UNIVERSIDADE ESTADUAL DE CAMPINAS

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# Vasectomia e próstata ventral de gerbilos: proliferação celular, morte celular e interação estroma-epitélio

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Orientador: Prof. Dr. Wílson de Mello Júnior

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*"O homem não morre quando deixa de viver,*

*Mas sim quando deixa de amar."*

***Charlie Chaplin***

*"Não existe vento favorável para o marinheiro*

*Que não sabe aonde ir."*

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# SUMÁRIO

# RESUMO

Doenças como o câncer e hiperplasia benigna de próstata estão relacionadas à falha no mecanismo de regulação do equilíbrio funcional entre os processos de proliferação celular e apoptose nas células prostáticas. O equilíbrio entre esses processos é controlado por níveis séricos de andrógenos e por fatores de crescimento. A vasectomia pode alterar tal equilíbrio por mecanismo ainda desconhecido. Durante os processos de carcinogênese da próstata, as interações parácrinas entre epitélio e estroma podem ser perturbadas, causando prejuízos tanto para epitélio quanto para musculatura lisa, e resultando em progressão para um estado anaplásico. Assim, o presente trabalho teve como objetivo avaliar a influência da vasectomia sobre os processos de proliferação e morte celular, bem como avaliar a estrutura e a ultra-estrutura da próstata ventral de gerbilo após vasectomia. Foram realizados estudos estruturais (Hematoxilina-eosina; tricrômico de Masson, reticulina de Gömöri e reação de Feulgen), ultra-estrutural (Microscopia eletrônica de transmissão) e imuno-histoquímicos (anti-Ki67, anti-PCNA e anti-Caspase-9) na próstata ventral de gerbilos. Os índices de proliferação celular prostática aumentaram nos gerbilos vasectomizados, porém os índices de apoptose não sofreram alteração pós-vasectomia. O volume relativo do epitélio aumentou, enquanto o volume relativo da luz diminuiu após vasectomia. Contudo, a morfologia geral da próstata ventral de gerbilo não se alterou. Portanto, a vasectomia não provoca alterações estruturais ou ultra-estruturais significativas na próstata ventral de gerbilo. Porém, promove um desequilíbrio entre os processos de proliferação celular e apoptose, em favor da proliferação celular do epitélio na próstata ventral de gerbilo.

**Palavras-Chaves:** vasectomia, próstata, proliferação celular, apoptose e gerbilo.

## ABSTRACT

Diseases such as cancer and benign prostatic hyperplasia are related to the disruption in the mechanism of regulating the balance between both processes of cell proliferation and apoptosis in the prostatic cells. That balance is controlled by androgens and growth factors. Vasectomy might alter that balance by unknown mechanism yet. During process of prostatic carcinogenesis, the paracrine interaction between epithelium and stroma can be disrupted, causing damage to epithelium and smooth muscle cells. This results in progression to anaplastic state. Thus this study evaluates the influence of the vasectomy on both processes of cell proliferation and apoptosis in the epithelium of the gerbil ventral prostate, and evaluates structural and ultra-structural alterations induced by vasectomy on the gerbil ventral prostate. It was accomplished structural (Hematoxylin-eosin, Masson's trichrome, Gömöri's reticulin and Feulgen's reaction), ultra-structural (Transmission electron microscopy) and immunohistochemical (anti-Ki67, anti-PCNA e anti-Caspase-9) studies in the ventral prostate of the vasectomized and sham-operated gerbils. The indices of cell proliferation increased significantly in the vasectomized gerbil significantly after vasectomy. The epithelium relative volume increased, while the lumen relative volume decreased post-vasectomy. However the general morphology of the gerbil ventral prostate did not alter. Thus the vasectomy did not promote structural and ultra-structural alterations in the gerbil ventral prostate. But, cause imbalance between both processes of cell proliferation and apoptosis in favor cell proliferation in the epithelium of the gerbil ventral prostate.

**Keywords:** vasectomy, prostate, cell proliferation, apoptosis and gerbil.

# INTRODUÇÃO GERAL

## Vasectomia e câncer de próstata

A vasectomia é o método anticoncepcional masculino mais seguro (Schwingl & Guess, 2000), que tem se tornado cada vez mais comum em muitos países desde 1970. Na década de sessenta, já havia aproximadamente 50 milhões de homens vasectomizados no mundo. Nessa cirurgia, o ducto deferente é seccionado, impedindo que os espermatozóides sejam expelidos, no líquido seminal, durante a ejaculação (Linnet, 1983).

No Brasil, é possível realizar vasectomia gratuitamente em algumas instituições da rede pública de saúde, contudo a prevalência da vasectomia entre os métodos anticoncepcionais é menor que 4,4%. Segundo Manhoso & Hoga (2005), é necessária uma política pública integrada para envolver o homem na saúde reprodutiva.

Desde a realização das primeiras vasectomias, estudos têm sugerido que a vasectomia causa alterações na função prostática (Thaker *et al*., 1972). Além disso, nos últimos anos, surgiu polêmica sobre a correlação entre vasectomia e câncer prostático. Embora alguns estudos epidemiológicos tenham apontado aumento do risco de câncer de próstata em homens vasectomizados (Giovanucci *et al.*, 1993ab; Emard *et al*., 2001), também a vasectomia tem sido relacionada à redução do risco de câncer de próstata (Ross *et al.*, 1983); outros autores, ainda, inferiram que não há qualquer tipo de correlação (Bernal-Delgado *et al*., 1998; Lesko *et al*., 1999; Stanford *et al*., 1999; Patel *et al.*, 2005).

Analisando esses trabalhos, observamos que a polêmica persiste até hoje, principalmente quando a vasectomia é realizada precocemente (Cox *et al*., 2002).

O câncer de próstata é uma moléstia claramente associada ao envelhecimento masculino. Aos 50 anos de idade, 30% dos homens submetidos à biópsias podem apresentar focos histológicos da neoplasia, com ou sem significado clínico. À medida que o homem envelhece, essa incidência acentua-se, atingindo 60% a 70% dos homens com idade entre 80 e 90 anos e, hipoteticamente, todo aquele que ultrapasse os 100 anos de vida (Brawley *et al.*, 2000).

Das lesões que acometem a próstata humana, as mais comuns são: Hiperplasia Benigna Prostática (HBP), Neoplasia Intraepitelial Prostática (NIP) e Adenocarcinoma Prostático.

A HBP é um crescimento tumoral não neoplásico que resulta da hiperplasia do estroma e do tecido glandular em diferentes graus e, sempre, em arranjo nodular (Billis, 1997).

A NIP é uma lesão pré-maligna atípica do epitélio de revestimento dos ductos e ácinos, com potencial de serem precursoras do carcinoma invasivo. Essa lesão caracteriza-se pela presença de células atípicas, irregularmente dispostas, com núcleos volumosos e nucléolos proeminentes no epitélio prostático (Billis, 1997).

O Adenocarcinoma Prostático é uma neoplasia constituída de células carcinotamosas que se originam dos ácinos e ductos prostáticos, mostrando arranjo, graus de diferenciação e comportamento biológico variáveis. Essa lesão pode apresentar-se com diferentes aspectos. Habitualmente observam-se microácinos em estroma de tecido fibroso abundante ou ausente. Os ácinos neoplásicos podem, também, ter diâmetro de ácinos normais ou mesmo maior. Em ácinos neoplásicos, nunca se observa a camada de células basais, presentes em ácinos normais ou em nódulos hiperplásicos. Um dos aspectos peculiares do adenocarcinoma de próstata é o arranjo cribiforme, observado com muita

freqüência. O tecido neoplásico mostra glândulas no interior de glândulas, o que confere aspecto crivado (Billis, 1997).

Howards (1993) levantou quatro hipóteses para tentar relacionar vasectomia com o risco de câncer de próstata: (1) endocrinológica; (2) imunológica; (3) do inibidor do fator crescimento; e (4) do fator de crescimento. A hipótese endocrinológica diz que a vasectomia aumentaria os níveis plasmáticos de andrógenos, o que poderia favorecer o estabelecimento de câncer de próstata; a hipótese imunológica diz que a vasectomia aumentaria a produção de anticorpos anti-espermatozóide, o que poderia favorecer o aparecimento de lesões na próstata, por um mecanismo ainda desconhecido; A hipótese do inibidor do fator de crescimento diz que a vasectomia diminuiria a produção de inibidores do fator de crescimento, o que poderia favorecer o crescimento prostático; e a hipótese do fator de crescimento diz que a vasectomia aumentaria a produção local de fatores de crescimento na próstata, o que também poderia favorecer o crescimento prostático. Porém essas hipóteses foram pouco discutidas e não apresentaram estudos conclusivos.

