Quantification of the prostatic disorders

During the ageing process, there was an increase in the multiplicity of all lesions, except for prostatic calculi. For dysplasias, multiplicity was found to be 10 times higher in senescent prostates than in adult ones. Prostatic hyperplasia doubled its multiplicity, while microcalculi, PIN and adenocarcinoma were three times more frequent. The percentage of lesion per acinus showed a significant augmentation during ageing, while this increase was not significant in hyperplasia, prostatitis, microcalculi, PIN and adenocarcinoma. An elevation in the acinar profile was observed between adulthood and senescence (Table 3), but this change was not statistically significant.

Discussion

According to Santos and Taboga (2006), besides the biological implications related to this gland, the main focus in the female prostate emanates from its capacity to develop severe lesions during senescence. However, Zaviačič (1999) reported that many previous pathological diagnoses have been imprecise. These diseases have been referred to as urinary tract disorders and not as prostatic ones, due to the acceptance of the vestigial concept and disbelief in the importance of this gland to women's health. Studies on the gerbil prostate indicated that, apart from the early functionality presented by the female gland in relation to the male one (Custodio *et al.* 2004), discrete morphological changes were identified from adulthood (Custodio *et al.* 2008). In males, on the other hand, this occurs only in senescence (Pegorin de Campos *et al.* 2006).

The major proliferative disorder evaluated in the present study was epithelial and stromal hyperplasia. This process led to the glandular increase, evidenced by acinar expansion. The consequence was observed in some epithelial metaplasia regions. Folson and O'Brien (1943) reported that this disorder is very frequent in women but little recognized and

treated, and it should be included among the lower urinary tract symptoms, similar to those observed in men. This disorder causes obstruction and urinary retention as well as a greater injury of the urogenital system, as has been proven at autopsy (Zaviačič 1999). As well as in man, the prostatic hyperplasia is characterized by progressive glandular and stromal hyperplasia around the urethra, causing urodynamic obstructions (Untergasser *et al.* 2005). Marcelli and Cunningham (1999) reported that the cellular dynamic caused by increased number of cells, related to high proliferation and low apoptosis, is one of the consequences of lumen enlargement in the prostate.

Starting from adulthood the incidence, multiplicity and percentage of prostatic hyperplasia per assinus were expressed. However, impairment occurred in senescence, during which all the females expressed this lesion, thus strongly relating it to age. The same is observed in the male prostate and advanced age is one of the risk factors for the development of this disorder (Carson & Rittmaster 2003; Untergasser *et al.* 2005). Calculi, also diagnosed in the prostate, were classified according to their size. Small structures, with crystalloid aspect, scattered in the luminal acini were identified as microcalculi, while the real prostatic calculi had larger dimensions and occupied most acini. The incidence of both injuries showed no changes with ageing, but the multiplicity of microcalculi doubled and calculi showed a slight decrease. The calcification of the corpora amylacea or the precipitation of prostatic secretion may be the responsible for the formation of these structures that contribute to the symptoms of lower urinary tract diseases (Klimas *et al.* 1985). Corpora amylacea and prostatic calculi contain salts of magnesium and potassium as well as calcium phosphate, calcium carbonate and calcium oxalate that usually are found in male benign prostatic hyperplasia (Geramoutsos *et al.* 2004). However, in the female gerbil, these concomitant lesions were less frequent. These structures can obstruct ducts and acini leading to inflammatory reaction that can cause abscesses.

An increase in the incidence and multiplicity of inflammatory infiltrates also occurred along with ageing. This benign lesion was identified in the lumen as well as in the interstices around the acini and was sometimes associated with adenocarcinoma. Zaviačič (1999) reported that, for a long period, these human female prostate infections were known as female urethral syndrome and were treated as urethral diseases. After the importance of this gland became known, the infection, which is similar to the male one, was called prostatitis and thus, appropriate therapeutic strategies began. Currently, it is well established that the most common infection of the female urinary tract, cystitis, originates in the prostate on account of communication among the prostate, the anterior wall of the urethra and the vagina. Thus, when inflammation of this organ occurs (prostatitis or Skenitis), the infection can spread throughout the female reproductive tract, constituting the well known urethro-prostato-cystitis.

Another lesion observed in the female prostate was characterized by an anomalous proliferation of the epithelial cells which were PCNA positive. Although this disorder was observed in few animals, it was impaired with ageing since its multiplicity tripled compared to adulthood. Such altered cell proliferation is defined as a premalignant lesion (Lippman & Hong 2002) known as prostatic intraepithelial neoplasia (PIN) and classified as low or high grade (WHO 2004) due to its complex architecture and morphological cell abnormality. However, according to the WHO (2004), it is difficult to distinguish the low grade PIN from normal epithelium and hyperplasia. Moreover, in clinical reports, this type of cell proliferation can progress, but it may not signify a potential lesion to the gland. Thus, despite low-grade PIN having been identified in this study, only the high grade PIN was analyzed. These lesions are heterogeneous and consist of large cells with prominent nucleoli. According to the cellular arrangement, it is possible to describe four different morphological patterns of PIN including flat, tufting, micropapillary and cribriform (Brawer 1992; Shappell *et al.* 2004; WHO 2004). This latter morphological pattern was a common finding in our study and showed characteristics similar to those described by Brawer (1992), where intraepithelial arcs are organized to form micro-lumens in acini. According to Mostofi *et al.* (1992), this arrangement is an important lesion of premalignancy that can be confused with prostate cancer.

