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MODULAÇÃO ANDROGÊNICA DAS METALOPROTEINASES DE MATRIZ E DOS RECEPTORES DE ESTRÓGENO NA PRÓSTATA MASCULINA E FEMININA DE GERBILOS

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

Co-Orientador: Prof. Dr. Sérgio Luis Felisbino

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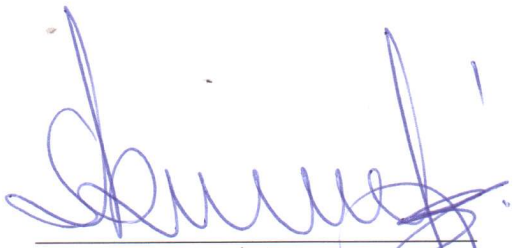

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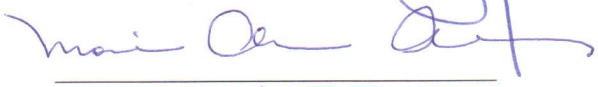

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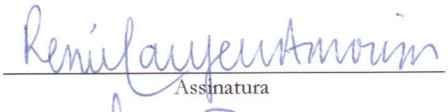

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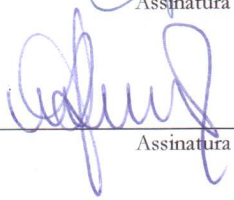

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"Quanto mais aumenta o nosso conhecimento,

mais evidente fica a nossa ignorância"

John F. Kennedy

Dedico

Ao Elison

meu amor, meu amigo, meu companheiro na alegria e na tristeza

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"Cada pessoa que passa em nossa vida é única, sempre deixa um pouco de si e leva um pouco de nós"

SUMÁRIO

RESUMO

O presente trabalho teve por objetivos: 1) Avaliar a atividade das MMPs na próstata de gerbilos machos e fêmeas adultos intactos, procurando comparar a glândula prostática de machos e fêmeas nas diferentes fases do ciclo estral (artigo I); 2) Avaliar o efeito da testosterona sobre o lobo ventral masculino e sobre a próstata feminina quanto à localização de MMP-2 e TIMP-2, enzimas envolvidas na remodelação estromal e na progressão do câncer (artigo II) e 3) Caracterizar a distribuição dos receptores de estrógeno, ERα e ERβ, nos tecidos prostáticos de gerbilos machos e fêmeas controles e submetidos à manipulação hormonal (artigo III). Foram realizados experimentos de castração (7 ou 21 dias) nos gerbilos machos adultos, além de experimentos de aplicação hormonal (7 ou 21 dias) em fêmeas adultas na fase de proestro (cipionato de T, 1 mg/kg ou DHEA, 1 mg/kg). Os tecidos foram avaliados por análises bioquímicas, histológicas e imuno-histoquímicas. A atividade gelatinolítica das MMPs obtida por zimografia difere entre os lobos prostáticos masculinos e a glândula feminina, que apresenta os níveis mais altos para MMP-2 e -9, provavelmente devido à constante remodelação estromal promovida pelo ciclo estral. Com relação ao ciclo estral, a maior atividade de MMP-2 e -9 foi encontrada na fase estro, indicando influência do estrógeno. A castração elevou a expressão de MMP-2 na próstata ventral masculina, e o mesmo efeito foi observado após 21 dias de aplicação de T nas fêmeas, indicando que MMPs e TIMPs são influenciados por andrógenos, mas de forma distinta entre machos e fêmeas uma vez que tanto a falta como o excesso de T levaram ao mesmo efeito, provavelmente, devido a machos e fêmeas apresentarem diferentes padrões de expressão para os receptores nucleares, responsáveis por regular a resposta hormonal pelos tecidos. Segundo o estudo há notável diferença na imunolocalização de ERα entre machos e fêmeas, cujas células estromais prostáticas apresentaram duas vezes mais ERα do que as células estromais da próstata ventral masculina. Esse número foi reduzido drasticamente após aplicação de T por 21 dias nas fêmeas, mas não após a castração dos machos. A reação ao ERβ foi similar entre machos e fêmeas intactos, mas a castração dos machos ou a aplicação hormonal nas fêmeas promoveu resposta específica, como total ausência após 21 dias de T nas fêmeas ou intensa imunoreação em algumas regiões da próstata após 21 dias de castração nos machos. Adicionalmente, lesões epiteliais presentes nos grupos experimentais tiveram reação ERα-positivas e ERβ-negativas, corroborando os estudos que relacionam o ERα com o desenvolvimento e progressão de doenças prostáticas e o ERβ como um fator de prevenção e tratamento do câncer. Os resultados obtidos reforçam o conceito de que o equilíbrio estrógeno/testosterona é essencial para a homeostase prostática, e que tanto andrógenos quanto estrógenos atuam sobre as células epiteliais, através dos receptores nucleares, mas também sobre o compartimento estromal, regulando componentes como as MMPs. No caso da próstata feminina, essa interação é ainda mais complexa pois envolve as alterações cíclicas do ciclo estral.

