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REPERCUSSÕES HISTOPATOLÓGICAS NA PRÓSTATA VENTRAL DO GERBILO DA MONGÓLIA *Meriones unguiculatus* APÓS SUPLEMENTAÇÃO POR HORMÔNIOS ESTERÓIDES

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

Orientador: Prof. Dr. Sebastião Roberto Taboga

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SUMÁRIO

RESUMO

O tecido prostático é susceptível aos desníveis hormonais provocados, principalmente pelo processo de envelhecimento. A hiperplasia benigna prostática e o câncer de próstata são doenças que acometem uma grande parcela da população masculina, e parecem estar envolvidas com alterações hormonais. Por isso, o esclarecimento dos processos celulares e teciduais envolvendo os hormônios sexuais: testosterona e estradiol são, sem dúvida, importantes para o entendimento da etiologia desses processos patológicos. O gerbilo *(Meriones unguiculatus)* foi utilizado como modelo experimental pois, segundo a literatura, é susceptível ao aparecimento de lesões autóctones e responde bem à carcinogênese experimental, mostrando-se um bom modelo experimental. Numa primeira etapa, foram utilizados animais de três idades diferentes: púbere, adulta e senil. Esses animais foram submetidos à suplementação androgênica e as próstatas ventrais foram destinadas a análises histopatológicas, quantitativas, imunocitoquímicas e ultraestruturais. Foi observado aumento no peso da glândula e também na altura das células epiteliais em todas as idades. Tal aumento reflete o aumento da capacidade sintética observada pela dilatação das organelas de síntese, às vezes de aspecto vesiculoso, ocupando toda a região supranuclear. Nos animais adultos e velhos foram notadas regiões hiperplásicas e displásicas freqüentemente associadas a Neoplasias Intraepiteliais de diferentes graus e a adenocarcinomas. Houve aumento na espessura da camada de células musculares lisas (CML) ao redor dos ácinos nos animais púberes e adultos, enquanto nos animais velhos houve diminuição dessa camada. Além disso, as CML se mostraram aparentemente hipertróficas e com maior atividade sintética nos animais púberes e adultos. Foi notado aparente aumento da vascularização periacinar, onde se observou a presença de freqüentes vasos sanguíneos em todas as idades após o tratamento. Ademais, em todas as idades foi observado aumento da densidade de marcação de receptores androgênicos após o tratamento, evidenciando a possível relação desses receptores com os efeitos observados. Em uma segunda etapa experimental, avaliou-se o efeito do estradiol sobre o tecido prostático intacto e hipoandrogênico em animais adultos, tentando com isso simular situações de descompensação hormonal, típicas da senilidade. As alterações epiteliais foram freqüentes nos animais tratados com estradiol onde se observou aumento na altura das células epiteliais, aparecimento de regiões de intensa displasia e hiperplasia, e a formação de PINs. Outro aspecto que independe da presença da testosterona é o arranjo dos elementos fibrilares e não fibrilares da matriz extracelular entre as CML, apontando para um possível papel dessas células no rearranjo e na síntese desses componentes após os tratamentos estrogênicos. Nos animais castrados observou-se acúmulo de elementos da matriz

extracelular sob o epitélio e em animais intactos presença desses elementos dispersos e escassos. Em ambos os grupos: intactos e castrados, notou-se que as CML e os fibroblastos apresentam fenótipo secretor acentuado após o tratamento com estradiol. Houve aumento na densidade de marcação ERα e AR positivos em regiões de hiperplasia apontando para um possível papel desses receptores na formação de lesões pré-malígnas e malignas. Portando, conclui-se que o gerbilo é susceptível a ação da testosterona e do estradiol, os quais provocam desarranjos estruturais e ultraestruturais de cunho patológico e funcional, mostrando-se um ótimo modelo para o estudo das doenças prostáticas de etiologia hormonal.

ABSTRACT

Prostatic tissue is susceptible to the hormonal imbalances, provoked mainly by the aging process. Benign prostatic hyperplasia and prostate cancer are diseases that attack a great portion of the male population, and they appear to be involved in hormonal alterations. Therefore, the explanation of the cellular and tissue processes involving the sex hormones testosterone and estrogen are important to understanding the etiology of these pathological processes. The gerbil *(Meriones unguiculatus)* was used as the experimental model because, according to the literature, it is susceptible to the emergence of autochthonous lesions and it responds well to experimental carcinogenesis, and thus is shown a good experimental model. In the first stage, animals of three different ages were used: pubescent, adult and aged. These animals were submitted to the androgenic supplementation and the ventral prostates were then submitted to hystopathological, quantitative and ultrastructural analyses. Increase in the weight of the gland and in the height of the epithelial cells at all of the ages were observed. This increase reflects the increase of the synthetic capacity observed by dilation of the synthesis organelles, sometimes of vesiculous aspect, occupying the entire supranuclear area. In the adult and aged animals were observed hyperplasic and dysplastic areas frequently associated with Prostatic Intraepithelial Neoplasias (PIN) of different degrees and adenocarcinomas. There was greater thickness of the layer of smooth muscle cells (SMC) around the acini in the pubescent and adult animals, while in the aged animals there was diminution of this layer. Besides, SMC appeared hypertrophic and with greater synthetic activity in the pubescent and adult animals. Increase was observed in the vessels of the subepithelial stroma at all ages after the treatment. Furthermore, at all ages higher density of demarcation of androgenic receptors was observed after the treatment, evidencing the possible relationship between these receptors and the observed effects. In the second experimental stage, the estradiol effect was evaluated on the intact and hypoandrogenic prostatic tissue in adult animals, thus attempting to simulate situations of hormonal imbalances. The epithelial alterations in the estradiol-treated animals were frequent. Increase in the height of the epithelial cells, emergence of areas of intense dysplasia and hyperplasia, and the formation of PINs were observed. Another aspect that does not depend on the presence of testosterone is the arrangement of the fibrillary and non-fibrillary components of the extracellular matrix among SMC, suggesting a possible role of these cells in rearrangement and synthesis of these components after the estrogenic treatments. In the castrated animals accumulation of extracellular matrix components under the epithelium was observed and in intact animals presence of these dispersed and scarce elements was noticed. In both groups: intact and castrated, the secretory phenotype SMC and fibroblasts was

accentuated after the treatment with estradiol. There was increase in the αER and AR-positive demarcation density in hyperplastic areas, implying a possible role of these receptors in the formation of premalignant and malignant lesions. Therefore, it was concluded that the gerbil is susceptible to the action of testosterone and estradiol, provoking structural and ultrastructural disarrangements of pathological and functional nature, and it is thus shown a great model for the study of prostatic diseases of hormonal etiology.

INTRODUÇÃO

1.1. A Estrutura Prostática

Dentre as estruturas acessórias do aparelho reprodutor masculino, a próstata apresenta-se como a glândula mais volumosa e a de maior expressividade funcional (NETTER, 1965). A próstata secreta um líquido fino de aspecto leitoso que aumenta o volume do sêmen. A secreção prostática apresenta propriedades importantes para que a fertilização tenha êxito; colaborando com a neutralização do meio e contribuindo sobremaneira para o aumento da motilidade e fertilidade dos espermatozóides (GUYTON, 1994).

Histologicamente, a próstata constitui-se de glândulas túbulo-alveolares. O epitélio secretor que reveste os alvéolos assenta-se numa membrana basal que repousa sobre um estroma conjuntivo, contendo fibras musculares lisas que circundam os alvéolos e ductos, e inúmeros vasos sanguíneos e linfáticos. No estroma podem ser encontradas terminações nervosas de vários tipos, freqüentemente entre as células musculares lisas (ICHIHARA *et al.,* 1978) e associadas ao epitélio (PRICE, 1963). No homem, as unidades glandulares dispõem-se em três camadas de forma concêntrica ao redor da uretra prostática, onde se abrem os ductos individuais. Perifericamente, uma fina cápsula fibromuscular rica em células musculares lisas envolve a próstata, enviando septos que penetram na glândula (JUNQUEIRA & CARNEIRO, 2004).

Na maioria dos roedores, a glândula prostática apresenta-se como uma estrutura complexa composta de três lobos distintos, designados pela localização que cada um deles ocupa em relação à uretra. Assim, são identificados o lobo ventral, no istmo da bexiga, e os lobos dorsal e lateral que circundam, dorso-lateralmente, a uretra, a base da bexiga, a vesícula seminal e as glândulas coaguladoras (PRICE, 1963; JESIK *et al.,* 1982; SUGIMURA *et al.,*1986). Assim como nas zonas da próstata humana, estes lobos diferem no que diz respeito à sua topografia, ao seu tamanho, à sua composição e organização estrutural, às suas secreções e aos respectivos processos secretórios (JESIK *et al.,* 1982; AUMULLER & SITZ, 1990; COLOMBEL & BUTTYAN, 1995).

O epitélio das unidades secretoras e seus ductos são constituídos de células geralmente cilíndricas e altas - as células epiteliais luminais – e entre as bases destas podem estar distribuídas células menores, achatadas ou arredondadas – as células epiteliais basais, que alguns autores consideram como células progenitoras e possuem marcadores específicos (KURITA et al., 2004; TIMMS et al., 2005).

Além das células musculares lisas e fibroblastos, outros tipos celulares também são encontrados no estroma, como mastócitos, células endoteliais e pericitos, juntamente com terminações nervosas e gânglios sensitivos. Cada célula desempenha um papel importante e específico na manutenção e função secretora da próstata ventral.

As células musculares lisas (CML) representam 22% da área total da próstata humana (SHAPIRO et al., 1992), predominando ao redor dos dutos, onde se encontram em íntimo contato com a membrana basal das células epiteliais. As CML têm um papel preponderante nos mecanismos de estimulação parácrina, especialmente sobre o epitélio (FARNSWORTH, 1999) e, provavelmente, também sobre as demais células estromais.

Entre as células epiteliais e o estroma encontra-se a membrana basal. Esta estrutura é extremamente importante no controle das atividades celulares e, principalmente, na manutenção da fisiologia das células epiteliais (HAYWARD et al., 1998), pois expressa proteínas adesivas e é responsável pela comunicação entre o estroma e o epitélio, principalmente no que diz respeito a nutrição.

1.2. Desenvolvimento da Próstata

A estimulação por andrógenos é absolutamente necessária para o desenvolvimento da próstata, assim como para as demais estruturas sexuais masculinas (CUNHA et al., 1987). A morfogênese prostática é dependente da produção de andrógenos pelos testículos do feto (POINTIS et al., 1980). O desenvolvimento da próstata não é determinado pelo sexo genético, mas sim pela exposição aos andrógenos, tendo sido demonstrado que o seio urogenital (UGS) de fêmea ou macho podem formar tecido prostático funcional, caso eles sejam estimulados por andrógenos no período adequado (TAKEDA et al., 1986).

O brotamento prostático é iniciado pela ação androgênica pré-natal (TIMMS et al., 1994). As subseqüentes morfogêneses ductal, canalização e citodiferenciação epitelial também precisam de estimulação androgênica e estão associados a um aumento perinatal transitório na concentração de testosterona (DONJACOUR & CUNHA, 1988). Embora a testosterona seja o primeiro andrógeno produzido pelos testículos fetais, a diidrotestosterona (DHT) é a responsável pela morfogênese prostática (TAPLIN e HO, 2001). A DHT é produzida no seio urogenital (UGS) pela redução da testosterona pela enzima 5α-redutase. Esta enzima foi detectada no UGS e na genitália externa de ratos, coelhos e humanos (WILSON et al., 1983).

Eventos distintos que modifiquem esse microambiente rico em andrógenos, podem decorrer em diferenciação anormal do tecido prostático ou ausência de diferenciação, como ocorre em fetos expostos a estrógenos semi-sintéticos (químicos) durante o período gestacional (TIMMS et al., 2005).

A diferenciação do epitélio prostático ocorre paralelamente à maturação do estroma prostático. Andrógenos atuam sobre receptores de andrógeno (AR) no mesênquima urogenital (UGM) para induzir a proliferação epitelial, ramificação ductal e citodiferenciação nos subtipos celulares basal e luminal (CUNHA et al., 1987; 1992). Por sua vez, o epitélio próstatico em desenvolvimento direciona os padrões de diferenciação do músculo liso próstatico (HAYWARD et al., 1998). Nem o epitélio prostático nem o músculo liso prostático são capazes de desenvolverem-se na ausência do outro tecido (HAYWARD & CUNHA, 2000).

No período pós-natal, o desenvolvimento prostático é também dependente de andrógenos já que a castração de ratos neonatos inibe o crescimento e desenvolvimento da próstata durante a puberdade, um efeito que pode ser revertido com a administração de testosterona (CUNHA et al., 1987; CORBIER et al., 1995).

A próstata de neonato é sensível aos andrógenos. Assim, a administração de testosterona acelera o crescimento da próstata, sendo possível atingir precocemente o crescimento máximo (BERRY and ISAACS, 1984). Na puberdade apresenta-se o início do crescimento prostático que é caracterizado por um aumento do peso seco da próstata e por um pequeno incremento no número das ramificações (SUGIMURA et al., 1986). Isto indica que a próstata em desenvolvimento é sensível às baixas concentrações de andrógenos para a ramificação ductal, e que a sua resposta aos níveis de andrógenos altos na puberdade (incremento de peso seco) é diferente da resposta inicial (HAYWARD & CUNHA, 2000).

1.3. Fisiologia e modulação hormonal na próstata

O crescimento normal , a diferenciação e a manutenção da integridade funcional (secretora) e estrutural da próstata e dos demais órgãos acessórios do sistema reprodutor masculino são dependentes de níveis constantes de andrógenos circulantes e ocorrem através de interações recíprocas entre o estroma e o epitélio (PRICE, 1963; AUMÜLLER & SEITZ, 1990; ROSAI, 1996; HAYWARD et al., 1998; THOMSON et al., 1997).

A produção de andrógenos está sob controle endócrino, sendo regulada pelo eixo hipotalâmico-hipofisário-gonadal (DEBES & TINDALL, 2002). Em humanos, o principal andrógeno é a testosterona, com as células de Leydig dos testículos produzindo mais de 95% e a glândula adrenal menos de 5%

desse esteróide sexual (HSING et al., 2002). Somente a testosterona livre é hábil para entrar nas células prostáticas e esse processo ocorre por difusão passiva (RUIJTER et al., 1999), uma vez que, mais de 90% desta é convertida no principal andrógeno prostático, a dihidrotestosterona (DHT), pela ação da enzima 5-α-redutase tipo 2 (HSING et al., 2002).

As funções da testosterona e DHT na próstata são mediadas por AR, os quais controlam respostas androgênicas distintas no epitélio e estroma (WANG et al., 2001). Na ausência de hormônio esteróide, o AR mantém-se associado a proteínas *heat-shock,* porém quando a DHT liga-se ao hormônio ligante de andrógeno, há dissociação das proteínas *heat-shock* e o AR é hiperfosforilado e dimeriza. Seu domínio de ligação ao DNA então se liga a genes de resposta a andrógenos, os quais estão envolvidos com o controle da divisão celular prostática (GALBRAITH & DUCHESNE, 1997).