Recentemente, Pereira *et al.* (2006) verificaram que houve aumento da proliferação celular na próstata ventral de ratos adultos 7 dias após vasectomia. Isso despertou interesse em estudar a possível influência da vasectomia na próstata ventral de gerbilo para melhor entender as alterações prostáticas pós-vasectomia.

**Morfofisiologia da Próstata**

A secreção prostática é um líquido fino, leitoso, alcalino contendo ácido cítrico, cálcio, fosfatase alcalina e fibrinolisina. A característica alcalina é importante para neutralizar a acidez do líquido do ducto deferente e, também, das secreções vaginais. Essa neutralização aumenta a motilidade e a fertilidade dos espermatozóides (Aumüller, 1989).

Na maturidade sexual, a próstata de roedores é uma glândula multilobulada arranjada ao redor da uretra na base da bexiga urinária. Os três pares de lobos da próstata são: ventral, dorsolateral e anterior ou glândula de coagulação (Jesik *et al.*, 1982). Devido às diferenças lobo-específicas no padrão de morfogênese ductal, a forma final de cada lobo é diferente. Além disso, os lobos têm algumas características histológicas distintas (Lee *et al*., 1990).

A próstata humana tem morfologia compacta sem lobos distintos e apresenta forma e tamanho aproximado de uma noz. A organização da próstata humana comumente é descrita em três regiões distintas: zona central, zona de transição e zona periférica, refletindo em três grupos de ductos presentes na próstata humana (McNeal, 1983; Hayashi *et al.*, 1993). Observações comparativas do desenvolvimento prostático demonstram que a morfogênese, processo dependente de testosterona, ocorre de maneira análoga em roedores e humanos (Timms *et al.*, 1994). Contudo evidências moleculares para homologia entre lobos específicos da próstata de roedores e regiões da próstata humana ainda não foram identificadas (Marker *et al.*, 2003).

A secreção da próstata é produzida e conduzida até a uretra através de um sistema ductal, que é definido como uma unidade morfofuncional prostática. Os principais

tipos de células epiteliais presentes no sistema ductal são: células basais ($CK5^+$, $CK8^-$, $p63^+$, $Ki67^+$ e $Sca\text{-}1^-$), células intermediárias ($CK5^+$, $CK8^{+-}$, $p63^-$ e $Ki67^+$), células luminais secretoras ($CK5^-$, $CK8^+$, $p63^-$ e $Ki67^-$), células neuroendócrinas e "stem cells" ($CK5^+$, $CK8^-$, $p63^+$ e $Sca\text{-}1^+$) (Wang *et al*., 2006).

A distribuição relativa dos tipos celulares é diferente entre a próstata humana e a de ratos. Porém, em ambas as espécies, a luz ductal é revestida por células epiteliais secretoras colunares altas. Essas células apresentam uma polaridade ápico-basal e secretam proteínas e fluídos de sua superfície apical na luz prostática (Lee *et al.*, 1990). Na próstata humana, as células epiteliais basais formam uma camada quase contínua entre as células secretoras e a membrana basal, enquanto na próstata de rato poucas células basais estão dispersas formando uma camada descontínua ao redor dos ductos (Marker *et al.*, 2003).

O estroma da próstata humana e de rato é em grande parte composta por células musculares lisas. Essa camada é tão espessa na próstata humana quanto na de rato. Porém, a razão entre as células estromais e epiteliais é mais alta na próstata humana. A camada estromal também contém fibroblastos, linfócitos, células endoteliais e neuronais (Marker *et al.*, 2003).

O sistema ductal prostático de ratos pode ser dividido em três regiões: proximal, intermediária e distal, em relação à abertura na uretra. As características morfológicas das células epiteliais são diferentes nessas regiões. Na região distal, as células epiteliais são colunares altas e seu núcleo está freqüentemente localizado na parte apical dessas células; figuras mitóticas podem ser identificadas ocasionalmente nas células dessa região. As células epiteliais da região intermediária também são colunares altas e seu núcleo, localizado basalmente, está mitoticamente quiescente. Células mortas não são evidentes

nem na região distal, nem na região intermediária. As células da região proximal, região imediatamente próxima à uretra, são colunares baixas ou cúbicas e estão em processo de morte celular ou apoptose (Nemeth & Lee, 1996; Janulis & Lee, 1998).

O crescimento normal, a diferenciação e a manutenção da integridade funcional (secretora) e estrutural da próstata e das demais glândulas sexuais acessórias são dependentes de níveis constantes de andrógenos circulantes e ocorrem através de interações recíprocas entre o mesênquima e o epitélio (Pour & Stepan, 1989; Kyprianou *et al*., 1996). Os andrógenos mantêm a morfologia e a função no tecido prostático, controlando a circulação sangüínea, bem como atuando em mecanismos de proliferação e morte celular (Kyprianou & Issacs, 1988; Marker *et al.*, 2003).

Os andrógenos possuem três funções principais nas células prostáticas que contribuem para o desenvolvimento, crescimento e manutenção da próstata: (1) podem estimular a proliferação, especialmente nas células epiteliais; (2) estimulam a diferenciação das células epiteliais secretoras; (3) inibem a apoptose de células prostáticas (Buttyan *et al.*, 1999).

A testosterona, principal hormônio androgênico, atua nas células epiteliais secretoras luminais via receptor nuclear. Porém, nas células basais e estromais, a testosterona é convertida em di-hidrotestosterona pela 5-α-redutase. A di-hidrotestosterona é cerca de 10 vezes mais potente que a testosterona e também se liga ao receptor nuclear de andrógeno (William, 2001).

**Proliferação e Morte Celular**

Os processos de proliferação e de morte celular (apoptose) são importantes no desenvolvimento neoplásico. Ambos processos são atividades biológicas fundamentais tanto para processos fisiológicos como patológicos. A regulação do ciclo celular é dependente de proteínas quinases (Wang *et al*., 2003) e outras proteínas como a p16, p27 e p53 (Fernandez *et al.*, 2002). Assim, o ciclo celular pode ser estudado em diferentes fases através do estudo de marcadores moleculares relacionados à proliferação celular – como Ki67, PCNA, BrdU e bcl2 (Leite *et al.*, 1999) e a apoptose – como DNA fragmentado, p53 e caspases (Wang *et al.*, 2005).

O PCNA (Proliferation Cell Nuclear Antigen) é a proteína acessória da DNA polimerase e é sintetizada nas fases G1 e S do ciclo celular. O PCNA, entretanto, correlaciona-se com o estado proliferativo da célula. A detecção imuno-histoquímica do PCNA indica que a célula está em divisão e não na fase G0 do ciclo. Já o Ki67, outro marcador imuno-histoquímico para proliferação celular, é encontrado no exterior dos nucléolos, especialmente nos componentes granulares durante as fases G1, G2 e M (Marker *et al*., 2003).

O componente central do processo de apoptose é um sistema proteolítico que envolve uma família de proteases intracelulares envolvidas na iniciação e na execução da apoptose chamadas caspases. Há duas classes de caspases: inicializadoras (8, 9 e 12) e executoras (2, 3, 6 e 7). As inicializadoras são capazes de ativar executoras ou amplificar a cascata de caspases pela ativação aumentada das inicializadoras. Enquanto que as

executoras clivam substratos citoplasmáticos (como actina e citoqueratinas) e nucleares (como polimerase e lamininas), culminando na apoptose (Kim *et al*., 2001).

A hiperplasia e o carcinoma prostático estão relacionados à falha no mecanismo de regulação do equilíbrio funcional entre os processos de proliferação celular e apoptose (Xie *et al*., 2000; Carson & Rittmaster, 2003). Assim, os marcadores moleculares de proliferação celular e apoptose estão sendo alvo de grande interesse, pois a modificação no padrão de expressão representa um desequilíbrio entre tais processos, o que chave na compreensão da carcinogênese (Berges *et al*., 1995; Claus *et al*., 1997; Davis & Day, 2002).

**Interações Estroma-epitélio na Próstata**

A organogênese da próstata é dependente de interações estroma-epitélio, de tal modo que a morfogênese e a diferenciação, tanto do epitélio quanto do estroma, fracassam se o epitélio e o estroma crescem separadamente (Farnsworth, 1999; Marker *et al.*, 2003).

Durante o desenvolvimento da próstata, os complexos processos morfogenéticos exigem uma mediação ativa das células estromais, enquanto a manutenção da diferenciação do tecido epitelial adulto pode ser regulada, em grande parte, pela matriz extracelular. Alguns trabalhos sugerem que o estroma seja o primeiro alvo da ação dos andrógenos, sendo a reação do epitélio mediada por fatores estromais (Nemeth & Lee, 1996).