ABSTRACT

The present study aimed to: 1) Acess the activity of MMPs in the prostate gland of intact adult male and female gerbils, trying to compare the male prostate lobes with the female gland in different phases of the estrous cycle (Article I); 2) Evaluate the effect of testosterone on ventral prostate of males and on female prostate in the proestrus phase over the location of MMP-2 and TIMP-2, enzymes involved in stromal remodeling and cancer progression (Article II); and 3) Characterize the distribution of estrogen receptors, ERα and ERβ in prostatatic tissue of male and female controls and under hormonal manipulation (Article III). Castration experiments (7 and 21 days) were conducted in adult male gerbils as well as hormone injection experiments (7 and 21 days) in adult females in the proestrus phase (T cypionate, 1 mg/kg or DHEA, 1 mg/kg). Tissues were evaluated by biochemical, histological and immunohistochemical methods. The gelatinolytic activity of MMPs, measured by zymography gel, differs from male prostatic lobes and the female gland, which presents the highest levels of MMP-2 and -9, probably due to its constant stromal remodeling promoted by the estrous cycle. Regarding the estrous cycle, the highest activity of MMP-2 and -9 was found in the estrus phase, indicating the influence of estrogen. Castration increased the expression of MMP-2 in the male ventral prostate, and this same effect was observed after 21 days of T injection in females, indicating that MMP and TIMP regulatory mechanism is also influenced by androgens, although differently between males and females since both the failure as the excess of T lead to the same effect, probably due to males and females presenting different patterns of expression for nuclear receptors, which are responsible for regulating the hormonal response by the tissues. According to our results, there is a remarkable difference in the immunolocalization of ERα between males and females, whose stromal cells presented two times more ERα than stromal cells of the male ventral prostate. This number was drastically reduced after 21 days of T injection in females, but not after castration of males. The immunoreaction to ERβ was similar between intact males and females, but the castration of males or the hormonal injection in females promoted specific response, such as total absence of reaction after 21 days of T treatment in females or intense immunoreaction in some regions of ventral prostate after 21 days of castration. Additionally, epithelial lesions found in the experimental groups, were ERα-positives and ERβ-negative, corroborating studies that relate ERα with the development and progression of prostatic diseases and ERβ as a factor in preventing and treating cancer. The results obtained in this study therefore reinforce the concept that the estrogen/testosterone balance is essential for prostate homeostasis, and that either androgens or estrogens have an effect on the prostatic epithelial cells, via nuclear receptors, but also on the stromal compartment by regulating components such as MMPs. In the case of the female prostate, this interaction is even more complex since it involves the cyclical changes of the estrous cycle.

INTRODUÇÃO

Próstata masculina e feminina do gerbilo da Mongólia

Figura 1: *Meriones unguiculatus*

O gerbilo (*Meriones unguiculatus*), também conhecido como esquilo da Mongólia (Fig. 1), é um murídeo da subfamília Gerbillinae, proveniente das regiões áridas da China e Mongólia (Schwentker, 1963). Devido às suas características especiais, comportamento dócil e facilidade de manejo, os gerbilos têm sido extensivamente adotados em experimentos biológicos e biomédicos nas áreas da fisiologia, farmacologia, parasitologia e endocrinologia (Neumann *et al.*, 2001). Além disso, o gerbilo têm se demonstrado um excelente modelo experimental para estudos do sistema reprodutor, tanto masculino (Rochel *et al.*, 2007; Pinto et al, 2010), como feminino (Nishino e Totsukawa, 1996; Santos *et al.*, 2006; Fochi *et al.*, 2008; Custódio *et al.*, 2010; Lv e Shi, 2010), bem como estudos sobre envelhecimento (Cheal, 1986; Campos *et al.*, 2010) e regulação hormonal da próstata (Góes *et al.*, 2007; Scarano *et al.*, 2008; Corradi *et al.*, 2009; Taboga *et al.*, 2009).

A próstata masculina do gerbilo, semelhantemente à de outros roedores, é uma glândula tubuloacinar composta, formada por um complexo sistema de ductos que partem da uretra e terminam em ramos, distalmente. Apresenta-se dividida em quatro lobos pares (ventrais, dorsais, dorso-laterias e anteriores) localizados abaixo da bexiga urinária, circundando a uretra (Sugimura *et al.,* 1986; Hayashi *et al.*, 1991; Rochel *et al.*, 2007).