As defined by cellular characteristics, adenocarcinoma was also detected in female prostate, and its incidence doubled with ageing. Moreover, this histopathology may be more intense, since its multiplicity was three times higher than in adulthood. This result, despite contradicting what was reported by Zaviačič (1999), reinforces his hypothesis. According to this author, the low incidence of female prostate cancer is probably due both to delayed recognition of its glandular functionality and to inaccurate diagnosis since other female genital tissues, such as the urethra, are involved in these disorders. In addition, as previously reported, immunohistochemical studies found that this tissue is the origin of many urogenital cancers (Huffman 1952; Dodson *et al.* 1994; Ali *et al.* 1995; Sloboda *et al.* 1998; Islam *et al.* 2001; Kato *et al.* 2005). Because of the morphological characteristics of the female prostate, neoplastic cells may easily spread to other urogenital organs. Furthermore, given the characteristics

of malignant cells such as migration, uncontrolled proliferation and loss of cell differentiation, the prostate may experience unregulated malignant growth (Reynolds & Kyprianou 2006). The similarities between the human (Zaviačič 1999) and gerbil gland (Santos *et al.* 2003, 2006; Custodio *et al.* 2004, 2008; Santos & Taboga 2006) suggest that both consist of a set of glands and ducts, positioned laterally to the urethra and inserted into the fibromuscular stroma. However, despite the existence of stromal constituents, the female gland does not present an effective protection as occurs in male prostate, whether in humans or rodents, where the dense layer of smooth muscle cells and surrounding collagen form a prostate capsule (McNeal 1983; Pegorin de Campos *et al.* 2006). Regardless of the severity of lesions, the ultrastructural analysis of epithelial secretory and stromal cells of the female gland showed no impairment of their activity since the endomembrane system was intact and functional.

While an important increase in the number of nuclei with more than two nucleoli was noted in the epithelial cells of the senescent female prostate, at the three ages of male postnatal development these cells showed no nucleolus or only a nucleolar corpuscle (Pegorin de Campos *et al.* 2006). However, these secretory cells of the female gland showed reductions in area, perimeter and nuclear form factor in old age (Custodio *et al.* 2008). According to Taboga *et al.* (2003), analysis of the nuclear form factor constitutes part of standard evaluation of prostatic lesions. Nevertheless, this factor was not analyzed in the present study due to the variety of lesions identified, which could hinder execution of this analysis. On the other hand, it cannot be hypothesized, based on the reduced nuclear measurements, that there was a decrease in the transcriptional activity during lesion development or even postnatal development in general. On the other hand, the ultrastructural features combined with the quantitative analysis of the nucleolar percentage per nucleus demonstrated significant cellular activity. In light of this result, it can be verified that the DNA content in the nucleoli remains determinant in the degree of cell proliferation (Trére 2000); and the use of nucleolar phenotype is an indicator of metabolic activation, as found in previous cancer studies (Derenzini *et al.* 2000).

The histopathological impairment in the gerbil female prostate during the ageing process was characterized by increased incidence, multiplicity and percentage of lesions per acinus in most of lesions diagnosed in the gland. This result is probably related to the hormonal levels found in those animals. Studies in women (Miller 2001) confirmed a reduction in the steroid levels during senescence in association with many pathological disorders that are not diagnosed in women of reproductive age. However, analysis of this experimental model showed that the histopathology was detected from the adult age which coincides with the elevation of the hormonal levels and the increase of the ration between T/E and T/DHEA levels. On the other hand, any type of lesion was identified in early life when, despite the low steroid levels. Risbridger *et al.* (2003) using E administration in hypogonadal mice and a model of aromatase knockout mice (ArKO) to study, respectively, the actions of E and T in the prostate noted that the action of these hormones individually caused only proliferative processes without malignant alterations. A balance between these steroids is critical to both normal prostatic function and the development of histopathologies in this gland. In this work, the relationship between T/E and T/DHEA levels decreased over the ageing process. Probably, this reduction in the steroid levels is associated not only with impaired ovarian function that is a hallmark of this phase, but also with reduced DHEA levels. This steroid precursor is converted into androgen and/ or E in the peripheral tissues (Labrie *et al.* 2005), which may also occur in the prostate. Thus, an individual action of these steroids cannot be the main cause of such changes. On the contrary, a combined hormonal action may have a potential function in this gland as indicated by studies showing the decreased ratio between T/E and T/DHEA levels and its relation to prostatic diseases.

The present investigation definitively shows, in the female prostate, lesions which have long been identified in male gland (McNeal 1983; Brawer 1992; Mostofi *et al.* 1992) and which present a higher incidence in senescence. Moreover, it defines the quantitative reality of these prostatic histopathologies through the analysis of incidence, multiplicity and percentage of lesion per acini. The knowledge of the pathological processes that affect the prostate in both sexes requires a combination of multidisciplinary expertise, especially in relation to cancer (Hsing *et al.* 2002). This approach includes epidemiological, urological, pathological, biochemical, endocrinology genetic and molecular data as well as the primary condition of extreme importance: the total acceptance of the prostate as a gland of the female genital apparatus which is predisposed to develop different type of lesions. Despite the surprising reality of the female prostate presented herein, all the clinical attentions, until now, have been focused on other urogenital organs. Information on the biology of this female gland, provided by experimental models and combined with basic preventions performed during routine urological examination, could be introduced into the clinical context.

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