As interações parácrinas entre epitélio e estroma, mais especificamente entre o epitélio e a musculatura lisa ao seu redor, são de importância fundamental durante a morfogênese no embrião e estão claramente envolvidas na homeostase do órgão adulto.

Durante os processos de carcinogênese da próstata, essas interações seriam perturbadas, causando prejuízos para o epitélio e a musculatura lisa e resultando em progressão para um estado anaplásico (Cunha *et al.*, 1996; Hayward *et al.*, 1998).

Além das células estromais como fibroblastos e células musculares lisas, atuam na interação estroma-epitélio alguns componentes da matriz extracelular, como o colágeno tipo I e tipo III, a elastina e outras proteínas não colagênicas (Carvalho & Line, 1996; Carvalho *et al.*, 1997ab; Vilamaior *et al.*, 2000, Vilamaior *et al.*, 2005).

**O Modelo Animal**

Os gerbilos (*Meriones unguiculatus*) são pequenos roedores que naturalmente habitam savanas, estepes, desertos e regiões semidesérticas da África, Ásia Central, Europa Oriental, Índia e Oriente Médio. De anatomia similar às do rato e do camundongo, os gerbilos machos adultos variam entre 11,5 e 14,5 cm de comprimento corpóreo e pesam em torno de 100 gramas (Harkness & Wagner, 1995).

No início da década de 60 foram introduzidos como animais de laboratório, tornando-se importantes para o desenvolvimento de diversos modelos biológicos para pesquisas científicas (Rich, 1968). Uma das vantagens desses animais, para estudos experimentais, reside no fato deles serem consideravelmente menores que os ratos, mas essencialmente maiores que os camundongos e hamsters (Williams, 1974). Esses animais têm sido amplamente utilizados para estudos de natureza científica principalmente pelo fato de terem comportamento extremamente dócil em cativeiro. Outra característica importante a ser considerada é o controle da micção; por serem de origem desértica, os gerbilos

consomem pouca quantidade de líquido, facilitando a manutenção da limpeza das gaiolas em cativeiro e promovendo grande asseio nas salas de biotérios (Almeida, 1997).

Algumas características metabólicas destes animais os tornam essenciais em certos modelos experimentais. O interesse científico nesta espécie iniciou-se através de estudos sobre colesterol e arteriosclerose, visto que os gerbilos não formam placas arterioscleróticas mesmo com níveis altos de colesterol no sangue (Gordon & Cekleniak, 1961). Os gerbilos têm sido utilizados em diversos estudos nas áreas de parasitologia (Gray & Pudney, 1999), oncologia (Iimuro *et al.*, 2002), neurociências (Kang *et al.*, 2003), reprodução (Segatelli *et al.*, 2000; Clark & Galef, 2002 Segatelli *et al.*, 2002; Custódio *et al*., 2003; Santos *et al*., 2003; Segatelli *et al.*, 2004), comportamento sexual (Portillo & Paredes, 2003;) e envelhecimento (Sohal *et al*., 1995; Zanetoni & Taboga, 2001), dentre outras.

**Próstata de gerbilo**

A próstata ventral de gerbilo está constituída de dois lobos amarelados, imediatamente ventrais à bexiga urinária, exatamente no ponto em que a uretra recebe os dois ductos ampulares. Esses lobos estão ligados na junção da bexiga urinária com as vesículas seminais (Pinheiro *et al*., 2003).

A próstata desse roedor apresenta ácinos glandulares com epitélio prismático simples e altamente secretório. Entre as partes glandulares, encontra-se um estroma conjuntivo ricamente vascularizado, com poucas fibras conjuntivas e elásticas, além de abundantes células musculares lisas dispostas concentricamente aos ácinos. Ultra-

estruturalmente o epitélio prostático apresenta heterogeneidade entre os seus tipos celulares e o estroma glandular mostra esparsas células musculares lisas com disposição concêntrica e entremeada às camadas de colágeno (Zanetoni & Taboga, 2001).

O período reprodutivo de gerbilos machos inicia-se com aproximadamente 3 meses de idade e estende-se por 12 a 17 meses (Harkness & Wagner, 1995). Em estudos recentes verificou-se que a próstata de gerbilos desenvolve espontaneamente neoplasias intra-epiteliais e adenocarcinomas invasivos e não invasivos e, também, hiperplasia estromal em decorrência da idade (a partir de 12 meses de idade), fato que não pode ser constatado em ratos e camundongos (Zanetoni & Taboga, 2001).

A próstata de gerbilo desperta interesse biológico e biomédico pela sua morfologia semelhante à próstata humana, no que se refere à compacidade e fusão dos seus lobos. Esse tipo de conformação anatômica não está presente no rato e no camundongo, onde os lobos são bem distintos (Price, 1963). O gerbilo foi escolhido como modelo animal para estudo da próstata, devido às características morfofisiológicas já apresentadas.

# OBJETIVO

O objetivo do presente trabalho foi:

- ✓ Avaliar os componentes estromais da próstata ventral de gerbilos vasectomizados precoce e tardiamente;
- ✓ Avaliar os processos de proliferação e morte celular epitelial da próstata ventral de gerbilos vasectomizados precoce e tardiamente.

**ARTIGO: Proliferative alterations in the gerbil ventral prostate induced by vasectomy**

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**ABSTRACT**

**BACKGROUND:** Diseases, such as cancer and benign prostatic hyperplasia, are related to disruption of the mechanism regulating the balance between cell proliferation and apoptosis in prostatic cells. This study evaluates the influence of the vasectomy on both processes of cell proliferation and apoptosis, and evaluates structure and ultra-structure of the gerbil ventral prostate.

**METHODS:** It was accomplished structural (Hematoxylin-eosin, Masson's trichrome, Gömöri's reticulin and Feulgen's reaction), ultra-structural (Transmission electron microscopy) and immunohistochemical (anti-Ki67, anti-PCNA e anti-caspase9) studies in the gerbil ventral prostate.

**RESULTS:** Cell proliferation indices increased, however apoptotic indices did not alter post-vasectomy. The epithelium relative volume increased, while the lumen relative volume decreased post-vasectomy.

**CONCLUSIONS:** Thus the vasectomy promote structural alterations in the gerbil ventral prostate, and cause imbalance between both processes of cell proliferation and apoptosis in favor of cell proliferation in gerbil ventral prostate.

**KEYWORDS:** prostate, cell proliferation, apoptosis, vasectomy and gerbil.

## INTRODUCTION

Vasectomy was related to risk of carcinoma of the prostate in epidemiological studies [1, 2, 3]. Other studies have presented contradictory results [4, 5, 6, 7, 8]. Even though there is no consensus among researchers, it appears that vasectomy realized prematurely was the factor of greatest concern [9]. The paucity of experimental studies have contributed to this epidemiological impasse.

Furthermore, we verified in a previous work [10], that there was an increase in cellular proliferation in the ventral prostate of rats 7 days after vasectomy. This incited interest in studying the influence of vasectomy on the ventral prostate after a longer post-operative period, to understand better post-vasectomy prostatic alterations.

Cancer of the prostate is one of the most frequent carcinomas that afflict men. Its incidence has increased startlingly as the quality of life provides progressively greater longevity. Benign hyperplasia of the prostate, also is a common pathology among men. Both diseases are related to failure of the mechanism of functional balance between the processes of cellular proliferation and apoptosis in prostatic cells [11]. The balance between these two processes is controlled by androgens and by a series of growth factors such as KGF (keratinocyte growth factor), EGF (epidermal growth factor), IGFs (insulin like growth factors) e TGF-β (transforming growth factor- β), which establish reciprocal interactions between stroma and epithelium [12, 13].

Normal growth, differentiation and maintenance of functional and structural integrity (secretory) of the prostate and the rest of the accessory sex glands, are dependent

on constant levels of circulating androgens and occur through reciprocal interactions between the mesenchyma and epithelium [14].

Androgens act by controlling local blood circulation and the mechanisms of proliferation and cellular death. In other words, androgens possess three principles in prostatic cells: (1) they can stimulate proliferation, especially in epithelial cells; (2) stimulate differentiation in secretory epithelial cells; (3) inhibit apoptosis of prostatic cells [15, 16].

During the development of the prostate, the complex morphogenetic processes require active mediation of stromal cells, while maintenance of differentiation adult epithelial tissue can be regulated, in large part, by the extracellular matrix. Some works suggest that the stroma would be the primary target of the action of androgens, with the reaction of the epithelium being mediated by stromal factors [17].