Histologicamente, todos os lobos prostáticos do gerbilo, bem como de outros roedores, são compostos por um componente epitelial circundado por um estroma fibromuscular. A camada epitelial prostática apresenta como principal tipo celular as células colunares secretoras. Estas células estão dispostas em uma única camada de revestimento epitelial e são responsáveis por sintetizar e secretar, no lumen acinar, diversas proteínas, incluindo antíngeno prostático específico (PSA, do inglês, *prostatic specific antigen*) e fosfatases específicas da próstata, que irão compor os constituintes líquidos do ejaculado masculino. O epitélio prostático contém ainda células basais, menores e menos numerosas que as secretoras, geralmente restritas ao compartimento basal do epitélio, atuando como fonte progenitora das células secretoras, células neuroendrócrinas e algumas células do sistema imune, como macrófagos e linfócitos (McNeal, 1997; Chatterjee, 2003; Rochel *et al.*, 2007). Uma membrana basal altemante organizada e composta principalmente por colágeno tipo IV

e laminina separa fisicamente a camada epitelial do componente estromal. A composição celular do estroma prostático inclui fibroblastos e células musculares lisas, formando uma densa camada que circunda os ácinos, além de células endoteliais, células nervosas e células imunológicas infiltradas como mastócitos e linfócitos. As células estromais encontram-se imersas em uma matriz extracelular rica em componentes estruturais incluindo proteínas fibrilares (como colágenos e elastina), proteoglicanos e glicoproteínas (como fibronectina e laminina), além de enzimas específicas como as metaloproteinases de matriz (Carvalho e Line, 1996; Vilamaior *et al.*, 2000; Chatterjee, 2003).

Apesar de apresentarem características semelhantes com relação à morfologia e estrutura, os lobos prostáticos diferem quanto à ultraestrutura das células secretoras, mecanismo de secreção do fluido prostático e componentes da matriz extracelular (Rochel *et al.*, 2007). O lobo ventral vem sendo o mais estudado por suas características similares à próstata humana e por ser o mais responsivo às alterações hormonais (Pegorin de Campos *et al.*, 2006; Cordeiro *et al.*, 2008).

As fêmeas de *Meriones unguiculatus*, assim como de outros mamíferos (Shehata, 1980; Gross e Didio, 1987; Flamini *et al.*, 2002), apresentam em seu sistema reprodutor uma glândula prostática funcionalmente ativa. Segundo Santos e colaboradores (2003), a próstata feminina de *Meriones unguiculatus* apresenta cerca de 10% do peso prostático masculino e possui localização parauretral, exibindo íntimo contato com a parede da uretra nas porções distal e mediana. Análises estruturais e ultra-estruturais da próstata feminina adulta de gerbilos indicam que as células secretoras encontram-se em plena atividade e revelam semelhanças morfológicas com o lobo ventral do gerbilo macho adulto (Santos *et al.*, 2003; Pegorin de Campos *et al.*, 2006). Adicionalmente, a próstata feminina do gerbilo apresenta similaridade com a próstata feminina humana descrita por Zaviačič (1999), no que se refere à estrutura alveolar e tecidual (Santos e Taboga, 2006), e tem sua atividade modulada de acordo com as alterações hormonais do ciclo estral, que no gerbilo é de 4 a 5 dias (Nishino e Totsukawa, 1996; Fochi *et al.*, 2008).

As similaridades estruturais existentes entre a próstata do gerbilo e a humana, tanto em machos como em fêmeas, sugerem que esse roedor é um excelente modelo experimental para investigar os aspectos funcionais do órgão normal e a instalação de desordens patofisiológicas.

Modulação hormonal prostática: equilíbrio andrógeno/estrógeno

A próstata é uma glândula sexual acessória do aparelho reprodutor que nos homens é responsável pela produção da maior fração do fluido seminal. O grande interesse em se

compreender a biologia desta glândula deve-se tanto ao seu fascinante processo de desenvolvimento, quanto à alta incidência de doenças prostáticas, como adenocarcinoma e a hiperplasia prostática benigna (HPB) nos homens (Marker *et al.*, 2003).

Os andrógenos, esteróides sexuais masculinos, desempenham um papel obrigatório no desenvolvimento embrionário e diferenciação da próstata, além de serem os responsáveis pela manutenção e suporte do tecido prostático no adulto (Thomson, 2001; Chatterjee, 2003; Prins e Putz, 2008). Além disso, os andrógenos regulam o desenvolvimento e progressão das patologias prostáticas associadas à idade, incluindo hiperplasia benigna e câncer (Dehm e Tindall, 2007; Richter *et al.*, 2007; Balk e Knudsen, 2008).

A testosterona (T) é o principal andrógeno atuante na próstata e sua demanda no organismo origina-se da produção testicular (95%) e da produção das glândulas adrenais (5%) (Aumüller e Seitz, 1990). Porém, na próstata a T é convertida através da ação da enzima 5α-redutase em dihidrotestosterona (DHT), um andrógeno cerca de 10 vezes mais potente (Wilson, 1996). Esses dois andrógenos atuam de forma distinta no sistema reprodutor, sendo que a T regula a diferenciação sexual, além de manter as funções sexuais e a libido, enquanto que a DHT tem papel fundamental durante a virilização embrionária puberal, além de ser responsável pela funcionalidade da próstata do indivíduo adulto (Soronen *et al.*, 2004).

Apesar de apresentarem funções distintas, tanto a T como a DHT exercem seus efeitos biológicos através do mesmo receptor de andrógeno (AR, do inglês *androgen receptor*). O AR, membro da superfamília dos receptores nucleares, pode ser encontrado tanto em células prostáticas epiteliais como estromais, onde age como fator de transcrição, sendo responsável por regular a expressão de mais de 100 genes na próstata, bem como de sequências específicas de DNA conhecidas como elementos andrógeno-responsivos (Nelson *et al.*, 2002; Chatterjee, 2003; Black e Paschal, 2004).