Embora a função dos andrógenos seja importante para a homeostase prostática, estes sozinhos são insuficientes para manterem-na. Tal processo requer interações entre fatores de crescimento peptídicos e moduladores de crescimento que são regulados por andrógenos ou por outras vias moleculares (LEE et al., 1997). Esses fatores são secretados por células epiteliais e estromais de maneira autócrina ou parácrina, atuando no controle de proliferação e morte celular, em ambos compartimentos celulares (UNTERGASSER et al., 2001)

As necessidades de se estudar as respostas deste órgão aos hormônios, sob várias condições, deve-se ao fato de ser a glândula prostática, em humanos, o sítio de um grande número de doenças relacionadas à idade, sendo que as de maior importância médica são o câncer prostático e a hiperplasia nodular, esta última conhecida também como BPH (benign prostatic hyperplasia). Os hormônios, entre outros fatores, exercem papel na etiologia destas lesões. Além disso, estas lesões, malignas ou não, podem ser tratadas por estratégias de remoção de andrógenos (PRICE, 1963; COLOMBEL & BUTTYAN, 1995; DROLLER, 1997; RAUCH et al., 1997) e/ou utilização de estrógenos semi-sintéticos (químicos) como o dietilestilbestrol (DES) (HELLERSTEDT & PIENTA, 2002).

A diferenciação e a proliferação celular do epitélio prostático foram primariamente consideradas andrógeno-dependente, já que terapias anti-androgênicas vêm sendo utilizadas no tratamento das doenças prostáticas (KOZLOWSKI et al., 1991).

Ainda que a próstata seja um tecido andrógeno dependente, os estrógenos influenciam as funções normais e mudanças patológicas. Isto pode ser devido à presença dos dois receptores de estrógeno no órgão (WEIHUA et al., 2001).

As evidências de uma ação direta dos estrógenos na próstata surgem da observação da existência de receptores para estrógenos (ERα e ERβ) nas células prostáticas estromais e epiteliais, respectivamente. Um efeito direto do estrógeno, estimulando o crescimento prostático, foi demonstrando

em camundongo hypogonadais (*hpg*), que não possuem andrógenos circulantes. O crescimento induzido foi bem menor que os controles e estava associado a diferentes neoplasias (BIANCO et al., 2002).

Lesões epiteliais, como a metaplasia escamosa, têm origem a partir de tratamentos estrogênicos e necessitam da interação entre receptores ERα estromais e epiteliais (CUNHA et al., 2002).

Estudos recentes em nosso laboratório demonstraram que o estradiol é capaz de estimular o crescimento fibromuscular e provocar lesões displásicas intraepitelais em diferentes idades, principalmente após a puberdade (SCARANO et al., 2004; 2005).

Além disto, há também um mecanismo denominado *imprinting* estrogênico, que se caracteriza pela exposição perinatal ao estrógeno e a manifestação de efeitos na adolescência e na vida adulta. Os efeitos do estrógeno têm ação não normotônica, ou seja, efeitos opostos quando diferentes dosagens são empregadas. Dosagens baixas resultam em estímulo do crescimento, enquanto dosagens mais elevadas resultam em redução do crescimento. Os efeitos do *imprinting* estrogênico são múltiplos e melhores conhecidos através de seus efeitos feminilizantes por ação direta no hipotálamo, além de atuar antecipando a puberdade em ratos (PUTZ et al., 2001b). Altas doses de estrógeno neonatal induzem também uma quase completa eliminação da expressão do receptor de andrógeno, como determinado por imunocitoquímica (PUTZ et al., 2001a). Foram observados ainda rearranjos das junções comunicantes (HABERMANN et al., 2001) e alterações na expressão dos receptores para ácido retinóico (PRINS et al., 2002). O estrógeno parece exercer suas ações através do receptor de estrógeno do tipo alfa, presente no estroma prostático.

O estrógeno pode atuar como um fator predisposicional para BPH ou câncer prostático. Em ratos, o tratamento neonatal com 17 Beta-estradiol induz alterações metaplásicas irreversíveis no epitélio das regiões periuretrais da glândula de coagulação, ductos ejaculatórios, e regiões da parede dorsal da uretra (ARAI et al., 1977). TIMMS et al. (2005) relataram considerável aumento no índice de proliferação em células epiteliais secretoras e basais, consideradas como progenitoras, em fetos de ratos, sendo este um indicativo de possíveis alterações no controle celular que poderiam levar a distúrbios proliferativos durante o desenvolvimento pós-natal.

O crescimento prostático parece estar associado à expressão de receptores para estrógeno e andrógeno nos compartimentos epitelial e estromal e à fatores de crescimento específicos produzidos pelo estímulo destes hormônios em células como os fibroblastos e as células basais do epitélio (HUYNH et al., 2001; WEIHUA et al., 2001).

1.4. O gerbilo como modelo experimental

Os gerbilos, também conhecidos como esquilos da Mongólia ou "clawed jirds", são roedores murídeos da subfamília Gerbillinae provenientes das regiões áridas da China e da Mongólia (SCHWENTKER, 1963). Introduzidos nas Américas como nova proposta de animal experimental nos anos cinqüenta por Victor Scwentker, os gerbilos, durante muito tempo, ficaram limitados ao Estados Unidos como animais de excelência para a pesquisa biomédica (ROBINSON, 1974). Nas últimas décadas, vêm sendo gradativamente introduzidos nos biotérios das universidades brasileiras e têm assumido importante papel nos experimentos biológicos e biomédicos juntamente com outras espécies clássicas como *Rattus rattus norvegicus* (rato), *Mus muscullus* (camundongo) e *Calomys callosus* (hamster).

De anatomia similar à do rato e do camundongo, os gerbilos adultos de ambos os sexos variam entre 11,5 e 14,5cm de comprimento corpóreo. Os machos pesam em torno de 100 gramas enquanto as fêmeas pesam cerca de 85 gramas (KRAMER, 1964).

A grande vantagem destes animais sobre os outros citados, para estudos experimentais, reside no fato deles serem consideravelmente menores que os ratos, mas essencialmente maiores que os camundongos e hamsters (WILLIAMS, 1974). Estes animais têm sido amplamente utilizados para estudos de natureza didático-científica principalmente pelo fato terem comportamento extremamente dócil em cativeiro. Outra característica importante a ser considerada é que apresentam comportamento de micção pouco freqüente e, por serem de origem desértica, consomem pouca quantidade de líquido, o que agiliza muito a limpeza das gaiolas no processo de manutenção desses animais em cativeiro, promovendo grande asseio nas salas de manutenção dos animais nos biotérios.

Na pesquisa científica, cada vez é maior a utilização dos gerbilos na experimentação biomédica, principalmente nas áreas da imunologia (NAWA et al., 1994; JEFFERS et al., 1984), fisiologia (MÜLLER et al., 1979; NOLAN et al., 1990) e crescentemente na área de morfologia (AOKI KOMORI et al., 1994; REDECKER, 1987; 1991).

Da morfologia do aparelho reprodutor, com ênfase no complexo glandular que envolve a próstata e vesículas seminais, poucos dados têm aparecido na literatura. Estudos de GROSS & DIDIO (1987) enfocam a morfologia e a ultra-estrutura de uma espécie muito próxima – *Praomys natalensis*. Estes autores referem-se principalmente ao compartimento epitelial da glândula prostática. Recentemente, CAMPOS et al. (2006) descreveram a estrutura dos compartimentos epitelial e estromal da próstata do gerbilo durante o desenvolvimento pós-natal. Adicionalmente, o modelo vem apresentando respostas significativas quanto a tratamentos hormonais em fêmeas (Santos et al., 2003), drogas contra

hiperplasia prostática humana (CORRADI et al., 2004) bem como, desenvolvimento de neoplasias espontâneas associadas ao envelhecimento (ZANETONI et al., 2001) e após carcinogênese química (ZANETONI et al., 2005).

Estudos adicionais sobre o aparelho reprodutor masculino de gerbilos foram realizados por Pinheiro e colaboradores (PINHEIRO et al., 2003a,b) que descreveram o sistema ductal dos lobos prostáticos, além da estrutura e ultra-estrutura da uretra peniana e pélvica.

A glândula prostática do gerbilo está constituída de dois lobos amarelados, imediatamente ventrais à bexiga urinária, exatamente no ponto em que a uretra recebe dois ductos espermáticos. Estes lobos estão ligados na junção da bexiga com as vesículas seminais (WILLIAMS, 1974; PINHEIRO et al., 2003a).

Vários trabalhos foram realizados no intuito de estabelecer os efeitos do estradiol (ISAACS & COFFEY, 1989; SCARANO et al., 2004) e da testosterona (RICKE et al., 2006) sobre a próstata intacta e após privação androgênica em ratos, camundongos e em cobaias, tentando com isso simular situações de descompensação hormonal.

A partir dos conhecimentos sobre a etiologia das doenças prostáticas e do papel dos hormônios: testosterona e estradiol, sobre a fisiologia e a estrutura da glândula prostática, bem como seu papel na indução de lesões de natureza maligna e pré-maligna, estudos que tragam informações complementares para a compreensão dos mecanismos teciduais frente as variações hormonais são, sem dúvida, muito importantes.

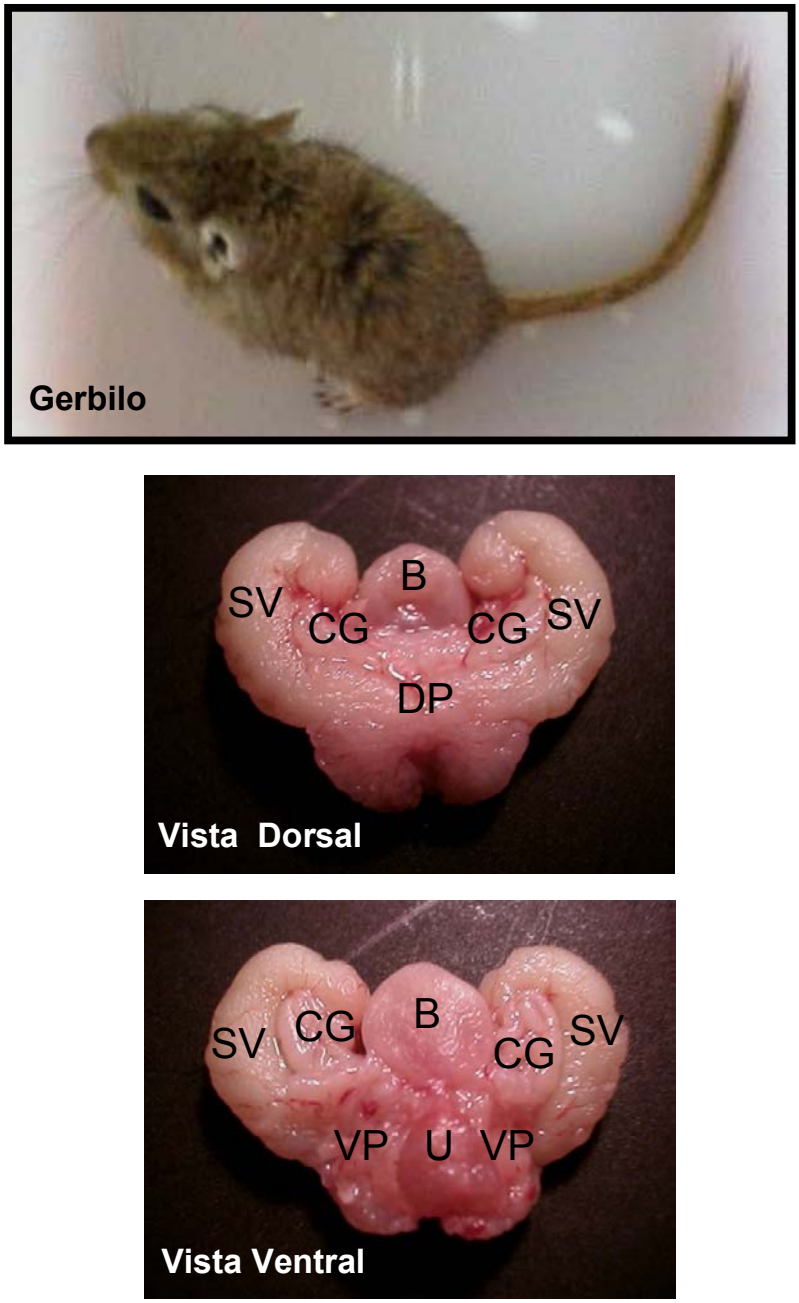

Figura 1. Gerbilo adulto e sua próstata, a qual se divide em lobos dorsais (DP), lobos ventrais (VP), lobos anteriores ou glândula coaguladora (CG). Podem ser visualizados: a bexiga (B), vesícula seminal (SV) e a uretra (U) – (Reproduzido com permissão de Sebastião Roberto Taboga).

OBJETIVOS

O presente trabalho teve por objetivos:

1. Avaliar os efeitos da testosterona sobre a próstata ventral do gerbilo em três diferentes idades: púbere, adulta e senil, através de análise histopatológica, quantitativa e ultraestrutural.

2. Caracterizar os efeitos do estradiol sobre a próstata ventral do gerbilo adulto intacto e após orquiectomia bilateral por métodos estruturais, imunocitoquímicos, histomorfométricos e ultraestruturais.

ARTIGOS

ARTIGO 1

STROMAL REMODELING AND HISTOPATHOLOGICAL ASPECTS IN THE INTACT AND CASTRATED GERBILS *(MERIONES UNGUICULATUS)* PROSTATE AFTER ESTROGEN SUPPLEMENTATION

Submitted to Cell and Tissue Research

Stromal remodeling and histopathological aspects in the intact and castrated gerbils *(Meriones unguiculatus)* prostate after estrogen supplementation

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Running title: Effect of estrogen on the intact and castrated gerbil prostate

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Abstract

The estradiol effect was evaluated on the intact and hypoandrogenic prostatic tissue in adult gerbils, trying with that to simulate situations of hormonal unbalances. The experimental animals were studied by histological, histochemical and immunocytochemistry techniques, morphometric–stereological analysis and transmission electron microscopy (TEM). The epithelial alterations in the estradiol treated animals were frequent where increase in the height of the epithelial cells, emergence of areas of intense dysplasia and hyperplasia, and the formation of PINs was observed. Another aspect that does not depend on the presence of the testosterone is the arrangement of the fibrillary and no-fibrillary elements of the extracellular matrix among SMC, appearing for a possible role of these cells in rearrange and in the synthesis of these components after the estrogenic treatments. In the castrated animals accumulation of extracellular matrix elements under the epithelium was observed and in intact animals presence of these dispersed and scarce elements was noticed. In both groups: intact and castrated, it was noticed that SMC and the fibroblasts present secretory phenotype accentuated after the treatment with estradiol. There was increase in the αER (estrogen receptor) and AR (androgen receptor) αpositive demarcation density in hyperplasia areas appearing for a possible role of these receptors in the formation of premalignant and malignant lesions.

Keywords: estradiol, prostate, castration, stroma, epithelium, gerbil

Introduction

Estrogens regulate the development and function of prostate at several stages by indirect and direct mechanisms. Prostate growth, differentiation and functions are primarily controlled by androgens but estrogens modulate these effects in several ways. The most important routes of indirect estrogen regulation are interference of androgen production by repression of the hypothalamic-pituitary-gonadal axis and direct effects on testis. Estrogens also clearly have direct effects on prostate, which may be elicited by external hormone or by estradiol produced by local aromatization of testosterone (Härkönen & Mäkelä, 2004).

Estrogen regulation has also been considered as one of the hormonal risk factors in association of development of benign prostatic hyperplasia and prostate cancer (Bosland, 2000; Henderson &

Feigelson, 2000). Exogenous estrogens given during the perinatal period elicit abnormalities in prostatic growth (Naslund & Coffey, 1986), differentiation (Arai et al., 1977), function (Prins et al., 1993), androgen metabolism (Santti et al., 1991), expression of androgens receptors (Prins et al., 1993), and may lead to prostatic cancer (Santti et al, 1990; Prins, 1997).