Paracrine interactions between epithelium and stroma, more specifically between epithelium and surrounding smooth musculature, are of fundamental importance during morphogenesis in the embryo and are clearly involved in homeostasis of the adult organ. During the processes of carcinogenesis of the prostate, these interactions are perturbed, causing damage to the epithelium and to smooth musculature, resulting in progression to an anaplastic state [14, 18].

The gerbil prostate has provoked biological and biomedical interest since its morphology is similar to that of the human prostate, which refers to its compact structure and fusion of its lobes. This type of anatomical form is not present in the rat or mouse, where the lobes are quite distinct [19]. Furthermore, recent studies verified that the gerbil prostate spontaneously develops intra-epithelial neoplasias, invasive and non-invasive

adenocarcinomas and stromal hyperplasia resulting from age [20], as well as demonstrate that the establishment of prostate cancer in gerbils occurs after a period of testosterone administration shorter than in other experimental models [21].

Therefore, the objective of the present work was to evaluate the influence of vasectomy on the processes of proliferation and cellular death, as well as to compare the structural and ultra-structural characteristics of the ventral prostate in vasectomized and sham-operated gerbils.

## MATERIALS AND METHODS

I – Experimental procedure:

All of the experimental procedure, described as follows, was realized in accord with the norms of the Brazilian College of Ethics in Animal Experimentation (COBEA).

1. Maintenance of gerbils in the biotherium:

Thirty-two adult male gerbils were utilized, *Meriones unguiculatus*, which were maintained in the Biotherium of the Department of Anatomy – Institute of Biosciences – UNESP – Botucatu Campus, in plastic cages, 4 gerbils in each cage, with bars of stainless steel, and floors covered with wood shavings, until they reached 4 months of age.

During this period, ambient conditions in the biotherium were controlled as follows: temperature (22±2$^{o}$C), relative air humidity (55±10%) and light-dark period of 12/12 hours. All the gerbils received solid diet and water "*ad libitum*". The gerbils were divided into two experimental groups – vasectomized (16 animals) and sham-operated (16 animals). Each experimental group was divided into two subgroups: Early and Late.

2. Experimental groups:

*2.1. Vasectomized group:*

The animals of the vasectomized group were divided into two subgroups: Early Vasectomized and Late Vasectomized.

*2.1.1. Early Vasectomized:*

The gerbils at 120 days of age were anesthetized with 1ml of *Dopalen*®, 0.5ml of *Rompun*® and 1ml of physiological solution (0.9%) per kilogram of body weight of the animal, by intraperitoneal route. The surgeries were realized under aseptic conditions, through median-sagittal abdominal incision. The ductus deferens were fastened at two points. The first point was located about 1.5 cm from the duct end in the urinary bladder base whereas the second point was 0.5 cm from the first. Afterwards the small fragment of the ductus deferens between fastenings was removed. The deferens blood vessels were fastened and removed also.

*2.1.2. Late Vasectomized:*

The gerbils of this group were maintained in the biotherium until they attained 240 days of age, when the surgical procedure identical to the anterior group was performed.

*2.2. Sham-operated group:*

The animals were divided into two subgroups: Early Sham-operated and Late Sham-operated.

*2.2.1. Early Sham-operated:*

The gerbils aged 120 days were anesthetized in conformity with the protocol already described for the vasectomized group. The surgeries were realized under aseptic conditions, through median-sagittal abdominal incision. After abdominal incision, manipulation of the genital organs and, subsequently, the suture was completed by layers: muscular and dermal.

*2.2.2. Late Sham-operated:*

The gerbils of this group were maintained in the biotherium until they reached 240 days of age, when the surgical procedure identical to the anterior group was performed.

The all of groups gerbils were observed during recuperation, looking for eventual existence of post-operative criptorchidism.

3. Collection of biological material:

When the animals from all experimental groups (sham-operated and vasectomized) attained 360 days of age, they were euthanized by an excess of anesthetic, and the ventral prostate of the gerbil was collected.

II – Analysis of biological material:

1. Light Microscopy:

Fragments of ventral prostate were fixed in solution of *Bouin,* included in *Paraplast*® and cut to 5µm thickness. The histological cuts were colored with Hematoxylin-Eosin, Masson's trichrome, and Gomori's reticulin. Also Feulgen's reaction was realized in histological sections. Next the histological slides were examined and photographed in a photomicroscope, the AXIOPHOT 2 - Zeiss.

2. Transmission Electron Microscopy:

Fragments of ventral prostate were fixed by immersion in 2.5% glutaraldehyde in phosphate buffer 0.1M pH 7.2 for twenty four hours. Next they were post-fixed in 1% osmium tetroxide in phosphate buffer 0.1M pH 7.2 for twenty four hours. Afterwards the fragments were dehydrated in a graded acetone series and embedded in Araldite. Ultra thin

silver sections were cut using a diamond knife and contrasted with 2% alcoholic uranyl acetate and then with 2% lead citrate in sodium hydroxide solution for 10 min. Grids were examined in transmission electron microscope.

3. Morphometry and stereology:

Utilizing five different histological sections, stained with Hematoxylin-eosin, obtained from each animal, 40 measures were completed of the epithelial height of the ventral prostate. These measures were realized through the AxioVision 4.1 (ZEISS) System of Image Analysis. Additionally, the relative volumes of the lumen, stroma and epithelium were evaluated through the Weibel Multipurpose System utilizing a grade of 168 points, in histological sections colored with Masson's trichrome.

It was evaluated in different histological sections per animal to determine the score of focal inflammation, focal intraepithelial neoplasia and adenocarcinoma. The score showing how many gerbils of the groups present these lesions in at least one histological section.

4. Immuno-histochemistry:

Immuno-histochemical reactions were realized as markers of cellular proliferation (Ki67 and PCNA) and apoptosis (Caspase-9), with monoclonal antibodies (*Novocastra Laboratories Ltd®)*. The histological cuts of the ventral prostate were placed

on histological slides pretreated with tissue adhesive *(Novobond Slide Tissue Adhesive/Novocastra Laboratories Ltd®)*. Next, they were removed from paraffin with xylene, hydrated in a series of alcohol with decreasing concentrations (100%, 90% and 70%), washed in phosphate buffer.

The stages of antigen recuperation, blockage of endogenous peroxidase activity, blockage of non-specific reaction (normal rabbit serum *Novocastra Laboratories Ltd®*), incubation with primary antibody (mouse anti-ki67, mouse anti-PCNA or mouse anti-Caspase-9), incubation with secondary antibody (*Biotinylated Rabbit Anti-mouse Immunoglobulins-DakoCytomation®*) and revelation (StreptABComplex-DakoCytomation® and di-amino-benzidine-Sigma®) were realized in accord with the protocol of the manufacturer, for each primary antibody *(Novocastra Laboratories Ltd®).*

5. Counting of cells:

The cell count was done through the AxioVision 4.1 of Image Analysis System (ZEISS) in three sections. In total, approximately 1,000 cells were counted in each section of prostate. The indices of cell proliferation (PCNA-positive cells and Ki67-positive cells) and apoptotic cell (caspase9-positive cells and apoptotic cells counted by Feulgen's reaction) were expressed as percentages. The results were expressed as mean and standard deviation. The apoptotic-cell indices by Feulgen's reaction were realized through morphological characteristics evidenced by this method.

6. Statistical analysis:

Statistical analysis of the variables – mean height of secretor epithelium of the ventral prostate (μm), relative volume of the lumen (%), relative volume of the epithelium (%), relative volume of the stroma (%), index of PCNA-positive cells (%), index of Ki67-positive cells (%), index of caspase9-positive cells (%) and the index of apoptotic cells by Feulgen's reaction – was realized through analysis of variance by 2 x 2 factorial scheme (2 groups and 2 moments of evaluation) and complemented with the bi-caudal Tukey test ($P<5\%$).

**RESULTS**

Structural analysis:

In general morphological analysis of all the groups, it was noted that the gerbil ventral prostate presented glandular ducts with simple columnar epithelium, supported by a thin basal membrane constituted, principally, of reticular fibers (collagen type III). In some regions of the ductile system, this epithelium presented papillae, also supported by the basal membrane. Basal epithelial cells, which were found interspersed among the luminal cells, also were supported in the basal membrane. Encircling the prostatic ducts, a continuous layer of smooth-muscle cells were intermingled by collagen and reticular fibers. Among the

glandular ducts, a richly vascularized connective tissue was observed with few fibers (figures 1, 2 and 3).