Durante o desenvolvimento prostático, os andrógenos estimulam os fibroblastos AR-positivos presentes no estroma a produzir e secretar diversos fatores de crescimento, os quais atuam de modo parácrino sobre as células epiteliais vizinhas, induzindo o crescimento celular e o desenvolvimento glandular (Fig. 2). Esse tipo de interação epitélio-estroma é essencial para a manutenção da próstata e encontra-se descrito mais detalhadamente a seguir. Já na próstata adulta, os andrógenos agem diretamente nas células epiteliais via AR, estimulando a síntese e secreção dos componentes prostáticos como PSA, auxiliando a manutenção estrutural e funcional da glândula (Nelson *et al.*, 2002; Chatterjee, 2003; Black e Paschal, 2004).

As células epiteliais secretoras, portanto, requerem constante estímulo androgênico para sobrevivência e integridade funcional. Quando o estímulo antrogênico cai abaixo do limiar necessário, como ocorre na castração por exemplo, as células secretoras entram em apoptose, causando involução glandular. As células epiteliais basais, no entanto, persistem após a castração uma vez que são, em sua maioria, negativas à este receptor, e dessa forma, parecem estar envolvidas no desenvolvimento de patologias andrógeno-independentes. Os ARs têm, portanto, fundamental importância para a fisiologia prostática uma vez que exercem papel essencial no desenvolvimento e manutenção da próstata normal e podem estar diretamente envolvidos no desenvolvimento e progressão do câncer prostático, tanto andrógeno-dependente como independente (Evans, 1988; Chatterjee, 2003; Dehm e Tindall, 2007; Richter *et al.*, 2007; Balk e Knudsen, 2008).

Além da T e da DHT, estudos apontam um outro composto androgênico, a dehidroepiandrosterona (DHEA) como tendo ação efetiva na biologia intra-prostática (Labrie, 2010). A DHEA é um pró-hormônio de origem adrenal e atua como um reservatório para a biossíntese tanto de andrógenos como de estrógenos nos tecidos periféricos (Labrie *et al.*, 2005). Embora a função biológica da DHEA como precursor de esteróides sexuais já tenha sido estabelecida, o mecanismo de ação celular ainda não foi totalmente elucidado (Arnold e Blackman, 2005, Labrie *et al.*, 2005; Labrie, 2010). Acredita-se, no entanto, que muitos dos seus efeitos ocorram via receptor de andrógeno e/ou estrógeno após sua conversão enzimática nesses hormônios sexuais (Labrie *et al.*, 2001).

Apesar dos andrógenos serem os principais hormônios atuantes na próstata, atualmente diversos estudos descrevem a importância dos estrógenos, especialmente o estradiol (E2), no desenvolvimento e na homeostase normal do tecido prostático (Chen *et al.*, 2008; McPherson *et al.*, 2008; Scarano *et al.*, 2008; Ellem e Risbridger, 2009). Os estrógenos desempenham importante papel na regulação do crescimento da próstata em todos os estágios da vida, porém, no homem os níveis estrogênicos precisam ser balanceados a um nível ótimo, uma vez que a falta do estrógeno pode levar a hipertrofia e hiperplasia prostática, e o excesso deste hormônio ocasiona respostas adversas ao epitélio prostático, como proliferação, inflamação e câncer (Chen *et al.*, 2008; Scarano *et al.*, 2008; Ellem e Risbridger, 2009).

O E2 é sintetizado via aromatização de andrógenos pela enzima aromatase (O'Donnell *et al.*, 2001; Risbridger *et al.*, 2003) e sua ação na próstata é mediada por dois tipos de receptores nucleares, receptores de estrógeno alfa e beta (ERα e Erβ, do inglês *estrogen receptor*), cada um atuando em um

determinado momento do desenvolvimento prostático e com respostas distintas frente ao estímulo hormonal (Chen *et al.*, 2008; Morani *et al.*, 2008; Prins e Putz, 2008). Segundo estudos recentes, o ERα parece ser o responsável pela construção da glândula prostática antes da puberdade enquanto o ERβ parece ter maior importância durante períodos mais tardios do desenvolvimento prostático, como a puberdade e a idade adulta, agindo juntamente com o AR na regulação da diferenciação e atividade funcional do tecido adulto (fig. 2) (Imamov *et al.*, 2004; Omoto *et al.*, 2005; McPherson *et al.*, 2008; Prins e Putz, 2008). Além disso, o ERα tem ação proliferativa, e quando ativado pode desencadear diversos efeitos adversos na próstata de roedores, como proliferação aberrante do epitélio, inflamação e desenvolvimento de lesões pré-malignas. Por outro lado, o ERβ está relacionado à quiescência e atua na sinalização celular epitélio-estroma e na regulação dos efeitos anti-proliferativos responsáveis por modular a ação hipertrófica dos andrógenos no epitélio prostático (Weihua *et al.*, 2002; Imamov *et al.*, 2004; Omoto *et al.*, 2005; McPherson *et al.*, 2008; Ellem e Risbridger, 2009).