Squamous metaplasia, hyperplasia and dysplasia are a direct effect of estrogen on the prostate induced by long-term exposure to high levels of exogenous or endogenous estrogens (Risbridger et al., 2001; Scarano et al., 2004). For example, the squamous metaplasia of the prostatic epithelium is characterized by the total replacement of the columnar secretory epithelium by layers of stratified squamous cells (Risbridger et al, 2001). Estrogenic induction is mediated through αER signaling. Stromal-epithelial interactions and a requirement of both epithelial and stromal αER to elicit estrogen-induced prostatic epithelial disorders. Presumably, the development of prostatic epithelial disorders involves the stimulation of epithelial proliferation mediated by stromal αER and an epithelial differentiation mediated by epithelial αERs (Cunha et al., 2002).

Previous studies have indicated that following the administration of estrogen the smooth muscle cells increased in size and number in the rat prostate (Thompson et al., 1979). The guinea pig prostate presented increased density and thickness of the collagen fibrils in castrated and intact adult animals after estradiol treatment (Neubauer & Mawhinney, 1981; Mariotti & Mawhinney,1982; Scarano et al., 2005). These effects are directly associated with the presence of estrogen receptors (αER) in the stroma and stimulation of the stromal cells through to autocrine and paracrine mechanisms (Droller, 1997).

In the present study it was evaluated the effect of the estradiol on the epithelial and stromal prostatic compartments of the intact and castrated gerbils, a new model of prostate study, with the intention of to establish the effects of this hormone in this rodent and to compare the results obtained with analyses accomplished in other experimental models.

Material and Methods

Animals and hormone treatments

To accomplish of the work, 20 adult (120 days) male *Meriones unguiculatus* gerbils were used. The animals were divided in 4 experimental groups: Intact Control (C), Intact estradiol-treated (E), Castrated (Ca) and Castrated estradiol-treated (CaE). The castrated group was submitted to bilateral orchiectomy by abdominal surgical incision. After the surgery the animals of this group were put in

individual boxes and they were submitted to the experiments after 7 days. The treated groups received in alternate days subcutaneous injections of Estradiol Benzoate (Sigma Chemical Co., St. Louis, Missouri) diluted in vegetal oil (10 mg/ml) at a dose of 0,1 ml/application/animal (1 mg/ application) for 21 days, while the control and castrated groups received only vegetal oil.

After 21 days of treatment, the animals of all ages and of both groups were anesthetized lightly by CO_2, weighted and killed by decapitation. The ventral prostate was removed, weighted (analytical balance) and submitted to light microscopy and ultrastructural procedures.

Hormonal serum levels

Blood were colleted after the decapitation and the serum was obtained after centrifugation and stored at -20ºC for subsequent hormone assay. The determination of serum levels of testosterone it was performed by luminescence-immunoassay (mouse antibodies anti-testosterone – Jhonson & Jhonson ®, USA) in automatic analyzer. The intra-assay and inter-assay variation was 4.6 and 4.3%, respectively.

Histochemistry

Ventral prostates of experimental groups were cut into fragments and immediately fixed by immersion, for 24 hours in Karnovski fixative (0,1M Sörensën phosphate buffer pH 7.2 containing 5% paraformaldehyde and 2.5%, glutaraldehyde). Fixed tissue samples were dehydrated in a graded ethanol series and embedded in glycol methacrylate resin (Leica historesin embedding kit). Histological sections (3-μm) were subjected to hematoxylin-eosin (H&E) staining for general studies, to Gömöri´s reticulin (Gömöri, 1937) staining for collagen and reticular fibers and to Feulgen (Mello & Vidal, 1980) staining for nuclear study. Microscopic analyses were performed on Zeiss-Jenaval or Olympus photomicroscopes, and the microscopic fields were digitalized using the Image-Pro®Plus version 4.5 for Windows™ software.

Morphometric and Stereological analysis

Using an analyzing system of images (Image Pro-Plus), H&E and Feulgen sections were analyzed. Images of 50 histological fields for each experimental group were analyzed, such that histological fragments of all animals were evaluated equally. The morphometric analyze was performed to evaluate to epithelium height, smooth muscle cells layer thickness, and nuclear area of the secretory

epithelial cells. For this comparative study were realized 200 measurements to each parameter. Stereologic analyses were obtained by Weibel's multipurpose graticulate with 120 points and 60 test lines (Weibel, 1979) to compare the relative proportion among the prostatic components (epithelium, stroma and lumen of acini) in the experimental groups (50 histological fields for group).

Statistical analysis

The estradiol effects on gerbil ventral prostate were evaluated by analyses of mean ± standard deviation (SD) in the parameters: epithelium height, smooth muscle cells layer thickness, nuclear area of the secretory and stereologic analyses. The statistical analysis was performed in the Statistica 6.0 software (Copyright©StatSoft, Inc. 1984-1996). The hypothesis test Anova, and Tukey HSD test were employed and, $p \leq 0.05$ was considered statistically significant.

Transmission Electron Microscopy

The ventral prostates of the experimental gerbils were processed for transmission electron microscopy as described previously (De Carvalho et al., 1994), employing the fixation procedure of Cotta-Pereira et al. (1976). Briefly, tissue fragments were fixed in 0.25% tannic acid plus 3% glutaraldehyde in Millonig's buffer, dehydrated in acetone, and embedded in Araldite resin. Silver sections obtained with a diamond knife were stained by uranyl acetate and lead citrate. Observation and electron micrographs were made with a LEO – Zeiss 906 transmission electron microscope.

Immunohistochemistry (IHC)

AR (SC-816, 1:100 dilution; rabbit polyclonal antibody), ERα (SC-542, 1:50 dilution; rabbit polyclonal antibody): Santa Cruz Biotechonology, Santa Cruz, CA, USA.; and Condroitin sulphate-anti SC56 (Sigma Chemical Co., St. Louis, Missouri) were used for IHC. Immunohistochemistry staining was performed using the avidin-biotin complex (ABC) kit (Santa Cruz Biotechnology, CA, USA). The tissue fragments to IHC were fixed by immersion in the formaldehyde solution at 10% and embedded in paraplast. The sections (5µm) were dewaxed and then rehydrated in graded alcohol and distilled water. Antigenic recuperation was realized in citrate buffer in high temperature (100ºC) for 45 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 45 min, followed by a quick rinse in distilled water and phosphate-buffered saline (PBS). Sections were incubated

with normal goat serum and primary antibody at 4ºC overnight. The slides were them incubated with the biotinylated anti-rabbit at 37ºC followed by peroxidase-conjugated avidin-biotin complexes and diaminobenzidine (DAB). The sections were then counterstained with hematoxylin of Harris. For negative control, the primary antibody was replaced with the corresponding normal isotype serum.

Results

Intact animals

The prostate of the intact adult gerbil presented glandular units with simple cylindrical epithelium, wrapped in a fine strip of vascularized conjunctive tissue and a smooth-muscle-cell (SMC) layer, delimiting the lumen of the gland (figs.1a and 1b). Among the acini was observed dispersed loose conjunctive vascularized tissue.

The epithelial cells of the gland possess evident secretory characteristics, presenting in some places extrusion granules, typical of apocrine secretory cells (1b) and presence of secretion organelles above the nucleus (fig. 13). Adjacent to the epithelium and intermixed with the smooth muscle cells, relative poverty in fibers of the extracellular matrix was observed (figs. 1b, 5a and 14).

Alteration was not verified in body weight among the experimental groups (Table 1)and in the seric testosterone levels after estradiol treatment (fig. 31).

In the estradiol-treated intact animals decrease in the absolute and relative weight of the prostatic compound in relation to the control group was verified (Table 1). However, increase in the height of the epithelium and of the muscular layer thickness was observed (fig. 2c) in the nuclear area and perimeter compared to the control animals (Table 1).

In general, the lumen of the acinun diminished due to the increased height of the epithelial secretory cells, which appeared with a large number of dilated cisternae (fig. 22) and in some acini there was occurrence of circular endoplasmic reticulum ER (fig 21). Areas with frequent hyperplasia and dysplasia presented elongated epithelial cells with nuclei of different heights (figs. 2a, 2b). In the epithelium, due to intense dysplasia, areas were observed where the cells were dislocated to the apical regions, simulating a possible detachment (figs. 2b). In some acini the presence of PIN was documented (Figs. 2d, 20).

In the stroma of the E animals the most oval nuclei were observed, while the smooth muscle cells were morphologically less elongated and more fusiform (fig. 2c). In the ultrastructure, SMC appeared hypertrophic, with well-developed secretory organelles (fig. 24). Besides, fibroblasts activated with large

quantities of endoplasmic reticulum and Golgi were observed (fig. 26). Fibers of the extracellular matrix presented expressive increase of collagen and elastic fibers mainly adjacent to SMC, besides intimate relationship between the synthesized fibrils and stromal cells and, in some areas, noticeable formation of cytoplasmic compartments through elongation of these cells (figs. 6a, 29 and 30). In accord with this fibrillary distribution, non-fibrillar components of chondroitin sulphate were observed as having predominantly inter-SMC demarcation and distribution (fig. 6b).

Castrated animals

The prostate gland in the castrated animals presented, predominantly, acini with cubic or short cylindrical epithelium, wrapped by a relatively thick conjunctive layer and smooth muscle cells (SMC) arranged concentrically (figs. 3a, 3b).

The epithelial cells were shown to be relatively short, assuming in some places a flat aspect (figs. 3a, 3b), thus increasing the nucleus-cytoplasm ratio. Furthermore, irregularity of the basal membrane with wavy aspect was observed in most of its extension, forming at times a semi-pleated arrangement (figs. 3b 7a, 15 and17).

Adjacent to the epithelium, filling out the irregularities of the basal membrane, was found a thick layer conjunctive subepithelial tissue (figs. 3b, 7a, 15 and 17). This conjunctive stroma is abundant in fibrillary constituents of the extracellular matrix, mainly collagen (figs.7a, 15 and 17) and reticular fibers (figs.7a) and non-fibrillary components such as chondroitin sulphate with strong immuno-reactivity in that area (fig. 7b). It is interesting that in this experimental group the constituents of the extracellular matrix, despite being found in every periacinar area, were concentrated mainly in the area adjacent to the basal lamina (figs. 7a, 15 and 17) where fibroblasts presented high synthetic activity (fig. 16).

The smooth muscle cells surrounding the acini were disposed concentrically (fig. 3a) and forming irregular layers, without defined orientation, amid the extracellular-matrix fibers (figs. 3b) of the subepithelial stroma. These cells assume an irregular phenotype (figs. 3b and 18), different from the elongated fusiform aspect usually found in the layers (fig. 3a).

After the treatment of castrated animals with estradiol for 21 days, it was observed that the prostatic regression process was less intense than in the castrated animals, when we compared the absolute and relative weights of the prostate (Table 1).

With respect to the relative proportion of the tissue components, an increase was verified in the amount of epithelium and decrease of the lumen in relation to the CaE group (Table 1). Regarding the

absolute volume for the same tissue components, increased amounts of epithelium, lumen and stroma were observed (Table 1)

Significant alterations in the secretor epithelium of CaE animals were observed. The epithelium is predominantly cylindrical, culminating in an increase in the epithelium height in relation to the Ca group (Table 1). Augmentation of the nuclear area and perimeter of secretor epithelial cells was also observed (Table 1).

After the treatment, frequently, dysplastic areas with increases in cellular density (hyperplasia) and cellular size were observed (figs. 4b, 4c and 4d). Besides, areas were evidenced with occurrence of Prostatic Intraepithelial Neoplasia (PIN) characterized by epithelial cells agglomerated with heterogeneous phenotypes (fig. 4a and 4c). Yet it was observed that the basal membrane assumes, as found in the Ca group, a wavy and semi-pleated arrangement (figs. 4b, 4d and 8a).

The distribution of the collagen fibers is represented in figure 8a, where an accumulation of these fibers was observed adjacent to the epithelium, accompanying the confluences of the basal membrane and intermixed with SMC. Apparently, a preferential deposition of collagen fibers occurred among the smooth muscle cells in the CaE group, as observed in figures 8a, 27 and 28, where discreet increases were observed in the fibrous bunches that permeate SMC and in chondroitin sulphate (fig. 8b).

The arrangement of the smooth-muscle-cell layer resembles that in the Ca group, where the smooth muscle cells assume elongated fusiform phenotypes forming concentric bunches of fibers (fig. 4a), and irregular spine-like cytoplasmic projections unwrapped by the extracellular matrix (fig. 4b, 25).

In relation to immunocytochemical expression of AR and αER., both intact and castrated animals presented positive demarcation in the epithelium and in the stroma (figs. 9a and 10a; 11a and 12a). That demarcation was heterogeneous, demonstrating that some cellular clones are more sensitive to the action of those hormones. After estradiol treatment, it was observed that the density of αER and AR-positive cells was larger in hyperplastic and dysplastic epithelial areas (figs. 9b, 9c, 10b, 10c, 11b,12b), when compared to areas of normal epithelium.

The immunocytochemistry assays for the demarcation of chondroitin sulphate, a glycosaminoglycan of the stromal extracellular matrix, demonstrated in the adult intact animals (figs. 7 and 8), diffuse demarcation in areas adjacent to the epithelial base and to the smooth muscle cells with moderate intensity of demarcation. Besides, in some acini specific demarcation in the epithelial cells (area of Golgi) was observed because there is production of sulphated residues there.

Demarcation of chondroitin sulphate in intact and castrated adult animals treated with estradiol showed (figs. 6b and 8b) localization less diffuse than in C and Ca groups, with strong demarcation mainly in areas adjacent to the basal lamina and among the smooth muscle cells. It is important to

emphasize that preferential deposition of chondroitin occurred among the SMC (figs. 6b and 8b), similar to that found in the collagen fibers of the estradiol-treated animals.

Discussion

Estrogen has been implicated in the pathogenesis of prostatic diseases by their direct and indirect effects. Prominent among the direct effects is the anti-androgenic action caused by repression of the hypothalamic-pituitary-gonadal axis and direct effect on the testis. Estrogens also clearly have direct effect on the prostate, which may be elicited by external hormone or by estradiol produced by local aromatization of testosterone (Härkönen & Mäkelä, 2004).

The effects of orchiectomy have been studied under several aspects in several experimental animals, mainly as a model of rearrangement of extracellular matrix architecture (Carvalho & Line, 1996; Vilamaior et al., 2000; Antonioli et al., 2004). Along with these studies, experiments using estrogen have been accomplished seeking to define the role of this hormone more precisely in hypoandrogenic prostatic tissue (Tam & Wong, 1991; Pelletier, 2002) and in physiologic conditions (Scarano et al., 2004).

According to Campos et al. (2006, *in press*) and Scarano et al. (2003), the prostatic acinun of the intact gerbil presents simple cylindrical epithelium, with presence of secretion granules in the luminal portion, besides areas with prominent chromophobes adjacent to the nucleus, identified as being the area of the Golgi apparatus. Such discoveries show the high secretor activity of that tissue in physiologic conditions.

The epithelium of the Ca animals was found short and cubic, with a scarcity of secretion vesicles in the luminal border and relatively high nucleus-cytoplasm ratio. This structure shows the regression of prostatic tissue faced with androgenic ablation, which agrees with the data obtained previously in mice by Pelletier, 2002, and after experimental chemical castration (Cordeiro et al., 2004, Corradi et al., 2004).