In all the groups evaluated, the presence of focal inflammation was characterized by inflammatory cells in both the prostatic lumen and stroma. Generally, these inflammatory infiltrates were encountered in the peripheral ducts. Furthermore, the presence of neoplastic lesions, such as prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma, was verified in all groups evaluated. PIN was characterized by presenting two or more layers of atypical luminal epithelial cells with pleomorphic characteristics, in other words, with variation in form and size. The focus of PIN also was characterized by the presence of basal cells (figure 4). Yet adenocarcinoma was characterized by the presence of a cribiform arrangement, in other words, glands in the interior of glands; or even by macroacini aspect. Table 1 shows the score of focal inflammation, focal intraepithelial neoplasia and adenocarcinoma.

Ultra-structural analysis:

In all groups evaluated, ultra-structurally the prostatic epithelium presented two cell types: secretory lumen cells and basal cells. Secretory lumen cells present the nucleus with nucleolus evident and loose chromatin. In its cytoplasm were observed few organelles involved in the process of cellular secretion, such as rough endoplasmatic reticulum and secretory vesicles, evidencing a low level of secretory activity. In the part apical of secretory luminal cells was observed the presence of microvilli and also “blebs”. Yet the basal cells, of reduced size presented characteristics of cellular undifferentiation (figure 5).

Morphometry and stereology:

The height of the prostatic secretor epithelium, in Early and Late Vasectomized groups, did not present a significant statistical difference compared to the Early and Late sham-operated groups. Also there was no significant statistical difference between the Early and Late Vasectomized groups. The same also occurred in comparison between the sham-operated groups (table 2).

The relative volume of the epithelium in the Early Vasectomized group was significantly greater than that of the Early Sham-operated group. The same occurred in comparison between the Late Vasectomized group and the Late Sham-operated group. When the Early Sham-operated group was compared to the Late Sham-operated group, it was verified that there was no statistically significant difference. However, it was noted that the relative volume of the epithelium in the Early Vasectomized group was greater than that in the Late Vasectomized group (table 2).

The relative volume of the stroma of the Early Vasectomized group did not present a statistically significant difference compared to the Early Sham-operated group. The same occurred in comparison between the Late Vasectomized group and the Late Sham-operated group. There was no statistical difference between the relative volume of the stroma in the Early and Late Sham-operated groups. There also was no statistical difference between the relative volumes in the Early and Late Vasectomized groups (table 2).

The relative volume of the lumen in the Early Vasectomized group was significantly less than that of the Early Sham-operated group. The same occurred in comparison between the Late Vasectomized group and the Late Sham-operated group. But there was no statistically significant difference in relative luminal volume between the Early and Late Sham-operated groups, or between the Early and Late Vasectomized groups (table 2).

Cell Proliferation and Apoptosis

The immunoreactivity for PCNA in epithelial cells was equally distributed through the nucleus. However, stained of all the cytoplasm was found when the cell was observed in the process of mitosis, as during cellular division there occurred a rupture of the nuclear envelope (figure 6).

The index of PCNA-positive cells was significantly greater in the Early Vasectomized group than in the Early Sham-operated group. The same occurred when the Late Vasectomized group was compared to the Late Sham-operated group, in other words, the index was significantly greater in the Late Vasectomized group. However, the index of PCNA-positive cells was greater in the Early Vasectomized group than in the Late Vasectomized group. There was no statistically significant difference in the PCNA-positive-cell index between the Early and Late Sham-operated groups (table 3).

The immunoreactivity for Ki-67 in epithelial cells was localized predominantly in the nucleolus, although diffuse stained of the nucleus could also be seen. In mitotic figures, it was distributed through all of the cytoplasm (figure 7).

The index of Ki67-positive cells was significantly greater in the Early Vasectomized group than in the Early Sham-operated group, and was also greater in the Late Vasectomized group than in the Late Sham-operated group; in other words, the index was significantly greater in the Late Vasectomized group. However, the index of Ki67-positive cells from the Early Vasectomized group was greater than that of the Late Vasectomized group. When the index of Ki67-positive cells was compared in the Early and Late Sham-operated groups, there was no statistically significant difference (table 3).

The index of apoptotic cells (by Feulgen's reaction) from the Early Vasectomized group did not present statistically significant difference when compared to that of the Early Sham-operated group. The same occurred when the Late Vasectomized group was compared to the Late Sham-operated group, there was not any statistical difference between the Early and Late Sham-operated groups. Also there was no statistical difference between the Early and Late Vasectomized groups (table 3).

The immunoreactivity for Caspase-9 in epithelial cells of the gerbil ventral prostate was distributed equally through the cytoplasm. The index of caspase9-positive cells in the Early Vasectomized group did not present a statistically significant difference compared to the Early Sham-operated group. The same occurred when the Late Vasectomized group was compared with the Late Sham-operated group. There was no statistical difference between the Early and Late Sham-operated groups, or between the Early and Late Vasectomized groups (table 3).

**Table 1**. Score of focal inflammation, focal prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma in the ventral prostate of the vasectomized and sham-operated gerbils (in proportion).

| Score | Group | Subgroup | |
|---|---|---|---|
| | | Early | Late |
| Focal inflammation | Sham-operated | 3/5 | 4/5 |
| | Vasectomized | 4/5 | 4/5 |
| Focal PIN | Sham-operated | 3/5 | 5/5 |
| | Vasectomized | 5/5 | 5/5 |
| Adenocarcinoma | Sham-operated | 2/5 | 3/5 |
| | Vasectomized | 4/5 | 3/5 |

**Table 2.** Height of the epithelium (μm), relative volume of the epithelium (%), relative volume of the stroma (%) and relative volume of the lumen (%)in the ventral prostate of the vasectomized and sham-operated gerbils, given as mean plus or minus one standard deviation.

| Parameter | Group | Subgroup | |
|---|---|---|---|
| | | Early | Late |
| Height of epithelium | Sham-operated | 18,5±4,8$^{Aa}$* | 18,9±4,6$^{Aa}$ |
| | Vasectomized | 20,0±5,6$^{Aa}$ | 20,2±4,8$^{Aa}$ |
| Relative volume of the epithelium | Sham-operated | 29,4±5,4$^{Aa}$ | 30,7±4,5$^{Aa}$ |
| | Vasectomized | 47,5±6,5$^{Ba}$ | 43,8±2,2$^{Ba}$ |
| Relative volume of the stroma | Sham-operated | 16,1±3,7$^{Aa}$ | 18,8±3,9$^{Aa}$ |
| | Vasectomized | 20,8±4,0$^{Aa}$ | 20,5±4,2$^{Aa}$ |
| Relative volume of the lumen | Sham-operated | 54,6±2,0$^{Ba}$ | 50,4±8,4$^{Ba}$ |
| | Vasectomized | 31,7±4,2$^{Aa}$ | 35,3±4,6$^{Aa}$ |

***Different upper-case letters indicate difference between groups within each region; different lower-case letters signify difference between subgroups; P ≤ 0.05.**

**Table 3.** PCNA-positive cells indices (%), Ki67-positive cells indices (%), Caspase9-positive cells indices (%) and apoptotic cells indices by Feulgen's reaction (%)in the ventral prostate of the vasectomized and sham-operated gerbils, given as mean plus or minus one standard deviation.

| Parameter | Group | Subgroup | |
|---|---|---|---|
| | | Early | Late |
| PCNA-positive cells indices | Sham-operated | $2,4\pm0,4^{Aa}$* | $2,1\pm0,5^{Aa}$ |
| | Vasectomized | $11,2\pm1,7^{Bb}$ | $6,7\pm1,7^{Ba}$ |
| Ki67-positive cells indices | Sham-operated | $1,4\pm0,4^{Aa}$ | $1,1\pm0,5^{Aa}$ |
| | Vasectomized | $8,6\pm2,8^{Bb}$ | $4,8\pm1,7^{Ba}$ |
| Caspase9-positive cells indices | Sham-operated | $1,04\pm0,18^{Aa}$ | $1,07\pm0,24^{Aa}$ |
| | Vasectomized | $1,00\pm0,25^{Aa}$ | $1,11\pm0,40^{Aa}$ |
| Apoptotic cells indices by Feulgen's reaction | Sham-operated | $1,61\pm0,24^{Aa}$ | $1,40\pm0,62^{Aa}$ |
| | Vasectomized | $1,14\pm0,44^{Aa}$ | $1,35\pm0,33^{Aa}$ |

***Different upper-case letters indicate difference between groups within each region; different lower-case letters signify difference between subgroups; $P \leq 0.05$.**