A ação dos estrógenos sobre a próstata é, portanto, importante e complexa, uma vez que exerce tanto efeitos benéficos como adversos via ERβ e ERα. Assim, não apenas a razão andrógeno/estrógeno influencia o desenvolvimento e a homeostase prostática, como também o equilíbrio entre a expressão de ERα e ERβ é crucial para determinar a resposta da próstata aos

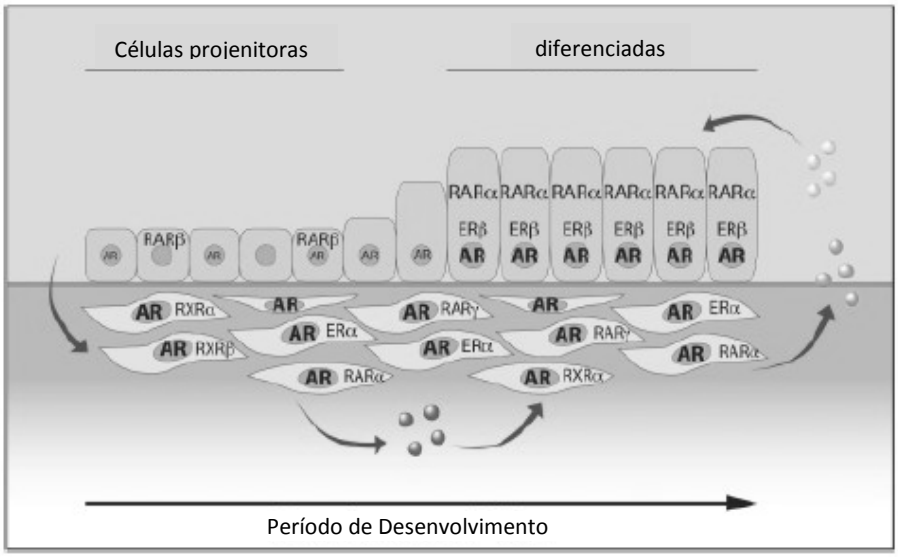

Figura 2: Expressão dos receptores nucleares AR, ERα e ERβ durante o desenvolvimento prostático. Modificado de *Prins e Putz, 2008*. Legenda: AR – receptor de andrógeno, ER – receptor de estrógeno, RAR – receptor de ácido retinóico.

estrógenos. Além disso, é o equilíbrio entre andrógenos e estrógenos, e não a ação isolada de cada um individualmente, que influencia o crescimento e desenvolvimento prostático normal, bem como a manutenção da homeostase prostática ao longo da vida adulta (McPherson *et al.*, 2008; Yatkin *et al.*, 2008). Um desequilíbrio na razão entre estes hormônios durante o desenvolvimento fetal ou pós-natal pode acarretar conseqüências permanentes e irreversíveis na vida adulta (Prins e Birch, 1997; Omoto *et al.*, 2005; McPherson *et al.*, 2008; Yatkin *et al.*, 2008).

Interação epitélio/estroma

Uma série de evidências tem atribuído um papel significativo ao compartimento estromal para a fisiologia prostática e para o desenvolvimento de patologias nessa glândula (Ricke *et al.*, 2005). O estroma prostático é constituído de células musculares lisas, fibroblastos e uma abundante matriz extracelular (MEC), rica em fibras colágenas e elásticas, cuja função é promover suporte mecânico ao epitélio adjacente. Além disso, o estroma é responsável por elaborar fatores de crescimento que modulam atividades secretórias e proliferativas dentro da população celular estromal, através de estímulos autócrinos, que por sua vez são transmitidos por secreção parácrina ao epitélio (Farnsworth, 1999). Esta interação parácrina entre o epitélio e o estroma ao seu redor tem importância fundamental durante a morfogênese prostática e na homeostase do órgão adulto, uma vez que tanto a proliferação como o fenótipo das células epiteliais dependem de alguma contribuição difusível do estroma (Cunha *et al.*, 1985; Nemeth e Lee, 1996; Hayward *et al.*, 1996; Taboga *et al.*, 2009). As interações epitélio-estroma na próstata são, portanto, fundamentais para entender o controle da atividade normal da glândula e a ocorrência de desordens patológicas (fig. 3).

Matriz Extracelular e as Metaloproteinases de Matriz

A matriz extracelular corresponde a um ambiente dinâmico formado por um complexo arranjo de proteínas fibrilares, glicoproteínas adesivas e proteoglicanos (Tuxhorn *et al.*, 2001). Os componentes estruturais da MEC tais como colágeno e fibras elásticas, proporcionam rigidez mecânica e flexibilidade ao tecido, servindo também como substrato para a adesão celular e migração (Carvalho *et al.*, 1997). Além disso, as macromoléculas que compõem a MEC atuam cooperativamente para regular uma gama de processos celulares, tais como proliferação, diferenciação e morte. Esses aspectos do comportamento celular são essenciais para o

desenvolvimento, a maturação e a homeostase dos órgãos, incluindo a próstata (Hay, 1991; Amălinei *et al.*, 2007).