Another characteristic of the castration process is the increase of the stromal compartment in relation to the intact animals. In the Ca animals, a large amount of extracellular-matrix fibers was observed in the subepithelial stroma , allied with structural modifications of SMC.

According to Vilamaior et al. (2000), the androgenic deficiency promotes a rearrangement of extracellular-matrix fibers, and possibly, synthesis of constituents of this matrix. That rearrangement occurs probably due to alterations in SMC synthetic capacity, which would start to play a role more synthetic than contractile.

Horsfall et al. (1994) demonstrated in guinea pigs that during the aging process, SMC increases its synthetic capacity modifying the morphologic structure. Similarly, experimental studies using orchiectomy and chemical castration showed an increase in extracellular-matrix components allied with phenotypic alterations of SMC that started to exhibit an irregular and thorny arrangement (Antonioli et al., 2004; Corradi et al., 2004). The phenotypic transition of CML, in this case, involves a process denominated dedifferentiation previously described in androgenic blockade processes (Corradi et al., 2004) and after estrogen treatments (Zhao et al., 1992), where the muscle cells assume an essentially secretory phenotype (Vilamaior et al., 2005).

In CE and CaE animals the epithelium tends to be long and cylindrical, which can justify the decreased luminal volume in those groups. The morphometric data revealed that even the E and CaE animals had increases in the nuclear area and perimeter, besides increase in epithelial height, agreeing with data obtained previously by Scarano et al. (2003). Furthermore, dysplastic processes with alteration of the epithelial structure, allied with hyperplasia in determinate regions, contribute to the relative increase of the epithelium verified in these animals.

Internally, the epithelial cells showed dilated endomembranes in some areas and presence of circular endoplasmic reticulum in intact animals treated with estradiol. That structure had been described previously by Kjaerheim et al., (1974) and compared to the structure found in castrated animals where reduction was observed in the size of synthesis organelles and number of ribosomes.

Scarano et al. (2004) described intraepithelial alterations in guinea pigs treated with estradiol, and observed the emergence of PINs with relative increase of the epithelial compartment.

Receptors specific for estrogen were identified in both the epithelium and stroma (Prins & Birch, 1997). Experimental studies have been demonstrating that estrogen is involved in the induction of premalignant and malignant alterations (Weihua et al., 2001, Scarano et al., 2004). According to Cunha (2002), the epithelial alterations, such as scamous metaplasia, require estrogen action through stromal αER (paracrine mechanism), as well as by the αER epithelial route (autocrine). Such discoveries agree with the results observed here, where higher density of αER-positive cells was found in hyperplasic areas, showing that estrogen has effector routes that do not depend on androgenic levels.

In spite of that, it is important to emphasize that the treatments in which estradiol is present show larger AR expression. According to Droller (1997), estrogen induces stromal fibroblasts to express receptors for both epidermal growth factor (EGF-R), and fibroblast growth factor (FGF-R), besides increasing the level of androgen receptors. The androgenic effects on normal epithelium are explained by paracrine factors produced by stromal AR-positive cells (Cunha et al., 2002). However, androgenic regulation of prostatic epithelial cells during malignant transformation of prostatic epithelial cells appears

to involve conversion from a paracrine to an autocrine mechanism of androgen-stimulated growth (Gao et al, 2001). Perhaps this mechanism of autocrine performance explains the increased density of AR-positive cells in the hyperplasic areas and in PINs.

Droller (1997) identified estrogen receptors in stromal cells. According to this author, estrogen induces receptor expression for specific growth factors, increasing the synthetic activity of those cells and also of the smooth muscle cells. This incentive may be responsible for the increase in synthesis of extracellular-matrix fibers and for the apparent increase in the stromal compartment of the estrogenized animals, observed in both intact and castrated animals. However, in a subtle way, the augmented synthetic capacity can be related to the sensitive decrease of intra-prostatic androgenic levels, despite not having shown a statistical difference among the serum testosterone levels in the intact animals, provoked by the estrogenic supplementation (Härkönen & Mäkelä, 2004), as observed in a very evident manner in castrated animals (Vilamaior et al, 2005).

Despite verifying that castration increased the stromal compartment as much as estrogenic treatments and that both stimulated the fibrillary and non-fibrillary constituent synthesis of the extracellular matrix, it should be emphasized that the distribution of those constituents in each case is different: In the castrated animals deposition or preferential accumulation of conjunctive constituents happens beneath the epithelium, while in the estrogenic treatments an accumulation of conjunctive constituents occurs around the smooth muscle cells. This observation exposes a possible role of SMC in the arrangement of the constituents of the extracellular matrix during the rearrangement process caused by estradiol (Scarano et al., 2005) and in BPH (Cardoso et al., 2004). Besides, it was observed that in the intact animals treated with estradiol, the frequency of elastic fibers was higher, in agreement with the data obtained previously by Scarano et al., 2005.

The results obtained in this work verified that estradiol has direct effects on gerbil prostate independent of the androgenic levels. Such effects are represented mainly by the proliferative and dysplastic epithelial alterations and by the architecture of the extracellular-matrix constituents, implying a possible role of smooth-muscle cells in the post-treatment arrangement. Besides, hormonal receptors were identified that seem to have fundamental role in the expression of altered epithelial phenotypes. Finally, this experimental model was shown to be important and promising in the study of the prostatic diseases of hormonal etiology.

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Legends of figures

Figures 1a-4d – Histologic sections stained by hematoxylin-eosin. **Figs. 1a and 1b**: Intact control group. 1a- General aspect of the prostatic acinun; 1b- Detail of the epithelium (e) and smooth muscle cells layer (smc) of adult prostatic acinun. **Figs. 2a-2d**: Estradiol-treated group (E). 2a- General aspect of a prostatic acinun with high epithelium and dysplasia. In 2b cells dislocated to the apical regions (arrow), simulating a possible detachment and secretory granules in the lumen were observed. 2c – Detail of the transition epithelium-stroma; arrow point the subepithelial stroma and the fusiforms smooth muscle cells. Between the SMC conjunctive tissue was observed. 2d- A high-grade Prostatic Intraepithelial Neoplasia (PIN).**Figs. 3a and 3b**: Castrated group. 3a- Prostatic acinun with a low epithelium (ep), the smooth muscle cells (smc) and evident subepithelial conjunctive stroma (arrow). 3b- Detail of the prostatic acinun where a prominent subepithelial conjunctive stroma (arrow) and smooth muscle cells (smc) with irregular aspect were noticed. **Figs. 4a-4d:** Estradiol-treated castrated group (CaE). 4a- Prostatic acinun showing the epithelial arrangement with cribriform aspect (ep). Arrow points to subepithelial conjunctive stroma. 4b- Arrows point to basal membrane with sinuous aspect e abundant conjunctive stroma. Arrow head show the SMC with irregular spine-like cytoplasmic projections. Notice the high epithelial cells (ep). 4c- Presence of PIN and abundant subepithelial stroma (arrow) and a substantial layer of SMC.4d- Detail of epithelium-stroma transition showing the subepithelial conjunctive tissue (arrow), the SMC and the dysplasic epithelium (ep). ep: epithelium; l:lumen; smc: smooth muscle cells

Figures 5a, 6a, 7a and 8a: Histologic sections stained by Gömöri's reticulin; **figures 5b, 6b, 7b, and 8b:** histological sections submitted to chondroitin sulphate IHC. **Figs. 5a and 5b:** intact control group; figs. 6a and 6b: Estradiol-treated group; **figs. 7a and 7b:** Castrated group; **figs. 8a and 8b:** Estradiol-treated castrated group. Arrows point positive demarcation to reticulin fibers and chondroitin sulphate, respectively. asterisk (*) : collagen fibers; ep: epithelium; l: lumen.

Figures 9a-10c: Histologic sections submitted to ERα IHC. **Fig. 9a:** Intact control group; **figs. 9b-c:** Estradiol-treated group; **fig. 10a:** Castrated group; **figs. 10b-c:** Estradiol-treated castrated group. Brown stain means positive demarcation.

Figures 11a-12b: Histologic sections submitted do AR IHC. **Fig. 11a:** Intact control group; **fig. 11b:** Estradiol-treated group; **fig. 12a:** Castrated group; **fig. 12b:** Estradiol-treated castrated group. Brown stain means positive demarcation.

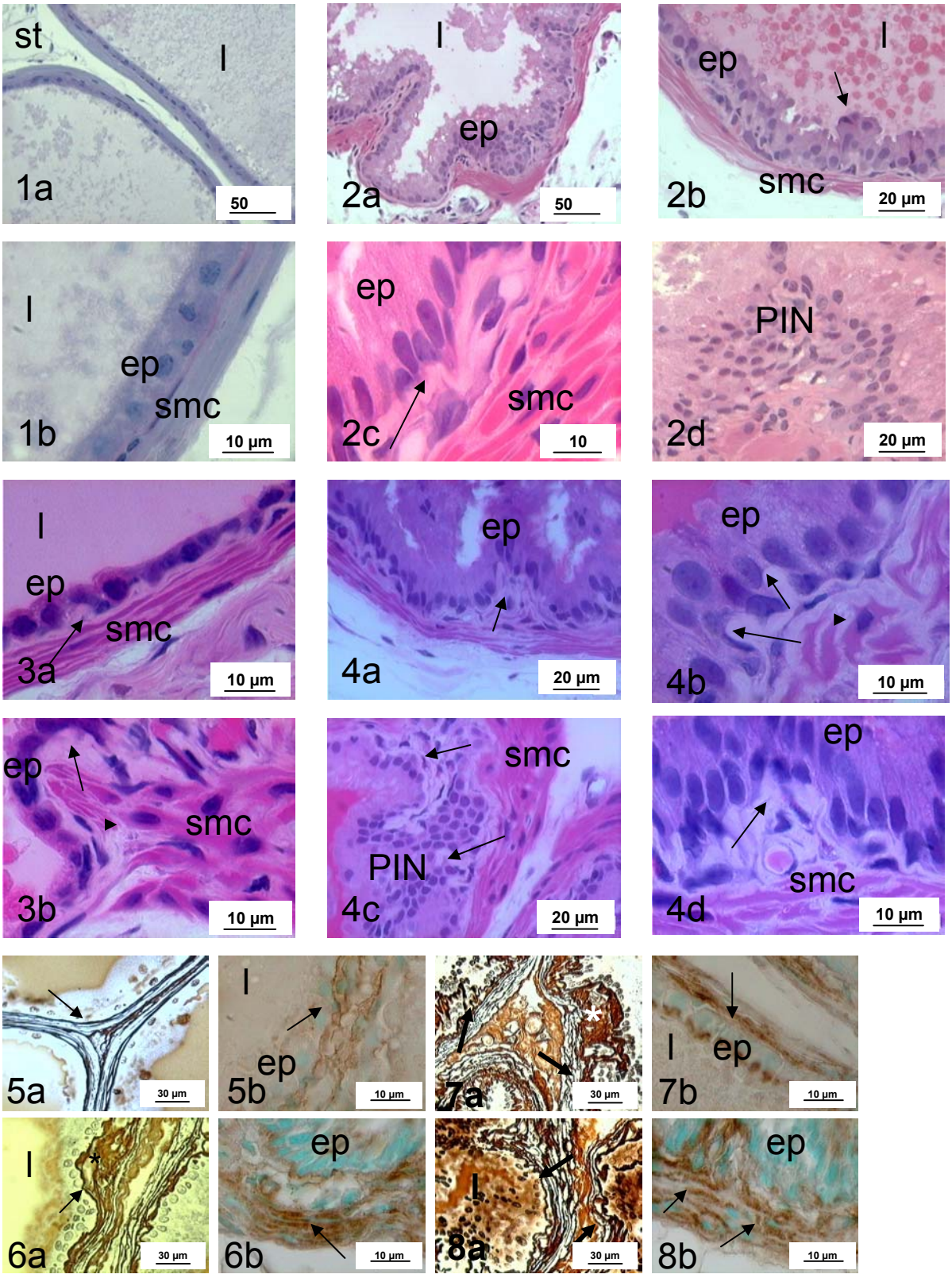
Figures 13-22: Ultrastructure figures. **Fig. 13:** Epithelial cells (ep) from intact control prostate. Arrow points to secretion organelles; bar: 2.60 µm. **Fig. 14:** Epithelium-stroma transition . Arrow point to basal laminae; in the stroma the smooth muscle cells (smc), fibroblasts (fib) and scarce collagen fibers (col); Intact control group; bar: 0.94µm. **Fig. 15:** Abundance of bunches of collagen fibers (col) in the subepithelial stroma and fibroblasts (fib); ep: epithelium; Ca group; bar: 1.56 µm. **Fig. 16:** Detail of the activated fibroblast (fib) with dilated endoplasmic reticulum cisternae (arrow); Ca group; bar: 0.72µm. **Fig. 17:** Sinuous aspect of basal laminae (bl) with collagen deposition (col) in the subepithelial stroma; Ca group; bar: 0.72 µm. **Fig. 18:** Irregular arrangement of SMC and collagen fibers (col); Ca group; bar: 1.56 µm. **Fig. 19:** Epithelial cells from estradiol-treated castrated prostate; bar: 2.60µm. **Fig. 20:** PIN in estradiol-treated prostate; bar: 3.36µm. **Fig. 21:** Detail of an epithelial cell from estradiol-treated prostate, showing the circular endoplasmic reticulum; bar: 0.56µm. **Fig. 22:** A high epithelial cell from estradiol-treated group. The arrow points to dilated cisternae; bar: 2.01µm. n: nuclei; ep: epithelium; st: stroma; smc: smooth muscle cells.

Figures 23- 30: Ultrastructure figures. **Fig. 23:** General aspect of stromal compartment showing the SMC with ER prominent in estradiol-treated castrated prostate; bar: 1.21µm. **Fig. 24:** The SMC with dilated Golgi apparatus cisternae (arrow); E group; bar: 0.56µm. **Fig. 25:** The SMC with irregular spine-like cytoplasmic projections surrounded by collagen fibers (col); CaE group; bar: 1.21µm. **Fig. 26:** The activated fibroblast replete of ER and with dilated Golgi apparatus cisternae (Ga). Notice the prominent nucleolus; E group; bar: 1.21µm. **Figs. 27 and 28:** Detail of SMC prolongations and collagen (col) arrangement between them. el: elastic fibers; CaE group; (27) bar: 1.56µm, (28) bar: 1.21µm. **Figs. 29-30:** Detail of SMC showing the intimate contact with elastic fibers (el) in cytoplasmic prolongations and collagen fibers (col); E group; (29) bar: 0.72 µm, (30) bar: 0.94µm. n: nuclei; nu: nucleolus.

Table 1. Quantitative analysis from experimental groups.