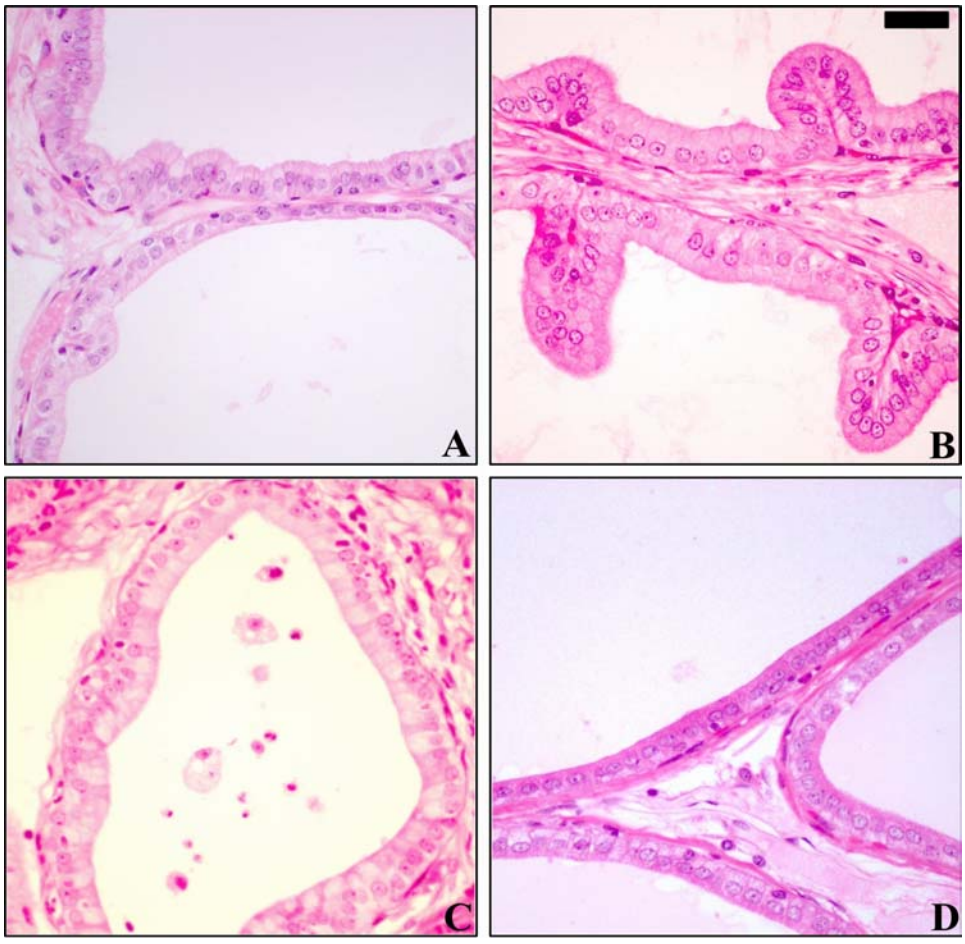


**Figure 1:** Photomicrographs of the gerbil ventral prostate stained with Hematoxylin-eosin. Bar=10μm. A) Early Sham-operated group. B) Early Vasectomized group. C) Late Sham-operated group. D) Late Vasectomized group.

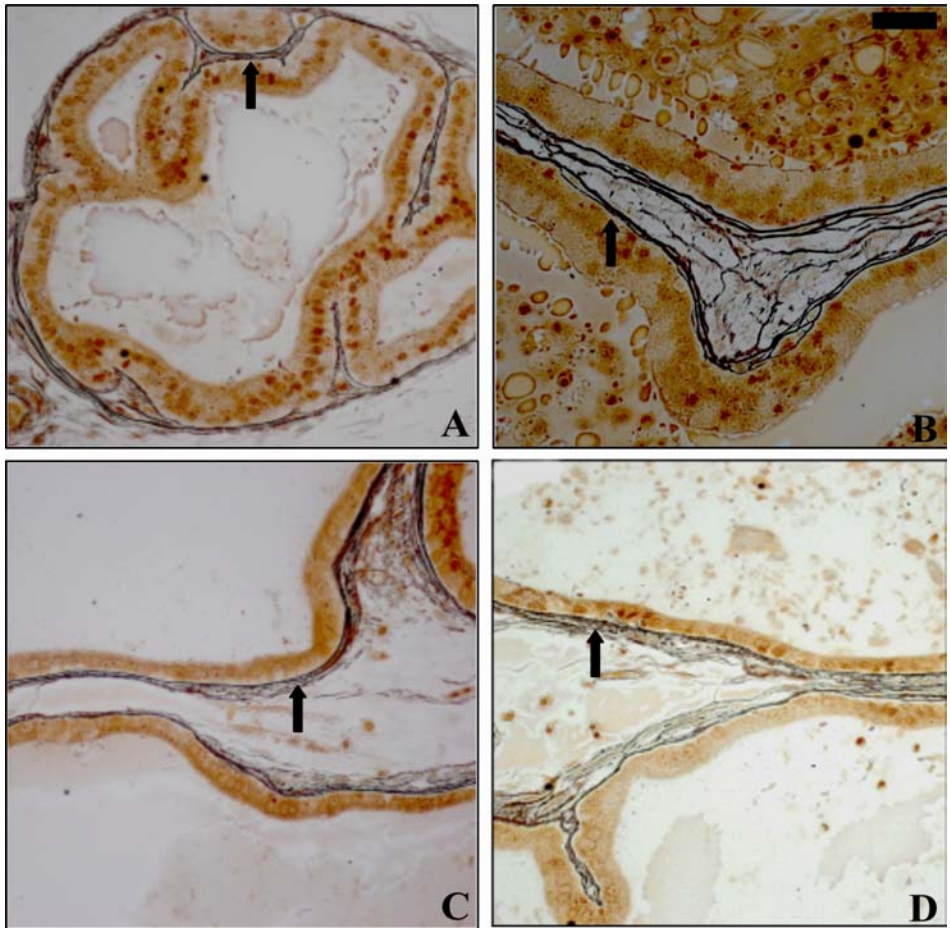


**Figure 2:** Photomicrographs of the gerbil ventral prostate stained with Gömöri's reticulin. Arrow: reticular fibers. Bar=25μm. A) Early Sham-operated group. B) Early Vasectomized group. C) Late Sham-operated group. D) Late Vasectomized group.

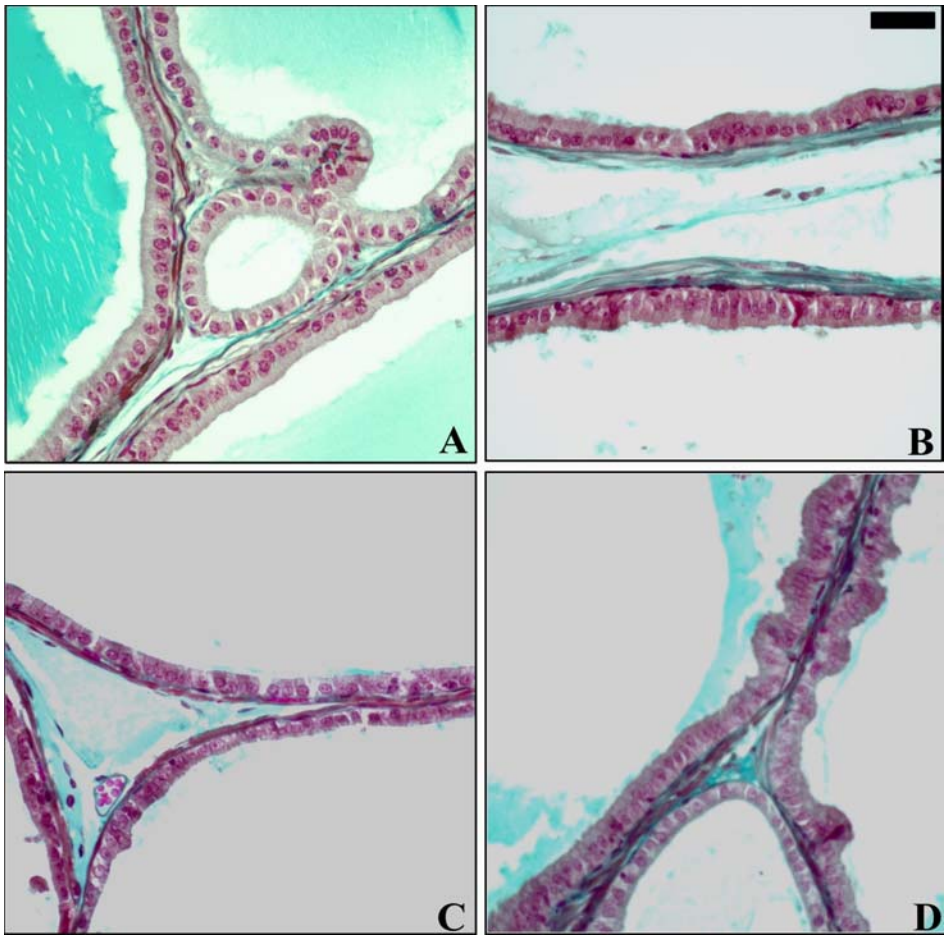


**Figure 3:** Photomicrographs of the gerbil ventral prostate stained with Masson's trichrome. Bar=25μm. A) Early Sham-operated group. B) Early Vasectomized group. C) Late Sham-operated group. D) Late Vasectomized group.