Conforme descrito anteriormente, um desequilíbrio na razão andrógeno/estrógeno pode provocar uma série de alterações patológicas na próstata, ocasionando remodelação do tecido conjuntivo prostático, o que requer tanto a degradação como a síntese de componentes da MEC (Hulboy *et al.*, 1997). Enzimas específicas secretadas no espaço extracelular, denominadas

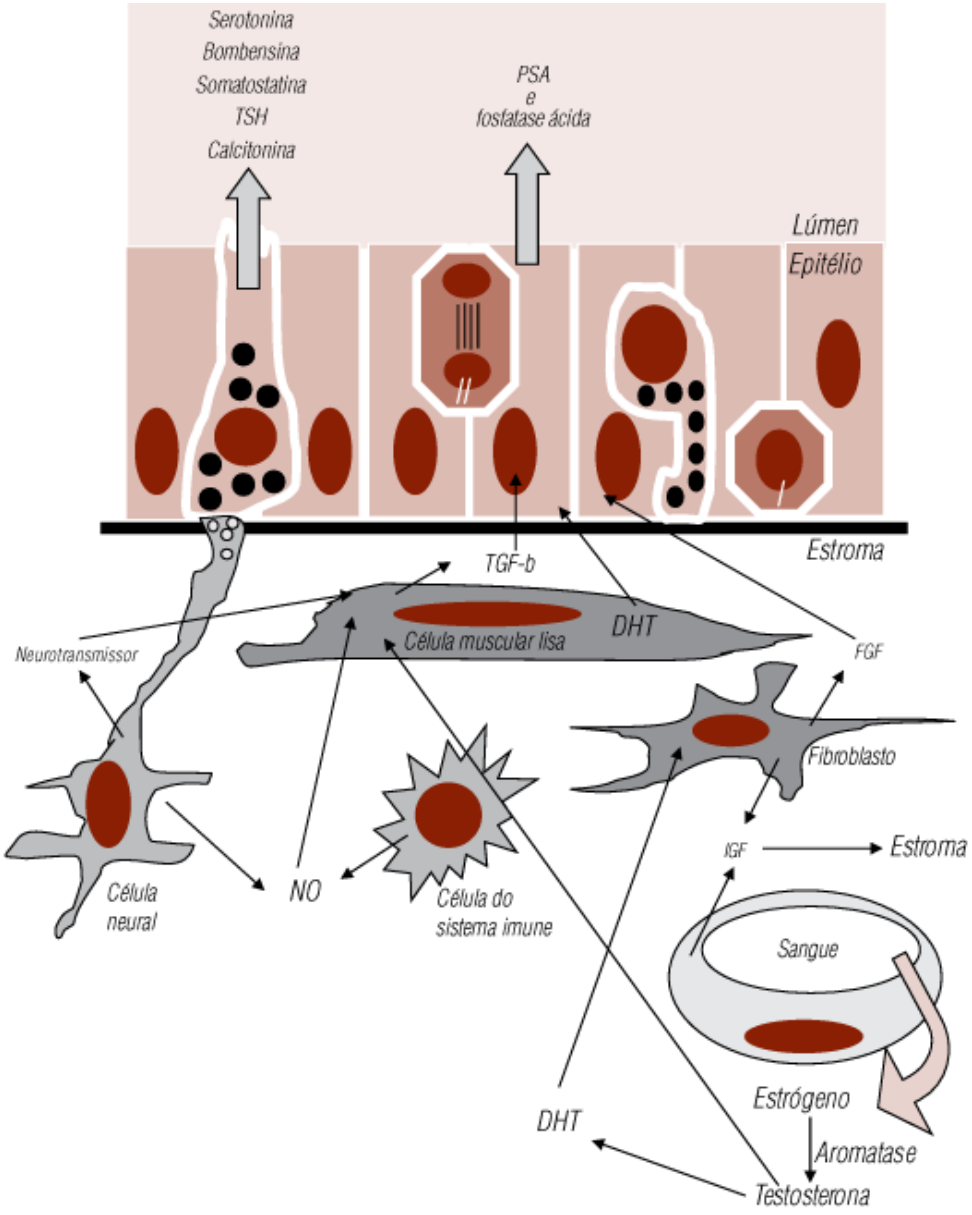

Figura 3: Complexas interações entre epitélio e estroma na próstata. FGF-b (fator de crescimento beta), DHT (diidrotestosterona), NO (óxido nítrico), FGF (fator de crescimento fibroblástico), PSA (antígeno prostático específico), I (célula basal), II (célula em divisão). Fonte: *Taboga et al., 2009*.

metaloproteinases de matriz (MMPs, do inglês *matrix metallo-proteinases*), atuam na degradação protéica da MEC, desnaturando colágenos e, assim, remodelando o estroma (Hulboy *et al.*, 1997). Além disso, por desempenharem um papel na perda de massa de tecido conjuntivo, as MMPs podem influenciar o fenótipo dos componentes celulares dos tecidos, alterando funções celulares básicas como proliferação, diferenciação e apoptose (Hulboy *et al.*, 1997).