Quantitative analysis	***Experimental Groups***			
	C	E	Ca	CaE
Morphometry (µm)				
Epithelium height	$11.08^{a} \pm 2.14$	$22.32^{b} \pm 4.80$	$10.16^{a} \pm 2.73$	$19.42^{b} \pm 3.24$
SMC layer thickness	$8.51^{a} \pm 1.85$	$11.87^{b} \pm 2.00$	$12.38^{b} \pm 2.43$	$11.75^{b} \pm 2.48$
Caryometry of secretory cells				
Nuclear area *(μm^2)*	$23.15^{a} \pm 3.84$	$30.06^{b} \pm 4.27$	$21.15^{a} \pm 4.51$	$27.80^{b} \pm 4.96$
Nuclear perimeter *(µm)*	$21.27^{a} \pm 2.80$	$22.96^{a} \pm 3.20$	$19.61^{a} \pm 2.61$	$21.94^{a} \pm 2.74$
Relative proportion of tissue components (%)				
Epithelium	$17.34^{a} \pm 2.64$	$28.15^{b} \pm 2.66$	$17.87^{a} \pm 4.64$	$33.90^{b} \pm 12.47$
Stroma	$38.77^{a} \pm 10.04$	$38.42^{a} \pm 5.34$	$50.13^{b} \pm 7.85$	$41.20^{a} \pm 10.25$
Lumen	$43.89^{a} \pm 10.52$	$33.46^{b} \pm 6.19$	$32.0^{b} \pm 11.99$	$24.90^{c} \pm 13.52$
Body weight (g)	80.0 ± 8.7	79.5 ± 7.0	78.8 ± 8.5	81.2 ± 6.5
Prostate weight (g)	$1.04^{a} \pm 0.25$	$0.55^{b} \pm 0.16$	$0.64^{b} \pm 0.11$	$0.76^{b} \pm 0.17$
Relative prostate weight (g prostate/g body weight)^2	$0.013^{a} \pm 0.002$	$0.007^{b} \pm 0.001$	$0.008^{b} \pm 0.001$	$0.009^{b} \pm 0.001$

Statistical analyze based to ANOVA and Tukey tests. Different superindices: a, b, c, d; indicate significant statistically difference: p≤ 0,05.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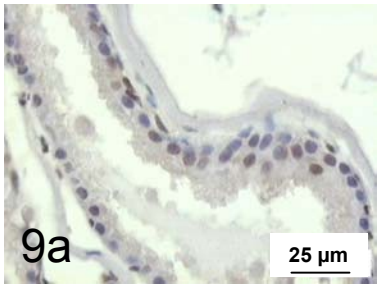
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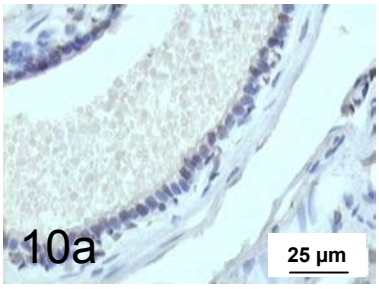
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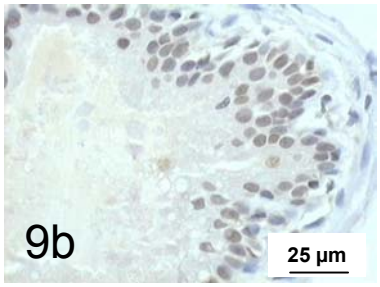
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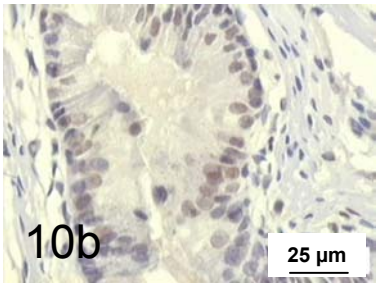
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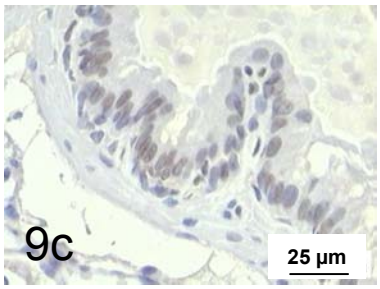
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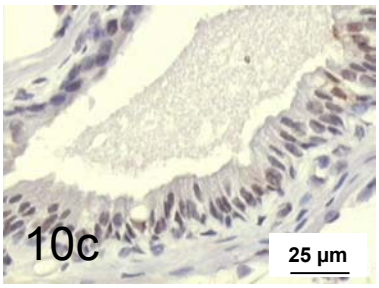
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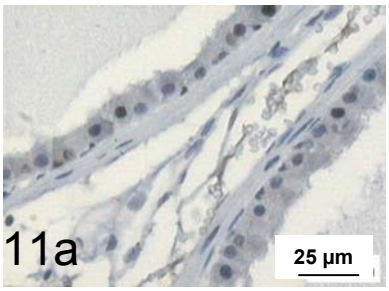
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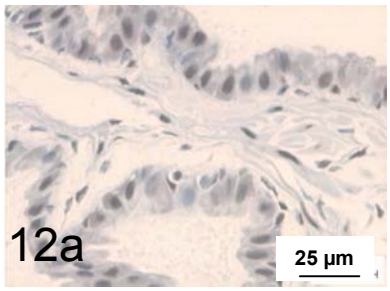
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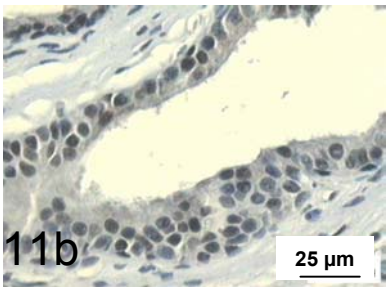
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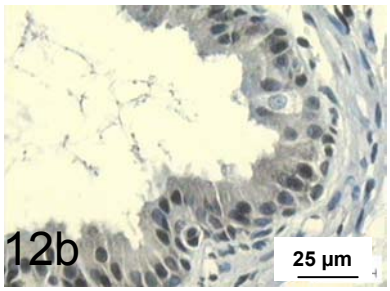
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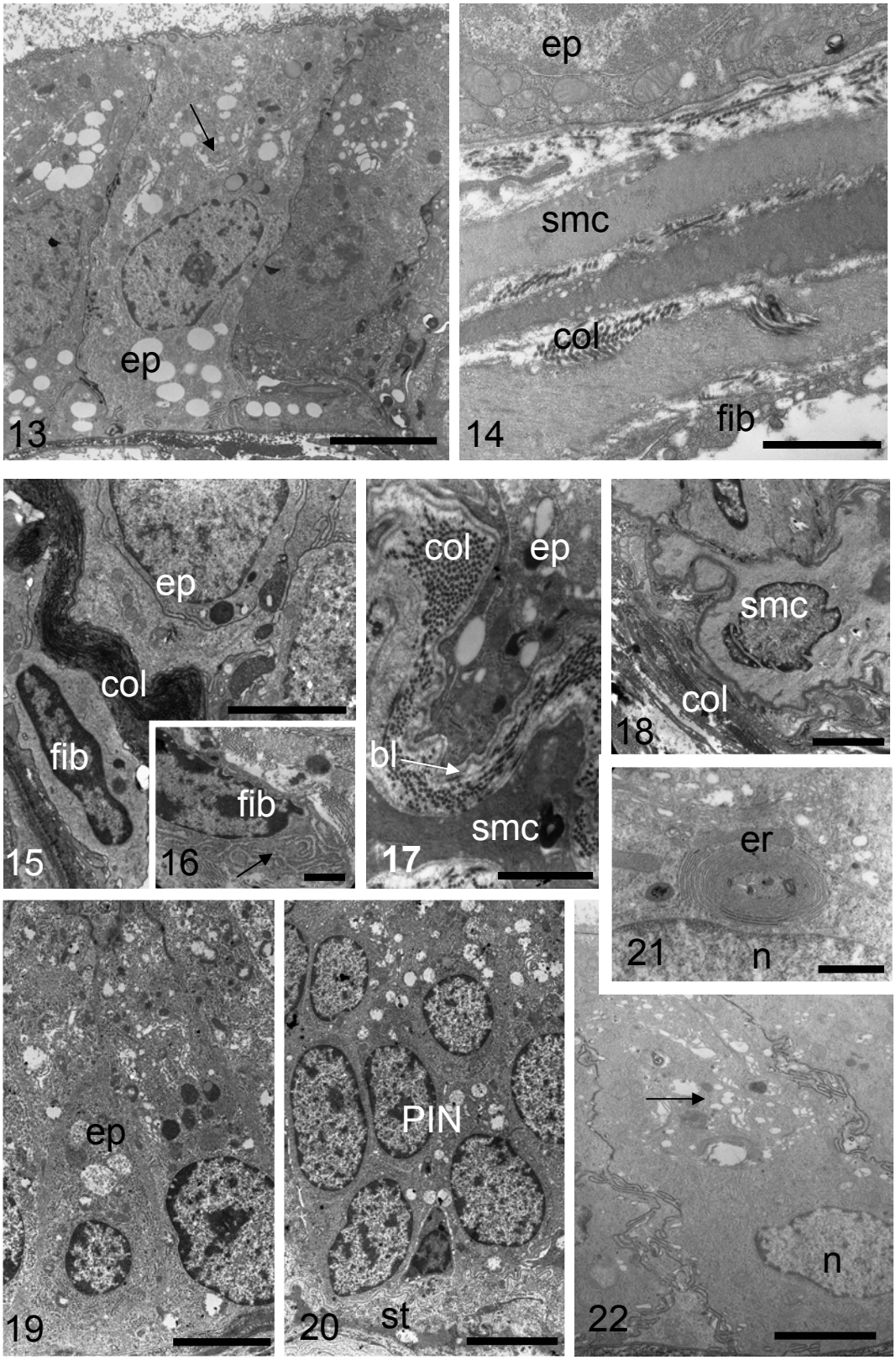
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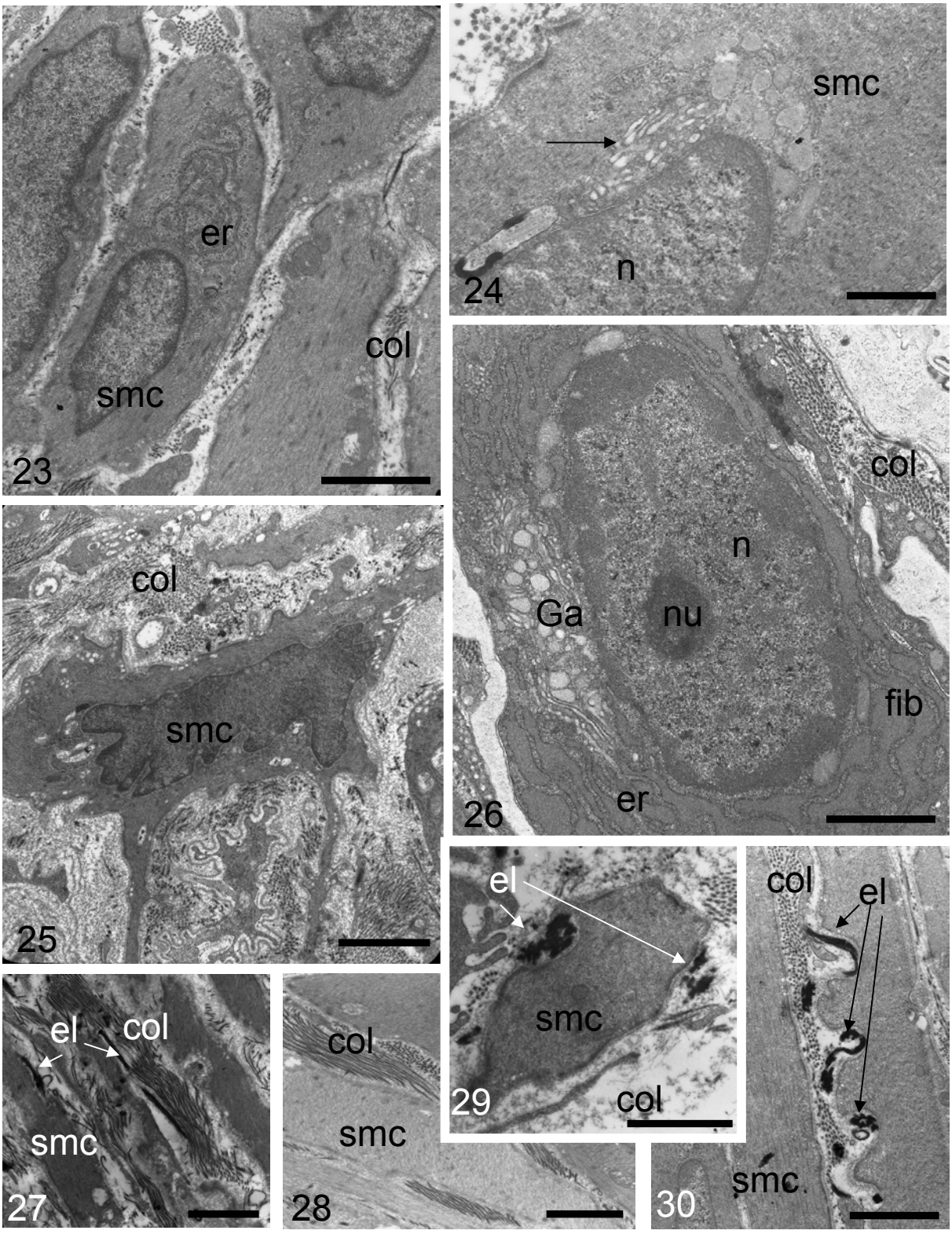
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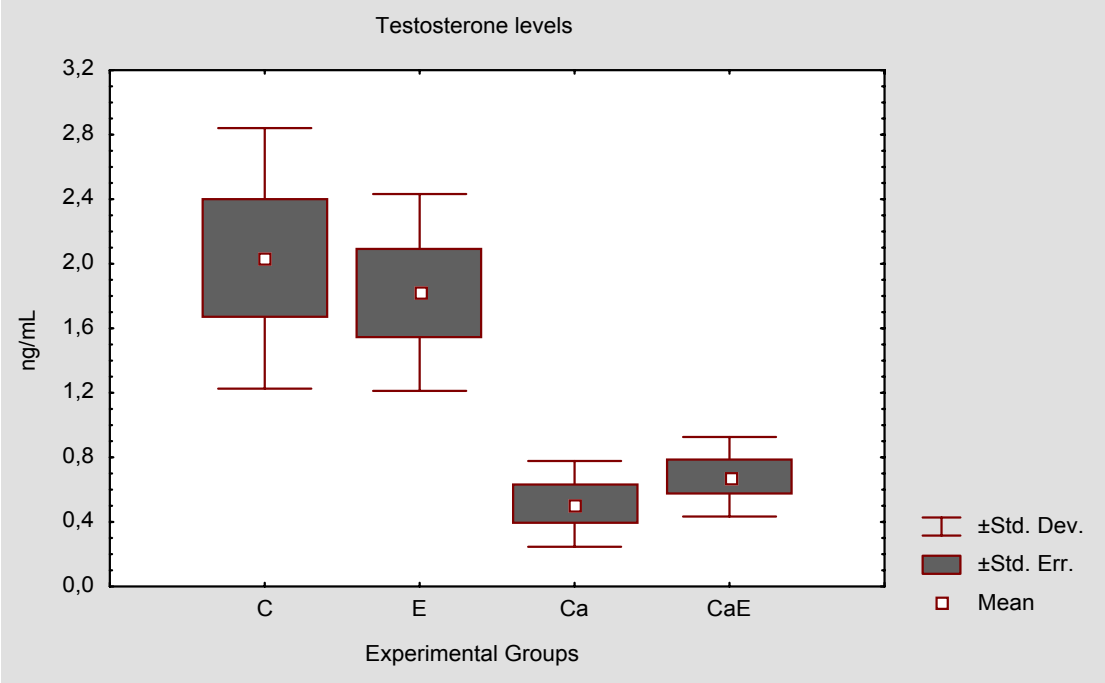
Figure 31 – Serum testosterone levels from experimental groups. Mean±SD.