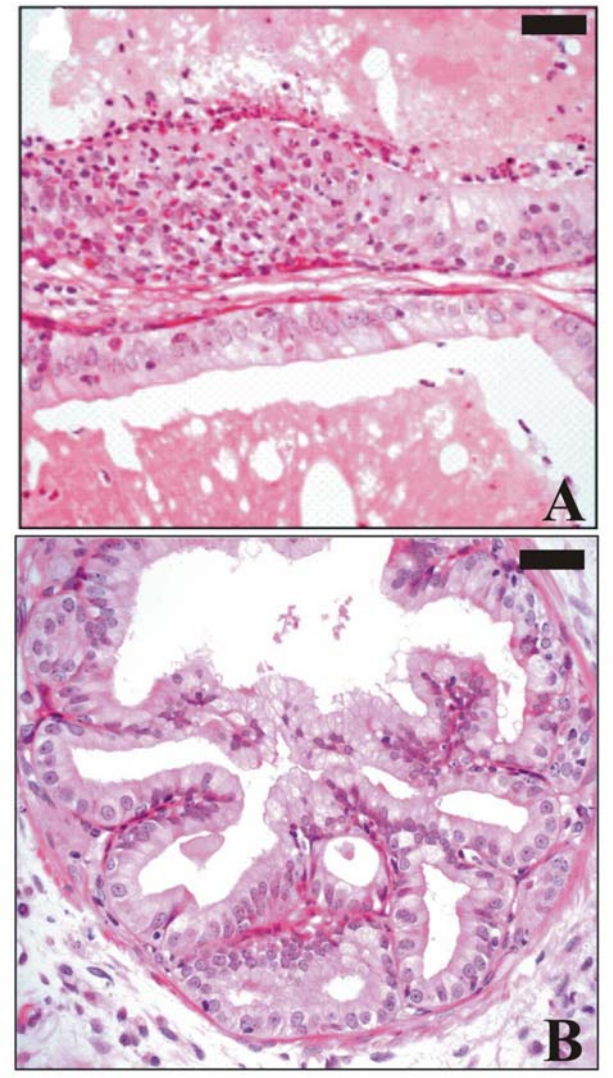


**Figure 4:** Photomicrographs of the gerbil ventral prostate stained with Hematoxylin-eosin A) Early Vasectomized group; showing the association between focal intraepithelial neoplasia and focal inflammation. Bar=25µm. B) Late Vasectomized group, showing focal intraepithelial neoplasia with cribiform aspect. Bar=25µm.

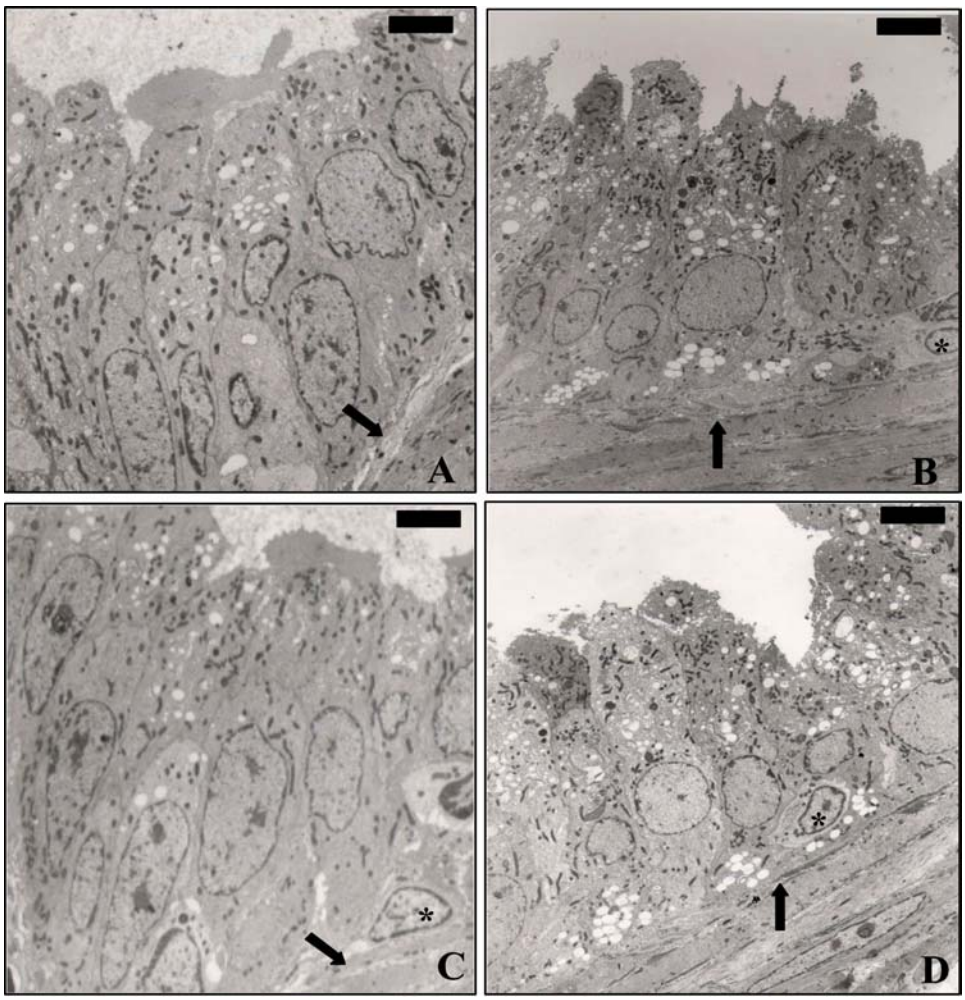


**Figure 5:** Eletromicrographs of the gerbil ventral prostate. Arrow: basement membrane. *: basal cells. A) Early Sham-operated group. Bar=4µm. B) Early Vasectomized group. Bar=3µm. C) Late Sham-operated group. Bar=3µm. D) Late Vasectomized group. Bar=3µm.

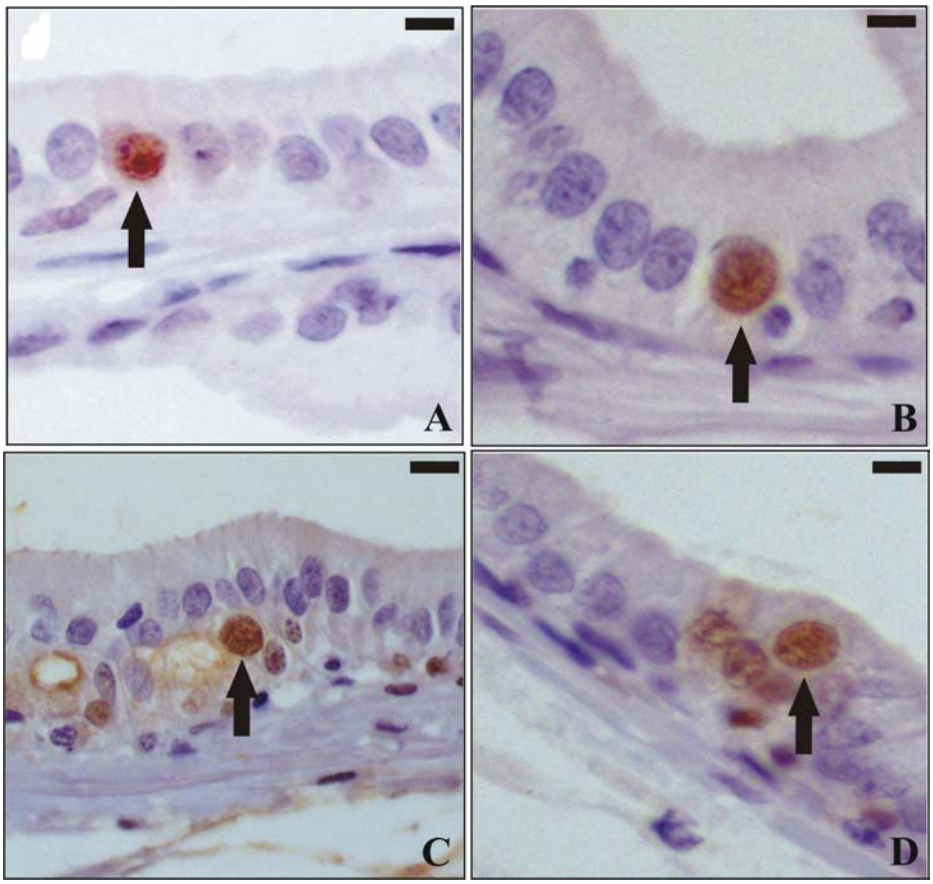


**Figure 6:** Photomicrographs of Immuno-histochemical (anti-PCNA) of the gerbil ventral prostate. Arrow: PCNA-positive cells. A) Early Sham-operated group. Bar=10µm. B) Early Vasectomized group. Bar=8µm. C) Late Sham-operated group. Bar=10µm. D) Late Vasectomized group. Bar=10µm.