MMPs são endopeptidades dependentes de metais, principalmente zinco e cálcio, capazes de degradar os componentes da MEC, como colágeno, elastina, laminina, fibronectina e proteoglicanos (Basset *et al.*, 1997; Hullboy *et al.*, 1997; Stamenkovik, 2000). Essas enzimas são secretadas na forma de um precursor latente, chamadas de zimógenos ou pró-MMPs, e necessitam de ativação antes de se tornarem capazes de clivar os elementos da matriz extracelular (Stamenkovik, 2000).

Atualmente são descritas mais de 20 tipos de MMPs, divididas em 6 grupos com base na especifidade do substrato, similaridade sequencial e organização dos domínios: 1) colagenases; 2) gelatinases; 3) estromelisinas; 4) matrilisinas; 5) metaloproteinases de membrana e 6) MMPs que não se enquadram nessas definições (Egeblad e Werb, 2002; Amălinei *et al.*, 2007).

As MMPs 2 e 9, integrantes do grupo das gelatinases, têm um papel importante na degradação e remodelação da MEC em vários estados fisiológicos como implantação e regeneração tecidual. As gelatinases degradam os colágenos tipo IV, V, VII, X, XI, e XIV, elastina, proteínas centrais dos proteoglicanos, além de fibronectina e fibrilina 1, entre outros fatores (Amălinei *et al.*, 2007). Além disso, são enzimas consideradas importantes por contribuírem no processo invasivo metastático e angiogênico de vários tumores, incluindo o câncer de próstata (Hornebeck *et al.*, 2002; Trudel *et al.*, 2008).

Por muito tempo acreditou-se que essas enzimas fossem produzidas por células malignas do câncer, contribuindo especificamente para a invasão tumoral, através de sua habilidade de degradar componentes da matriz extracelular. No entanto, estudos têm demonstrado que proteinases extracelulares implicadas na progressão de carcinomas humanos, incluindo a maioria das MMPs, são de fato predominantemente expressas por células estromais sadias (McDougall e Matrisian, 1996; Basset *et al.*, 1997).

Acredita-se que a expressão destas metaloproteinases seja influenciada por hormônios sexuais uma vez que estudos experimentais em animais revelaram um aumento na concentração de MMPs em decorrência da privação androgênica. Estudos demonstraram que durante a involução prostática que se segue à castração de ratos machos, há um aumento significativo da expressão de

MMP-7 (Powell *et al.*, 1996) bem como um aumento na atividade gelatinolítica das MMP-2 e MMP-9 na próstata destes animais (Wilson *et al.*, 1991; Justulin-Jr *et al.*, 2009). Esses dados sugerem que as MMPs estão envolvidas nos processos de atrofia glandular e remodelação tecidual da próstata após redução no nível de andrógenos.

Segundo Matrisian (1990), a atividade e a produção das MMPs pode ser controlada por uma série de inibidores endógenos conhecidos como inibidores teciduais de metaloproteinases (TIMPs, do inglês *tissue inhibitors of metallo-proteinases*. O balanço entre MMPs e TIMPs é, em grande parte, responsável pelo controle da degradação dos elementos formadores da MEC. Um desequilíbrio entre essas duas moléculas é responsável por diversas condições patológicas, como por exemplo, artrite reumatóide, doenças cardiovasculares e a progressão do câncer (Bode *et al.*, 1999). Quatro TIMPs foram identificados em mamíferos: TIMP-1, TIMP-2, e TIMP-4, que são proteínas secretadas, enquanto o TIMP-3 encontra-se ancorado na MEC. A expressão dos TIMPs é regulada durante o desenvolvimento e remodelação tecidual e o mecanismo de inibição parece ocorrer devido à ligação do domínio N-terminal do TIMP ao domínio catalítico da MMP (Visse e Nagase, 2003).

Além da sua capacidade de inibir a atividade de MMPs, os TIMPs também têm sido implicados em processos envolvendo crescimento celular. O TIMP-1, por exemplo, tem sido relatado como parte de um complexo que pode estimular a esteroidogênese nos testículos e ovários (Hulboy *et al.*, 1997). Condições patológicas associadas ao desbalanço na atividade das MMPs e alterações nos níveis dos TIMPs devem ser consideradas importantes uma vez que esses inibidores afetam diretamente a atividade das MMPs (Visse e Nagase, 2003).

OBJETIVOS

Objetivo pricipal:

Avaliar os efeitos da ação hormonal sobre a próstata de gerbilos machos e fêmeas adultos, especialmente com relação às alteraçoes da matriz extracelular e sua remodelação estromal.