Statistical analyze based to ANOVA and Tukey tests. Different superindices: a, b; indicate significant statistically difference: p≤ 0,05.

ARTIGO 2

TISSUE EVIDENCE OF THE TESTOSTERONE ROLE ON THE ABNORMAL GROWTH AND AGING EFFECTS REVERSION IN THE GERBIL *(MERIONES UNGUICULATUS)* PROSTATE

Accepted to Anatomical record

Tissue evidence of the testosterone role on the abnormal growth and aging effects reversion in the gerbil *(Meriones Unguiculatus)* prostate

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Running title: Effect of the testosterone on the gerbil prostate at different ages.

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Abstract

Prostate differentiation during embryogenesis and its further homeostatic state maintenance during adult life depend on androgens. Abundant biological data suggest that androgens play an important role in the development of the prostate cancer and other prostatic diseases. The objective of this work was to evaluate the effects of the testosterone supplementation in gerbil (a new experimental model) at different ages. Tissues from experimental animals were studied by histological and histochemistry procedures, AR-immunohistochemistry assay, morphometric-stereological analysis and transmission electron microscopy (TEM). After the treatment were observed increase of prostate weight, epithelium height in all ages studied. In some adult and aged treated animals, hyperplasic and displasic process were observed, including PIN´s and adenocarcinomas. Increase of the tickness of the smooth muscle cells layer (SMC) were observed in pubescent and adult animals and TEM revealed apparent SMC hypertrophy. An apparent increase in the frequency of blood vessels distributed by the subepithelial stroma in the treated animals was noticed. Reversion of the natural effects of aging on the prostate was observed in the aged treated animals in some acini of the gland. These data demonstrate that the gerbil prostate is susceptible to androgenic action at the studied ages and it can serve, for example, as experimental model to studies of prostate neoplasic process induction and hormonal therapy in aged animals.

Keywords: testosterone, prostate, stroma, epithelium, gerbil

Introduction

Androgens are steroid hormones that induce the differentiation and maturation of the male reproductive organs and the development of the male secondary sex characteristics.

Prostate differentiation during embryogenesis and its further homeostatic state maintenance during adult life depend on androgens. The normal prostatic epithelium is composed of different cells types that have varying androgen sensitivities, including androgen independent basal stem cells, androgen-dependent luminal secretory cells, and androgen-independent but androgen-sensitive transitional cells (Isaacs, 1999). Thus, the normal prostate is inherently heterogeneous in its sensitivity to androgens.

Testosterone enters the prostate cell by passive or active diffusion. Once in the cytoplasm it will remain as testosterone or transform to DHT by 5α-reductase and will be attached predominantly to a cytoplasmic receptor or androgen receptor (AR) or to a nuclear receptor (Rosner et al., 1999).

Epithelial ARs are required for expression of AR-dependent prostatic secretory proteins but many androgenic effects on epithelium, as regulation of proliferation of normal epithelia, are elicited by paracrine factors produced by AR-positive stroma (Donjacour and Cunha, 1993). Conversely, hormonal regulation of epithelial differentiation and functions requires direct hormonal action mediated epithelial hormone receptors (Buchanan et al., 1998; 1999). Androgenic regulation of prostatic epithelial cells during malignant transformation of prostatic epithelial cells appears to involve conversion from a paracrine to an autocrine mechanism of androgen-stimulated growth (Gao et al, 2001).

Abundant biological data suggest that androgens play an important role in the development of the prostate cancer. The growth and maintenance of the prostate are dependent on androgens, prostate cancer regresses after androgen ablation or anti-androgen therapy, and testosterone induces prostate tumors in laboratory animals (Shirai et al.,2000; So et al., 2003; Zanetoni et al., 2005).

Current evidence indicates that serum levels of sex hormones carry no relations to the development of prostate cancer, and there is either no change or only a modest increase in PSA after

testosterone administration (Nomura et al., 1988). The suspicion of prostate cancer is, however, an absolute contraindication for androgen therapy.

On the basis of these considerations, the aim of the present study was to investigate the effects of testosterone supplementation on the gerbils prostate at different phases of the postnatal development, trying to establish the possible model to experimental carcinogenesis.

Material and Methods

Animals and hormone treatments

To accomplish the work, 30 male *Meriones unguiculatus* gerbils of the following ages were used: pubescent (40 days after birth), adult (120 days after birth) and aged (12 months after birth).

For each age group, the animals were divided in two groups of 5 each for control and treated groups. In each case, the treated group received in alternate days subcutaneous injections of testosterone cipionate diluted in vegetal oil (10 mg/ml) at a dose of 0,1 ml/appication/animal (1mg/application) for 21 days, while the control group received only vegetal oil (SANTOS et al., 2006, *in press*).

After 21 days of treatment, the animals of all ages and of both groups were anesthetized lightly by CO_2 and killed by decapitation. The ventral prostate was removed and submitted to light microscopy and ultrastructural procedures.

Hormonal serum levels

Blood were colleted after the decapitation and the serum was separated by centrifugation and stored at -20ºC for subsequent hormone assay. The determination of serum levels of testosterone it was

performed by luminescence-immunoassay (mouse antibodies anti-testosterone – Jhonson & Jhonson ®, USA) in automatic analyzer to control group. The intra-assay and inter-assay variation was 4.6 and 4.3%, respectively.

Histochemistry

Ventral prostates of control and testosterone-treated groups were cut into fragments and immediately fixed by immersion, for 24 hours in Karnovski fixative (0,1M Sörensën phosphate buffer pH 7.2 containing 5% paraformaldehyde and 2.5%, glutaraldehyde). Fixed tissue samples were dehydrated in a graded ethanol series and embedded in glycol methacrylate resin (Leica historesin embedding kit). Histological sections (3-μm) were subjected to hematoxylin-eosin (H&E) staining for general studies, to Gömöri´s reticulin (Gömöri, 1937) staining for collagen and reticular fibers and to Feulgen (Mello and Vidal, 1980) staining for nuclear study. Microscopic analyses were performed on Zeiss-Jenaval or Olympus photomicroscopes, and the microscopic fields were digitalized using the Image-Pro®Plus version 4.5 for Windows™ software.

Morphometric and Stereological analysis

Using an analyzing system of images (Image Pro-Plus), H&E and Feulgen sections were analyzed. Images of 50 histological fields for each experimental group in the ages studied were analyzed, such that histological fragments of all animals were evaluated equally. The morphometric analyze was performed to evaluate to epithelium height, smooth muscle cells layer thickness, and nuclear area of the secretory epithelial cells. For this comparative study were realized 200 measurements to each parameter. Stereologic analyses were obtained by Weibel's multipurpose graticulate with 120 points and

60 test lines (Weibel, 1979) to compare the relative proportion among the prostatic components (epithelium, stroma and lumen of acini) in the different ages in both experimental groups.

Statistical analysis

The testosterone effects on gerbil ventral prostate were evaluated by analyses of mean ± standard deviation (SD). The statistical analysis was performed in the Statistica 6.0 software (Copyright©StatSoft, Inc. 1984-1996). The hypothesis test Anova, and Tukey HSD test were employed and, $p \leq 0.05$ was considered statistically significant.

Transmission Electron Microscopy

The ventral prostates of control and treated gerbils were processed for transmission electron microscopy as described previously (De Carvalho et al., 1994), employing the fixation procedure of Cotta-Pereira et al. (1976). Briefly, tissue fragments were fixed in 0.25% tannic acid plus 3% glutaraldehyde in Millonig's buffer, dehydrated in acetone, and embedded in Araldite resin. Silver sections obtained with a diamond knife were stained by uranyl acetate and lead citrate. Observation and electron micrographs were made with a LEO – Zeiss 906 transmission electron microscope.

Immunohistochemistry (IHC)

AR (N-20, 1:100 dilution; rabbit polyclonal antibody, Santa Cruz Biotechonology, Santa Cruz, CA, USA) was used for IHC. Immunohistochemistry staining was performed using the avidin-biotin complex (ABC) kit (Santa Cruz Biotechnology, CA, USA). The tissue fragments to IHC were fixed by immersion in the formaldehyde solution at 10% and embedded in paraplast. The sections (5µm) were dewaxed and then

rehydrated in graded alcohol and distilled water. Antigenic recuperation was realized in citrate buffer in high temperature (100ºC) for 45 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 45 min, followed by a quick rinse in distilled water and phosphate-buffered saline (PBS). Sections were incubated with normal goat serum and primary antibody at 4ºC overnight. The slides were them incubated with the biotinylated anti-rabbit at 37ºC followed by peroxidase-conjugated avidin-biotin complexes and diaminobenzidine (DAB). The sections were then counterstained with hematoxylin of Harris. For negative control, the primary antibody was replaced with the corresponding normal isotype serum.

Results

The prostate gland, in intact animals, presented acini with simple cylindrical epithelium, surrounded by a fine strip of vascularized conjunctive tissue and a layer of smooth muscle cells (SMC) (figs.1a; 2a; 3a). Among the acini loose vascularized conjunctive tissue was observed.

The epithelial cells of the gland have evident secretory characteristics, and in some places could be observed extrusion granules, typical of apocrine secretory cells (figs. 1a, 3a). Other secretory characteristics were based on the presence of clear supranuclear areas designated to be the area of the Golgi apparatus (1a, 2a and 3a). Dysplastic regions were observed in aged control animals in some acini (fig. 3a).

Ultrastructurally, the secretory glandular epithelium was formed by prismatic cells that vary from short to high, depending on the age, whereas the nucleus had basal polarity and a large amount of endoplasmic reticulum (figs. 7 and 8). Besides, it was possible to differentiate clearly between the secretor cells and the basal cells (fig. 7). The basal cells were located at the base of the epithelium in intimate contact with the basal laminae and they were poor in endoplasmic reticulum (fig. 7). Sometimes,

it was possible to identify electrondenses corpuscles, probably of ceramide, between the epithelial cells (fig.7).

Adjacent to the epithelium and intermixed among the smooth muscle cells were observed collagen and reticular fibers (fig. 9). However, the reticular fibers became denser at the epithelial base, adjacent to the basal membrane and intermixed among the SMC (figs. 1d, 2f, 3f). In the aged animals, there was a stromal rearrangement, where accumulation of collagen fibrils was observed adjacent to the epithelium and among the prostatic acini (fig. 3f , 10 and 11), and apparent hypertrophy of the SMC (fig. 10), as can be observed in table 1 which compared the control animals of the other ages in relation to thickness of the SMC layer and to the relative volume occupied by the stromal compartment. Besides, there was decrease in the serum testosterone levels in the aged group, in relation to the pubescent and adult animals (fig.20).

The testosterone-treated group presented significant increase in the weight of the prostate, but without suffering variation in the corporal weight when compared to the control groups (table 1). Increase was observed in the height of the secretor epithelium in relation to the control group at all of the ages (figs. 1b, 1c, 2b, 3e, 12, table 1), as well as in the volume occupied by the epithelium at the pubescent and adult ages. After treatment with testosterone the nuclear area and perimeter were unaltered only in the pubescent animals, while in the adult and aged animals there were increases of the nuclear area and perimeter in the treated animals (table 1).

The morphometric data indicated significant increase in the thickness of the SMC layer of the acini in relation to the control group at the pubescent and adult ages, while in the aged animals there was a decrease in the treated animals. In the stroma, there was no alteration in relation to the relative volume among the groups in the pubescent and adult animals, with a decrease only in the treated aged animals (table 1). The absolute data showed significant increases in all of the appraised parameters since there was increase of the prostatic weight, without alteration in the body weight (table 1).

The histopathological analysis showed that in the testosterone treated animals at all ages occurred an increase in the supranuclear area, where they concentrate in the synthesis organelles, mainly the Golgi apparatus, which appears prominent and dilated after the treatment (figs. 1b, 1c, 2b, 3e, 12, 13 and 15).

An enlargement of the endoplasmic reticulum cisterns was noticed, mainly in the treated adult animals, which appeared with a vesiculous aspect, in all cytoplasm of the secretory cells (fig. 14).

Mitotic figures were evidenced at all ages after the testosterone treatment (figs. 1c, 2d and 3c). In the pubescent animals, this treatment did not provoke relevant alterations, but dysplasias in some acini were observed (fig. 1f). In the adult animals, Prostatic Intraepithelial Neoplasia (PIN) of varied degrees was observed as having acted in figures 2c (high-grade PIN) and 2h (low to medium-grade PIN). Besides, some of these animals presented adenocarcinomas and presence of microacini surrounded by tumorous cells (figs. 2d and 2e). In some areas of PIN, some nuclei were observed with a differentiated chromatin distribution pattern (fig. 2h).

In the aged individuals the treatment with testosterone evidenced two different effects: in some acini reversal of the aging process was observed, where there were noticed increase of the epithelium, decrease of fibrillar elements of the extracellular matrix, decrease in the thickness of SMC layer and increase in the relative volume of the lumen (figs. 3d, 3e, 18, table 1), each resembling, phenotypically, the adult control animals (fig. 2a); in other acini hyperplasic processes and presence of PIN were evidenced (fig. 3b, 3c, 3h), as well as the presence of cells denominated here as basophiles of granular cytoplasmic aspect (fig. 3i).

An apparent increase in the frequency of blood vessels distributed by the subepithelial stroma in the treated animals was noticed (figs. 1b, 3g and 19). In the stromal compartment of the treated pubescent animals some populations of SMC appeared hypertrophic, but without clear cytoplasmic alterations (fig. 17). In treated adult animals it was common to observe hypertrophic cells with prominent

nucleoli and abundant endoplasmic reticulum (fig. 16). In general, alterations in the distribution and density of collagen and reticular fibers were not observed under light microscopy, although in some areas rapid accumulation of collagen fibrils was observed by electronic microscopy, when compared to the control group (figs. 1e, 2g, 9 and 16).

In the aged animals, the testosterone treatment showed a pattern of stromal organization similar to that observed in untreated adult animals (fig. 3g), differing from the pattern of the control group where abundance of components of the extracellular matrix was found (fig. 3f).

The data also show that with the advancement of chronological age the AR expression becomes less frequent, increasing the index of negative demarcation among the untreated animals (figs 4a, 5a and 6a). After the treatment, at all of the ages, increase in the expression of the androgenic receptors was observed, mainly in the atypical regions where the epithelium shown hyperplasic and dysplasic processes (figs 4a, 4b, 5a, 5b, 6a and 6b).

Discussion

The histology, histochemistry and ultrastructure of the gerbil prostate were described by Campos et al. (2006, *in press*). The described aspects indicated that the prostate of this rodent seems to be a good model for experimental studies, because it is susceptible to pathological alterations similar to those found in the human prostate gland, besides being animals easy to maintain in captivity.

Androgens are required in functional activities and the normal growth of the prostate to maintain homeostasis of the organ (Cunha et al., 1986; Debes and Tindall, 2002). The functions of testosterone in the prostate are moderated by androgen receptors (AR), which control androgenic responses in the epithelium and in the stroma (Wang et al., 2001).

The increase in the weight of the organ after testosterone treatment is associated with the anabolic factor exerted by the testosterone, in which there is production of growth factors favoring prostatic hypertrophy (Thomson, 2001).

The increase of the epithelial height, along with the prominence of the Golgi area, demonstrates the probable increase in secretory activity of the epithelial cells in the treated animals in all of the ages. Such association is directly related to an increase of the intracellular secretory machinery: RER and Golgi (Gross and Didio, 1987).