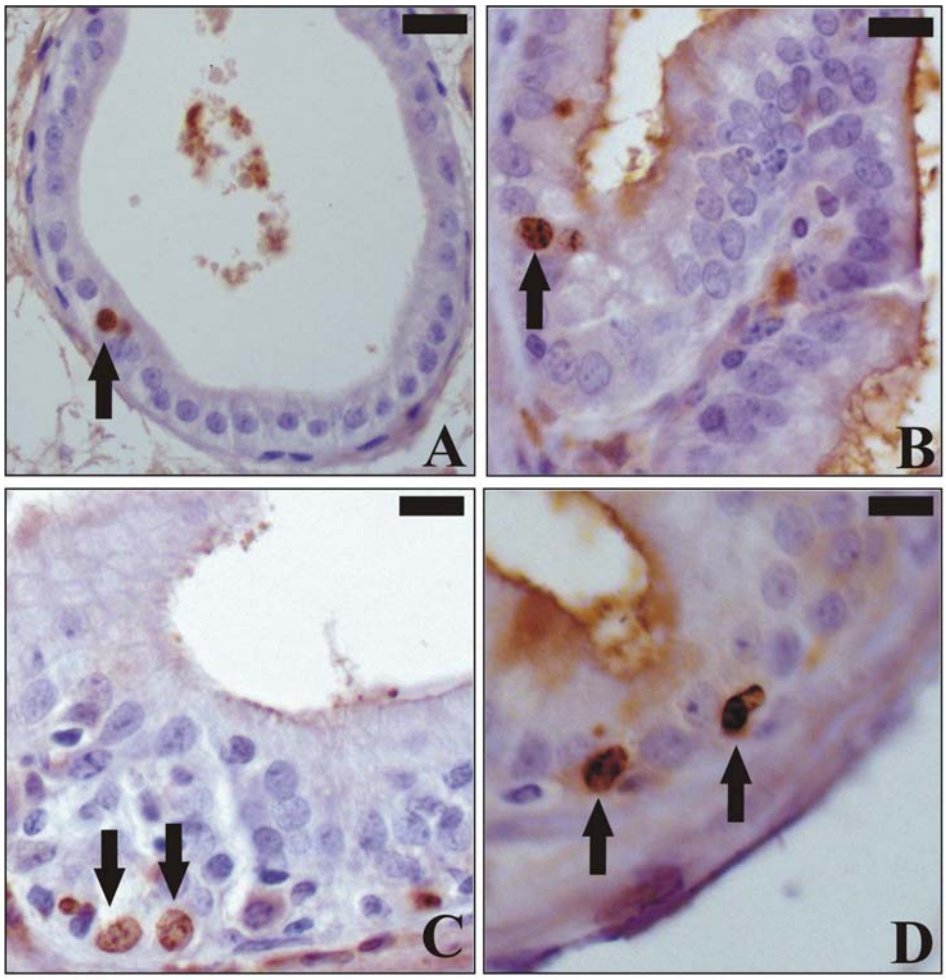


**Figure 7:** Photomicrographs of Immuno-histochemical (anti- Ki67) of the gerbil ventral prostate. Arrow: Ki67-positive cells. A) Early Sham-operated group. Bar=20µm. B) Early Vasectomized group. Bar=8µm. C) Late Sham-operated group. Bar=10µm. D) Late Vasectomized group. Bar=10µm.

**DISCUSSION**

The general morphology observed in the gerbil ventral prostate is similar to that described in the literature [20, 22]. The height of the epithelium of the gerbil ventral prostate is not altered after vasectomy, confirming data from the literature [10], where it was verified that the height of the epithelium in the rat ventral prostate is not altered after vasectomy. The neoplastic lesions found in this study were verified previously by Zanetoni and Taboga [20] in old gerbils. Also, the morphological characteristics of neoplastic lesions were similar to those described by Billis [23].

Evaluation of incidences of focal inflamation, focal intraepithelial neoplasia and adenocarcinoma suggests that the prostates of gerbils from the sham-operated groups present a lesser degree of disorders than the vasectomized groups. Howerer we believe that other analyses should be completed, and that a comparative conclusion from these variables in the present study is premature, due to the high incidence of lesions in all the groups on account of the high incidence of lesions in all the groups, which may be associated with age or characteristics of species, or not solely the influence of vasectomy.

However, it became evident that the neoplastic lesions are associated with focal inflammation. This confirms data from the literature that associates prostate cancer in humans with focal inflammation, but not with vasectomy [8]. This may occur because in ducts with inflammatory infiltrate, cellular proliferation is greater than in healthy ones, since in the inflammatory process proliferative mediators are released [24].

Benign Prostatic Hyperplasia in men is caused by an imbalance between the processes of proliferation and cellular death [12]. The imbalance between these two processes also can result in the transformation or progression of pre-malignant lesions in the prostates of rats from the lineage Noble [11].

Comparing the indices of PCNA-positive and Ki67-positive cells, there is greater specificity for Ki67 and it was verified that the interpretation of results is equal for both markers, although the absolute value for each marker was different. These results corroborate data in the literature that report immunoreactivity for PCNA in all phases of the cellular cycle except G0; and immunoreactivity for Ki67 only in phases G1, G2 and M of the cellular cycle [25]. Thus it can be affirmed that the expression of Ki67 occurs for a shorter period of the cellular cycle than that of PCNA, constituting a more selective marker.

The rise in indices of cellular proliferation, in epithelial cells of the gerbil ventral prostate after vasectomy, corroborates results obtained in our previous study that verified an increase in cellular proliferation in the rat ventral prostate 7 days after vasectomy [10]. The greater increase in cellular proliferation index in the Early Vasectomized group suggests that the longer the post-surgical period, the greater the proliferative alterations in the gerbil ventral prostrate. These results strengthen data in the literature that evidence increased relative risk of prostate cancer in men who realize vasectomy prematurely [9]. Factors still undetermined may be involved in prostatic alteration post-vasectomy, when this is performed at the beginning of the reproductive period.

Comparing the indices of apoptotic cells (by Feulgen's reaction) and Caspase-9-positive cells, it was verified that there were no differences among all the groups for either

of the two methods, although the absolute value for the indices was different. Therefore, vasectomy did not alter the index of apoptosis. The difference in absolute values is due to the specificity of caspases-9 as an initiator of the process of programmed cellular death [26], a process more rapid than the morphological alterations utilized in identification of apoptosis.

The increase in the cellular proliferation index is not accompanied by increase in the apoptosis index in either of the vasectomized groups, which indicates an imbalance between the processes of proliferation and cellular death, in favor of cellular proliferation. Yet this increase in cellular proliferation did not result in higher total volume of the prostate, because the reduction in relative volume of the lumen compensates for growth in relative volume of the epithelium (epithelial hyperplasia).

As suggested by Howards [27], vasectomy can impede the action of TGF-β or increase local production of growth factors that favor the development or progression of prostate cancer. This can be explained by the increase in cellular proliferation in vasectomized animals.

Thus vasectomy causes significant structural alterations in the gerbil ventral prostate how alteration in relative volumes of the epithelium (increase) and lumen (decrease). The vasectomy also promotes an imbalance between the processes of cellular proliferation and apoptosis, in favor of cellular proliferation in the epithelium of the gerbil ventral prostate.

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# CONCLUSÕES

- A vasectomia provoca alterações estruturais na próstata ventral do gerbilo como aumento do volume relativo do epitélio e diminuição do volume relativo da luz;
- A vasectomia causa um desequilíbrio entre os processos de proliferação celular e apoptose, em favor da proliferação celular no epitélio da próstata ventral de gerbilo.
- As alterações proliferativas são maiores quando a vasectomia é realizada precocemente.

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