The testosterone participates in the process of prostate development, including the secretory processes, stimulating the synthesis of constituent substances of the sperm (Price, 1963; Aumüller and Seitz, 1990; Rosai , 1996; Hayward et al., 1997; Thomson et al., 1997). Studies using castrated or aged animals with low androgenic levels show that testosterone is capable of increasing the height of epithelial cells and of the secretory apparatus (Scarano et al., 2003).

Heterogeneity was observed in the expression of ARs among the acini in all age groups. This fact is associated with the existence of cellular clones more sensitive to the androgenic stimulus and its effects. This justifies the presence of the acini with neoplastic lesions beside morphologically normal acini. In the treated groups, there was greater density of AR-positive cells suggesting higher susceptibility to the androgenic action in these animals (Brandes, 1966). Besides, it was noticed that the cells of areas of PIN and focal hyperplasia has larger index of positive marcation than in normal areas, inferring that these lesions are associated to AR expression and to the androgenic incentive (Gao et al., 2001).

Huynh et al. (2001) suggest that, in the prostate, the production of specific growth factors such as IGF-I is dependent on androgens. Such factors act in the activation or inhibition of genes that control the cellular cycle, favoring cellular proliferation Besides, genes that respond to androgens, through AR, are involved in control of cellular division (Galbraith and Duchesne, 1997). In animals whose cells possess predisposition or alteration genetic, such interaction can aggravate the proliferative character, such as

the inductor factor (Pollard and Luckert, 1986). This fact can justify the increase of the AR, mainly in hyperplasic and dysplasic regions of the epithelium.

Zanetoni et al. (2005) showed that the induction of tumor development is highly potentiated in the presence of testosterone in adult gerbils submitted to chemical carcinogenesis. This study points to histopathologic alterations similar to those found in our work such as PIN and adenocarcinomas. Furthermore, the gerbils possess a high index of spontaneous histopathologic alterations during the aging process (Zanetoni et al., 2001), similar to what happens in humans, suggesting the existence of genetic predisposition that can be potentiated after hormonal supplementation. Induction of invasive prostate carcinomas in the rat frequently requires long-term administration of a pharmacological dose of testosterone with or without application of a chemical carcinogen (Shirai et al., 2000)

Franck-Lissbrant et al. (1998) reported that testosterone stimulates angiogenesis in the ventral prostate of mice after castration, possibly from the metabolic necessity of cells after the hormonal incentive. The obtained data suggest apparent increase of the angiogenesis process in the prostate of treated animals, which possibly is involved in the increased energy consumption provoked by the process of cellular synthesis, since the activation of the compound AR-DNA is associated with transcriptional components and co-activators to promote gene transcription (Tsai and O'Malley, 1994).

The prominence of the smooth muscle cells, mainly in pubescent and adult animals, after treatment, can be involved with direct anabolic processes, such as cellular hypertrophy and increase of contractile filaments (McArdle et al., 2003). Besides, that fact can be linked to an increase in the synthesis of elements of the extracellular matrix, on account of increase of synthesis organelles including the endoplasmic reticulum, similar to what happens in animals after castration (Vilamaior et al., 2005).

In some areas, the collagen fibers appear in larger amounts in the treated pubescent and adult animals than in the group control, perhaps reflecting a discreet increase in the synthesis of that element of the extracellular matrix. The androgenic receptors are more abundant in the epithelium when compared to the stromal cells (Droller, 1997). Therefore, only an increase in the synthesis of the stromal

cells may happen that are responsive to testosterone, causing heterogeneity along the acini in the amount of fibril elements.

The decrease in the thickness of the muscular layer in treated aged animals is probably linked to alterations in the synthetic character of SMC. With the decrease in the testosterone levels, during aging, SMC start to develop greater synthetic activity, which justifies the increase in collagen fibers and morphologic alterations of these cells (Horsfall et al., 1994; Vilamaior et al., 2005; Campos et al., 2006, *in press*). With the increase in the testosterone levels, after the treatment, SMC reestablish a contractile and fusiform character, which promotes a decrease in the thickness of the muscular layer, similar to that in castrated animals treaties with testosterone (Sugimura et al., 1986).

Populations of basophilic cells were identified amid the acini epithelium, showing cytoplasmic granular aspect similar to that in neuroendocrine cells described by Capella et al., (1981).

These data demonstrate that the gerbils prostate is susceptible to androgenic action at the studied ages, showing proliferative and dysplastic effects mainly in adult and aged animals, perhaps suggesting a possible model for the study of induced neoplasias. Besides, they show reversal of some hypertrophic effects of aging mainly on the prostatic stroma, which calls for future studies of this rodent in terms of finding dose-dependent responses with the objective of elucidating the reversibility of aging effects of through hormonal therapy.

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Figures Legends

Figures 1a-1f. Histologic sections from pubescent animals. Figures 1a, 1b, 1c, 1f (H&E); 1d, 1e (reticulin). Control animals: 1a and 1d; Testosterone treated animals: 1b, 1c, 1e and 1f. Figure 1a show the general tissue aspect. In 1b the arrows point to the blood vessels (v) in the stroma and the (*) show the evident supranuclear clear area. In the figure 1c occur the presence of mitotic figures (fm), basal cells (bc) and prominent Golgi area (*). Figures 1d and 1e, the arrows point to reticular fibers of the stroma. The figure 1f show the displasic secretory epithelium. l: lumen; smc: smooth muscle cells; ep: epithelium.

Figures 2a-2h. Histologic sections from adult animals. Figures 2a, 2b, 2c, 2d, 2e and 2h (H&E); 2f and 2g (reticulin). Control animals: 2a and 2f; Testosterone treated group: 2b, 2c, 2d, 2e,2g and 2h. Figure 2a: general tissue aspect. In 2b, prominent supranuclear area (*) of the non-altered epithelium; The figure 2c show high-grade and low-grade of the Prostatic Intraepithelial Neoplasia (PIN). In 2d, adenocarcinoma

(ad) and presence of microacini (ma) surrounded by tumorous cells are observed. Figure 2e: detail of the carcinoma showing division of cells (mf). Figures 2f and 2g: arrows point to reticular fibers of the stroma. In 2h: presence of the PIN and some nuclei of the cells with a differentiated chromatin distribution pattern. l: lumen; smc: smooth muscle cells; ep: epithelium.

Figures 3a-3i. Histologic sections from aged animals. Figures 3a, 3b, 3c, 3d, 3e, 3h and 3i (H&E); 3f and 3g (reticulin). Control animals: 3a and 3f; Testosterone treated group: 3b, 3c, 3d, 3e,3g, 3h and 3i. Figure 3a: General tissue aspect where it is observed a displasic secretory epithelium. The prominent subepithelial stroma (st) and folds in the basal membrane. In 3b: a focal PIN and high epithelium. Figure 3c: detail of a PIN cell in division (figure mitotic: mf). Figures 3d and 3e: a high secretory epithelium and prominence of the supranuclear clear area (*). Figures 2f and 3g: arrows point to reticular fibers of the stroma. In 3f collagen fibers (col) are abundant in subepithelial stroma; and in 3g the evident presence of the blood vessels (v). Figure 3h: Hiperplasic process in some microacini with mitotic figures (mf). In 3i, presence of the basophilic cells of granular cytoplasmic aspect (bc). l: lumen; smc: smooth muscle cells; ep: epithelium.

Figures 4a-6b: AR immunohistochemistry. Pubescent animals: 4a (control) and 4b (treated); Adult animals: 5a (control) and 5b (treated); Aged animals: 6a (control) and 6b (treated). Brown stain means positive demarcation.

Figures 7-13: Ultrastructure figures. **7.** Pubescent control prostate. Detail of the epithelium (ep) and abundant endoplasmic reticulum cisternae (rer). Presence of ceramides granules between the secretory epithelial cells (arrow). st: stroma; bc: basal cell; n: nuclei; bar:2.01µm. **8.** Adult control prostate. Epithelial cell with secretion vesicles (arrow) and blebs (apocrine secretion). n: nuclei; st: stroma; bar: 2.01µm. **9.** Adult control prostate. Epithelium (ep)-stroma (st) transition. col: collagen fibers; smc: smooth

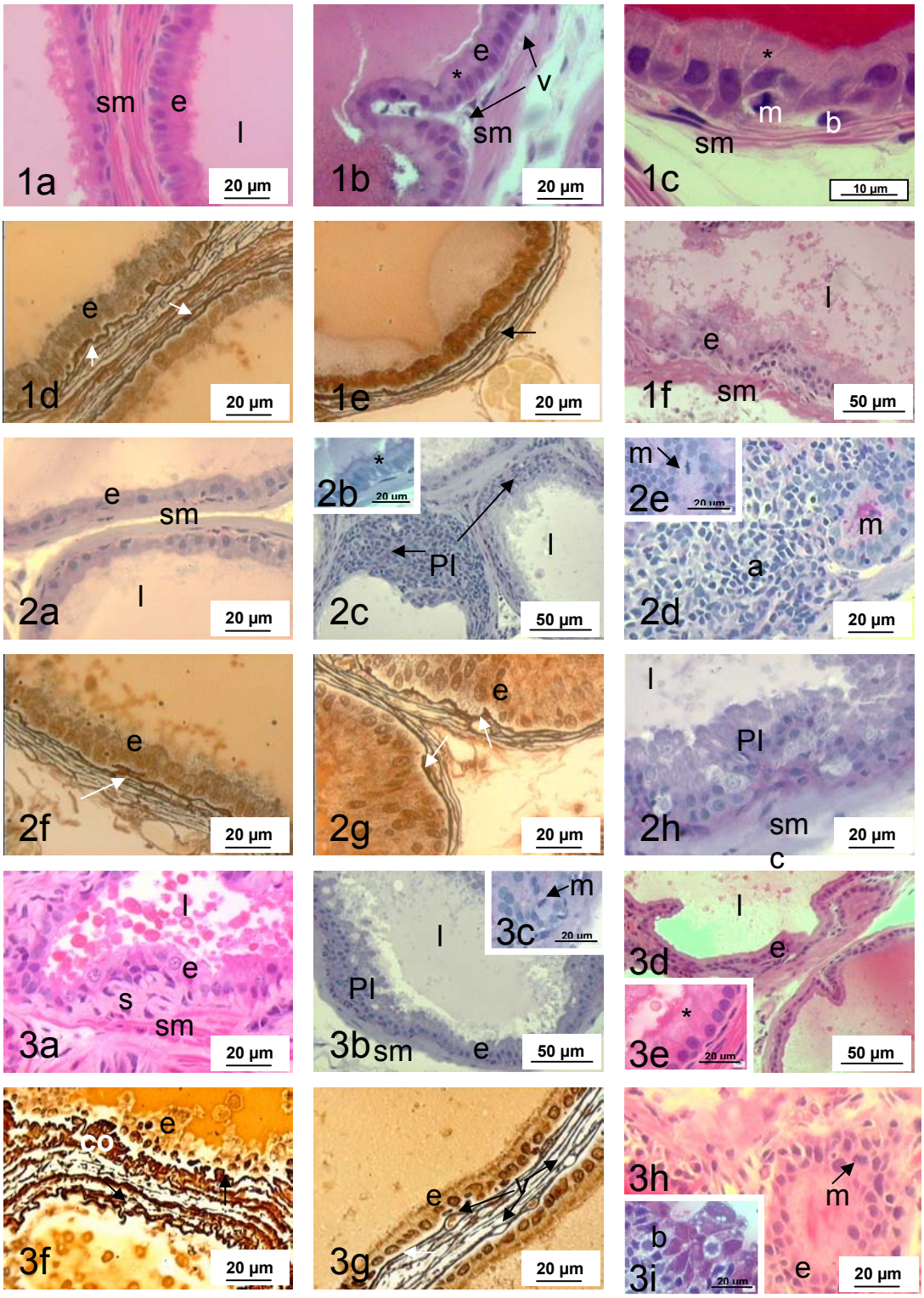
muscle cell; bar: 0.72µm. **10.** Aged control prostate showing the stromal compartment (st). Presence of bunches of collagen fibers in subepithelial stroma (col) and smooth muscle cells (smc); bar: 2.01µm. **11.** Aged control prostate. Subepithelial stroma with abundant collagen fibers; bar: 0.56µm. **12**. Adult treated prostate. A high epithelium (ep) with dilated cisternae (arrow). n: nuclei; bar: 4.34µm. **13.** Detail of the epithelial cell of adult treated prostate where it is possible to observe a prominent Golgi (arrows).n: nuclei; bar: 1.21µm.

Figures 14-19: Ultrastructure figures. **14.** Adult treated prostate. Secretory epithelial cells with dilated endomembranes of vesiculous aspect (arrows) for all cytoplasm. Presence of secretion vesicles (sv) and blebs (*). n: nuclei; nu: nucleolus; bar: 1.56µm. **15.** Aged treated prostate. Detail of the secretory epithelial cell with prominent Golgi (arrows) and secretion vesicles (*).n: nuclei; bar: 0.56µm. **16-17.** Pubescent and adult treated prostate, respectively. Detail of the stroma showing smooth muscle cells with prominent (rer) and evident nucleolus (nu). col: collagen fibers; n: nuclei; (16) bar: 1.56µm, (17) bar: 1.56µm. **18.** Aged treated prostate showing the fusiform smooth muscle cells (smc) arrangement in the stroma (st). ep: epithelium; bar: 1.21µm. **19.** Aged treated prostate. Subepithelial stroma (st) pointing blood vessels (v) adjacent to the basal laminae (bl); bar: 1.56µm. ep: epithelium.

Table 1 – Quantitative Analysis from experimental animals at different ages.

Quantitative data	***Experimental Groups***					
	Pubescent gerbil		***Adult gerbil***		***Aged gerbil***	
	Control	*Testosterone*	*Control*	*Testosterone*	*Control*	*Testosterone*
Morphometry (µm)						
Epithelium height	17.18 ± 3.66	23.90* ± 8.10	9.08 ± 2.14	17.81* ± 4.18	10.89 ± 2.21	14.69* ± 2.29
SMC layer tickhness	8.10 ± 3.08	10.49* ± 3.20	8.51 ± 1.85	10.61* ± 2.44	14.64 ± 5.10	11.39* ± 2.95
Caryometry of secretory cells						
Nuclear area (μm^2)	29.86 ± 7.57	29.38 ±5.49	23.15 ± 3.84	30.90* ± 6.46	19.81 ± 4.06	29.95* ± 5.70
Nuclear perimeter (µm)	22.30 ± 3.48	22.26 ± 2.90	21.27 ± 2.80	23.25* ± 3.61	19.78 ± 3.75	22.86* ± 3.22
Relative proportion of tissue components (%)						
Epithelium	16.50 ± 2.21	21.77* ± 2.21	17.34 ± 2.64	22.04* ± 2.26	19.08 ± 3.14	18.65 ± 2.52
Stroma	42.42 ± 7.91	42.35 ± 5.82	38.77 ± 10.04	41.77 ± 7.16	45.73 ± 7.00	35.73* ± 8.80
Lumen	41.08 ± 7.51	35.88* ± 5.04	43.89 ± 10.52	36.19* ± 6.14	35.19 ± 5.87	45.62* ± 9.27
Absolute proportion of tissue components (g)						
Epithelium	0.12 ± 0.02	0.21* ± 0.02	0.18 ± 0.03	0.34* ± 0.04	0.09 ± 0.01	0.23* ± 0.03
Stroma	0.30 ± 0.06	0.41* ± 0.06	0.41 ± 0.11	0.66* ± 0.11	0.21 ± 0.03	0.44* ± 0.11
Lumen	0.29 ± 0.05	0.34* ± 0.15	0.46 ± 0.11	0.57* ± 0.10	0.17 ± 0.03	0.56* ± 0.11
Body weight (g)	62.24 ± 5.82	61.92 ± 6.95	80.02 ± 8.77	79.42 ± 6.78	67.02 ± 7.51	77.62 ± 5.67
Prostate weight (g)	0.71 ± 0.06	0.96* ± 0.12	1.05 ± 0.04	1.57* ± 0.38	0.47 ± 0.19	1.22* ± 0.17
Relative prostate weight (g prostate/g body weight)	0.011 ± 0.002	0.016* ± 0.003	0.013 ± 0.001	0.193* ± 0.004	0.007 ± 0.003	0.016* ± 0.003

Values represent mean ± SD. Statistical analysis based on the Anova and Tukey Tests. * Significant ($P \leq 0.05$) vs. control group.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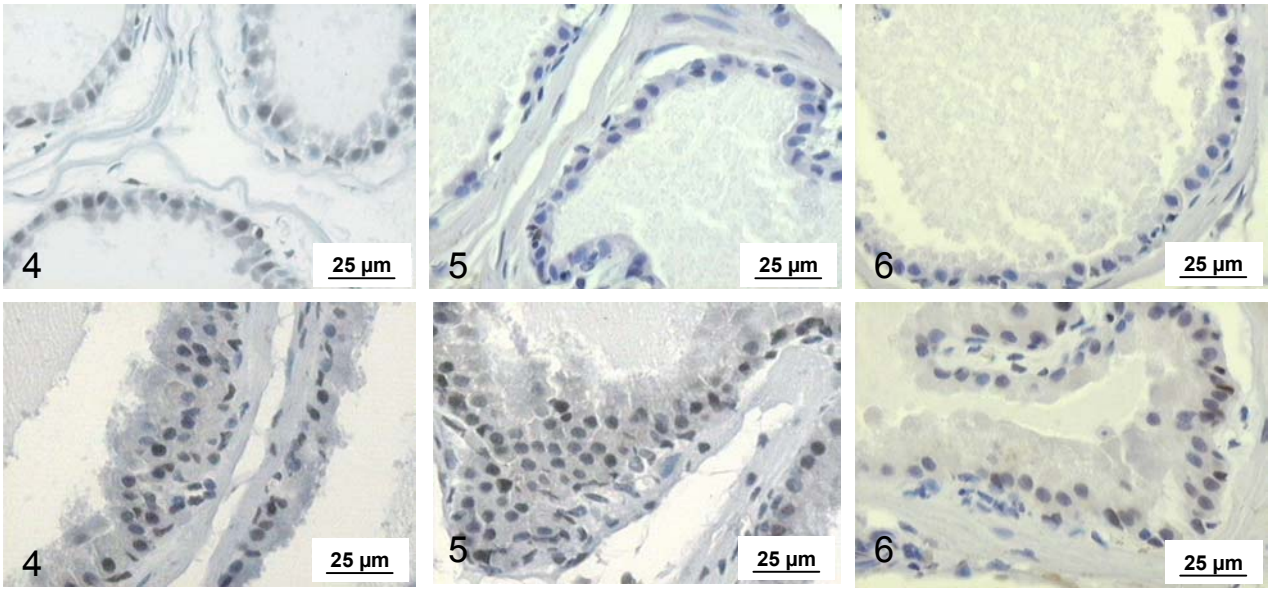
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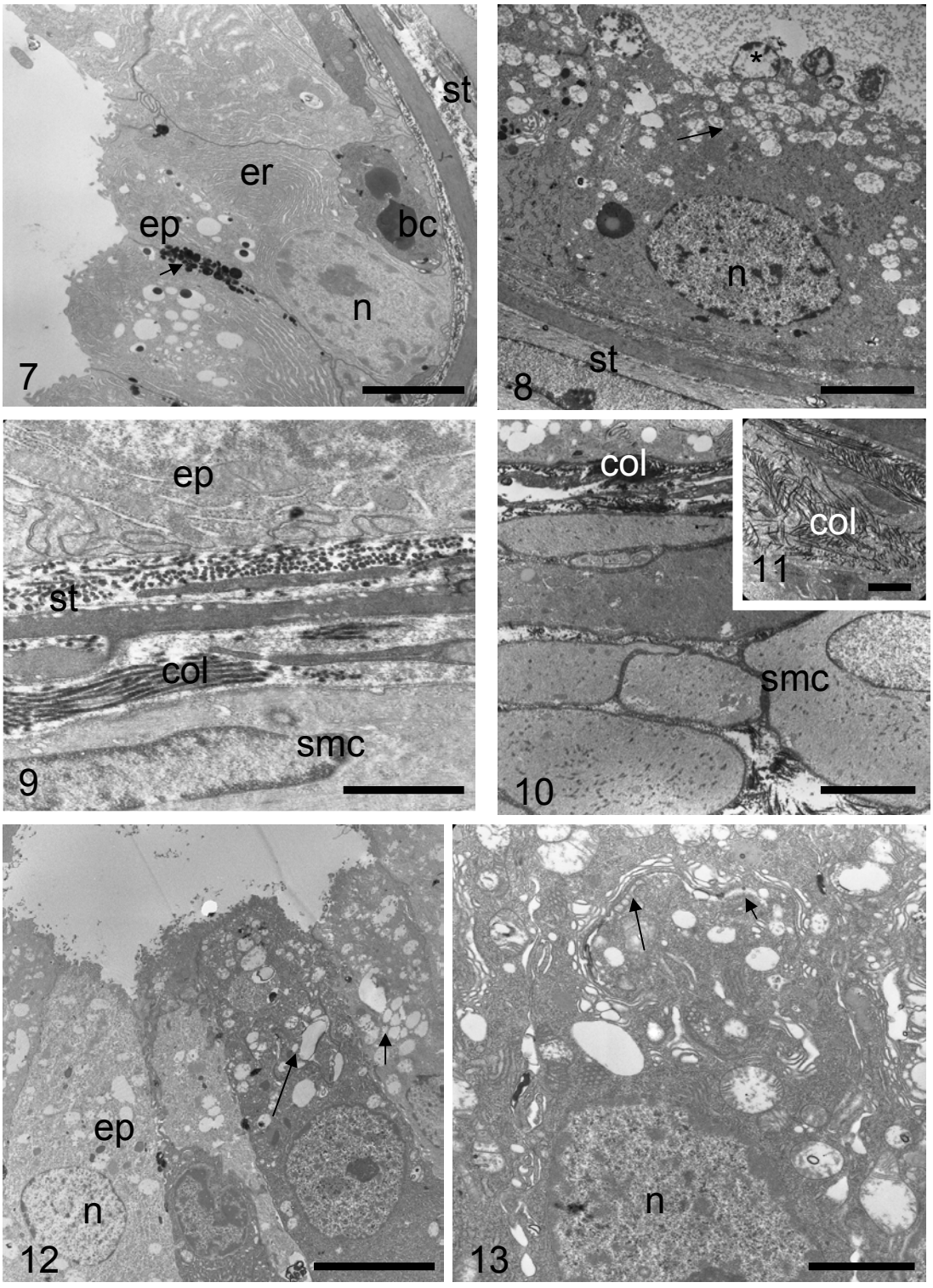
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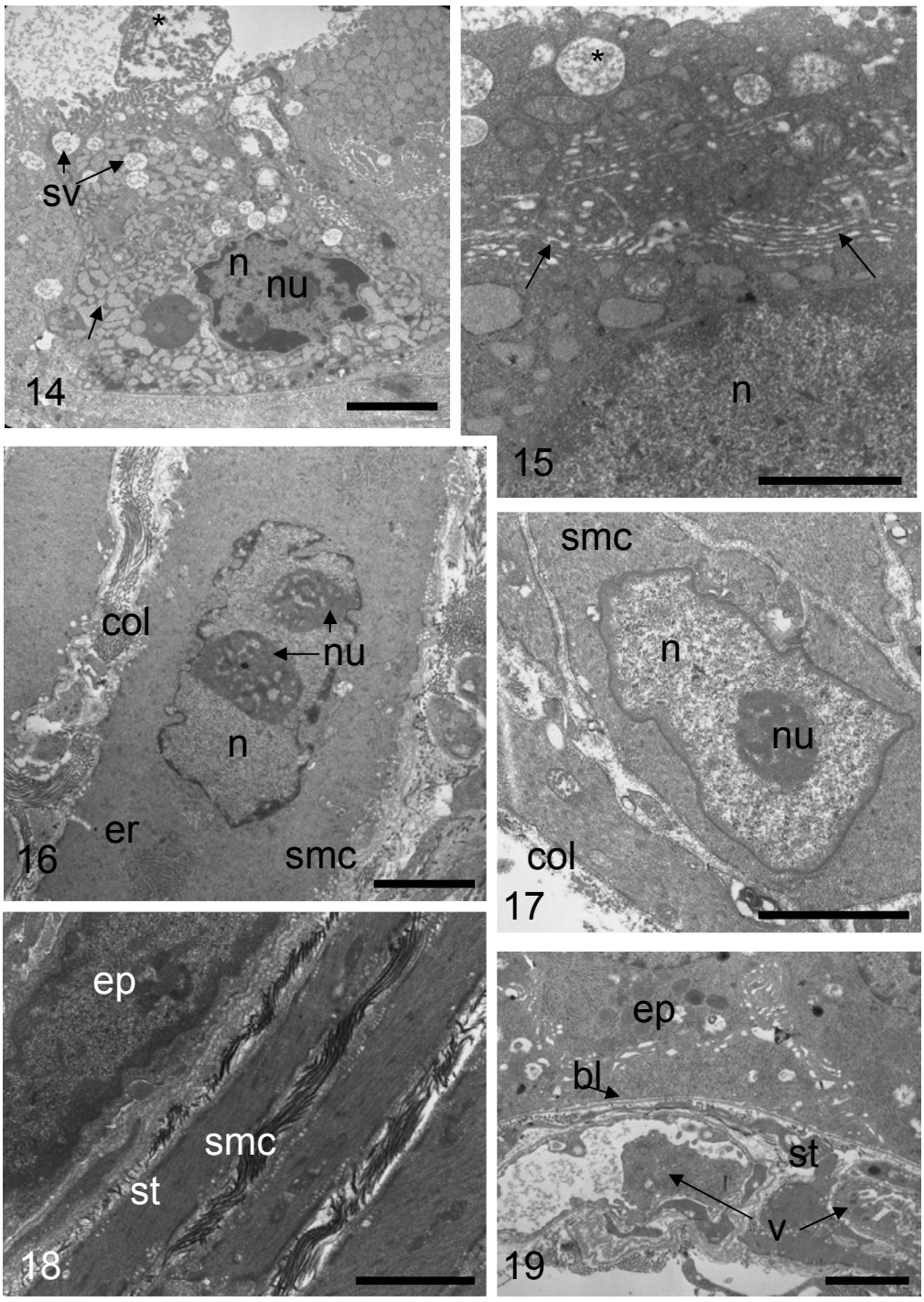
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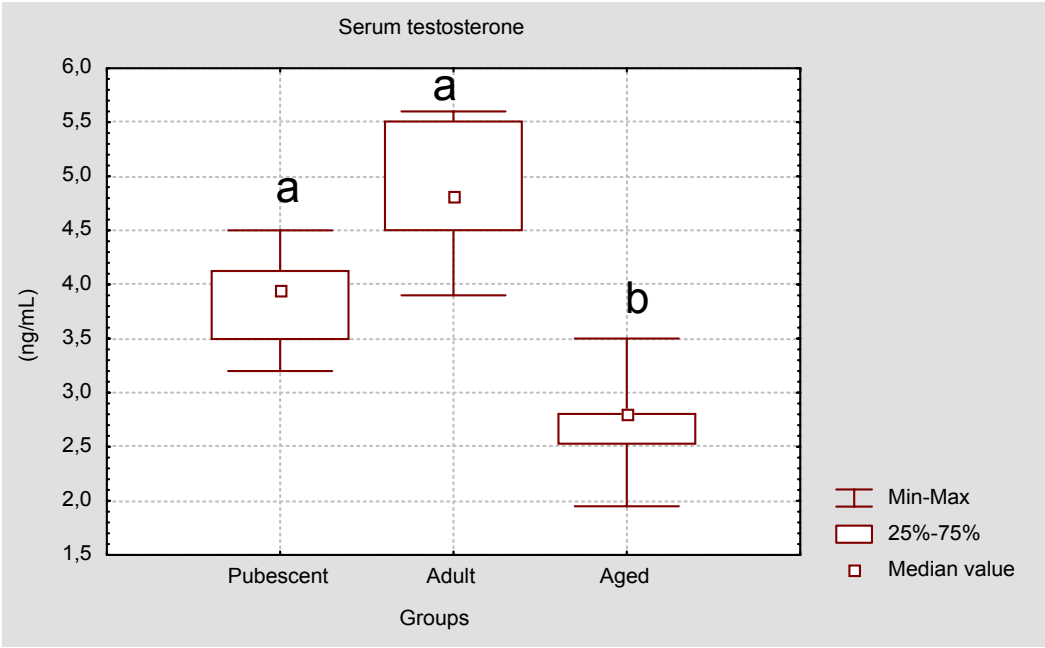
FIGURE 20– Serum testosterone levels at the three ages studied



Statistical analyze based to ANOVA and Tukey tests. Different superindices: a, b; indicate significant statistically difference: p≤ 0,05.

Conclusões Gerais

Levando em conta o modelo experimental e os métodos empregados, e a partir dos resultados obtidos, conclui-se que:

1. A próstata ventral do gerbilo é sensível a alterações hormonais como ablação androgênica e suplementação androgênica e estrogênica;

2. Os efeitos da suplementação estrogênica sobre o epitélio são independentes dos níveis androgênicos

3. O estrógeno tem papel importante sobre as células estromais, estimulando o processo de secreção celular;

4. As células musculares lisas parecem estar relacionadas com o rearranjo estromal após suplementação estrogênica;

5. Apesar de haver acúmulo de elementos de matriz tanto na castração como na suplementação estrogênica, os processos de arranjo desses elementos são diferentes em cada situação;

6. O aumento na densidade de células ER e AR positivas nos animais tratados com estradiol em regiões displásicas e hiperplásicas, atenta para um possível papel desse hormônio em alterações de natureza maligna e pré-malígna;

7. A castração e a suplementação estrogênica, independente de estarem associadas, alteram o aspecto morfológico das células musculares lisas, bem como seu arranjo na formação da camada concêntrica que circunda os ácinos prostáticos;

8. A suplementação androgênica aumenta a atividade secretora das células epiteliais em todas as idades estudadas;

9. A suplementação com testosterona não altera a morfologia das células estromais, mas parece estar envolvida na ativação e no aumento de atividade secretória das células musculares lisas;

10. Aparentemente, não há alteração de distribuição e densidade de fibras da matriz extracelular após o tratamento androgênico;

11. A suplementação androgênica parece atuar como fator importante no processo de tumorigênese da próstata do gerbilo, principalmente após a idade adulta;

12. A suplementação androgênica está relacionada com a reversão dos efeitos hipertróficos do envelhecimento sobre o estroma da próstata ventral do gerbilo;

13. Enfim, o gerbilo mostrou-se um bom modelo experimental para o estudo das alterações prostáticas frente a alterações hormonais, tornando-se importante em estudos de aplicação clínica e veterinária.

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