Objetivos específicos:

- ➔ Estabelecer o perfil de expressão de MMPs dos lobos prostáticos de gerbilos machos e da próstata de gerbilos fêmeas durante o ciclo estral;
- ➔ Avaliar o efeito da castração cirúrgica sobre o estroma da próstata ventral de gerbilos machos, com especial ênfase à MMP2 e seu inibidor tecidual TIMP2;
- ➔ Avaliar o efeito da Testosterona e DHEA sobre o estroma da próstata de fêmeas de gerbilos na fase de proestro, com especial ênfase à MMP2 e seu inibidor tecidual TIMP2;
- ➔ Analisar o padrão de expressão dos receptores de estrógeno alfa e beta (ERα e ERβ) na próstata ventral de gerbilos machos intactos, bem como na próstata de fêmeas na fase de proestro;
- ➔ Analisar as alterações causadas pela castração ou aplicação hormonal de Testosterona e DHEA sobre a expressão de ERα e ERβ na próstata masculina e feminina de gerbilos.

RESULTADOS

Para desenvolvimento deste trabalho diversos métodos de pesquisa foram empregados, entre eles a análise histológica, imuno-histoquímica, análise ultraestrutural e análise bioquímica, envolvendo a colaboração de três instituições de ensino:

- **Laboratório de Microscopia e Microanálise** - Ibilce/UNESP, São José do Rio Preto, SP – Brasil (experimentação animal, análise histológica, imuno-histoquímica e ultraestrutural);
- **Laboratório de Matriz Extracelular –** IB/UNESP, Botucatu, SP – Brasil (análise imuno-histoquímica e zimografia);
- **Centro de Receptores Nucleares e Sinalização Celular** – CNRCS/UH, Houston, TX – EUA (análise imuno-histoquímica e imunofluorescência).

Os resultados estão organizados na forma de 3 artigos científicos, apresentados na sequência.

ARTIGO I

INFLUENCE OF ESTROUS CYCLE ON THE ACTIVITY OF MMP-2 AND MMP-9 IN THE GERBIL FEMALE PROSTATE

Submetido para publicação na revista

"Reproduction"

Influence of Estrous Cycle on MMP-2 and MMP-9 Activity in the Gerbil Female Prostate

Short Title: ESTROUS CYCLE AND FEMALE PROSTATE MMPS

Key-words: female prostate, estrous cycle, MMPs, steroid hormones, ultrastructure.

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ABSTRACT

The prostate gland of female gerbils undergoes extensive morphological and physiological changes resulting from hormonal fluctuations that occur during the reproductive cycle. These changes include glandular growth with increased secretory activity during proestrus and estrus, and glandular regression with reduced secretory activity during diestrus I and II. These repetitive cycles of glandular growth and regression are followed by an extensive reconstruction and remodeling of prostate stroma throughout the reproductive life of the female gerbil. In this sense, the objective of this study was to evaluate the effect that the hormonal fluctuations of the reproductive cycle have on the stromal remodeling and the histological localization and activity of matrix metalloproteinases MMP2 and MMP9 in the adult female gerbil prostate. For this, serological, ultrastructural, and immunocytochemical methods were employed, as well as biochemical studies for MMP2 and MMP9. The results showed that the major stromal alteration coincide with the peak of estradiol, which occurs in estrus, and with the peak of progesterone, occurring during diestrus II. MMP2 and MMP9 presented a similar pattern of activity during estrous cycle. The estrus was the phase of greater activity of MMP2 and MMP9. On the other hand, in DI and DII, the immunolocalization and activity of MMP-2 and MMP-9 was very weak. These results are very important since they suggest the involvement of estradiol and progesterone in regulating MMP-2 and -9 activities in adult female gerbils prostate. These MMPs activity profile are in agreement with our previous results and confirm that female prostate gland undergoes a remarkable remodeling during estrus cycle, as occurs in uterus glands, guided by hormonal scenario.

INTRODUCTION

The reproductive organs undergo dramatic changes in its structure and function throughout adult life. These changes, which are controlled by hormones and cytokines, involve extensive connective tissue remodeling (Hulboy *et al.*, 1997).

Most of the processes of connective tissue remodeling are accomplished by extracellular components degradation by matrix metalloproteinases (MMPs). MMPs are zinc-dependent endopeptidases that degrade various extracellular matrix components such as collagen, elastin, laminin and proteoglycans (Visse & Nagase, 2003; Page-McCaw *et al.*, 2007).

The MMP family members are widely expressed in many reproductive processes, including menstruation, ovulation, and embryo implantation, and also during involutive processes of the uterus, breast and prostate (Wilson, 1995; Zhang & Nothnick, 2005; Ra & Parks, 2007; Jezierska & Motyl, 2009). In many organs, MMPs activity influences the celullar behavior by altering basic functions such as proliferation, differentiation, migration and apoptosis.

In normal prostate of male rodents, MMP-2 and -9 are produced by secretory epithelial cells and stromal cells. They act in the maintainance of glandular homeostasis and are also present in prostatic fluid (Wilson *et al.*, 1991; Wilson, 1995; Felisbino et al, 2007; Justulin *et al.*, 2010ab). Expression and activity of several MMPs have been described in early and late stages of progression of prostate cancer, and the increased activity of MMPs may be associated with poor prognosis of the disease (Lokeshwar, 1999; VanSaun & Matrisian, 2006).

Structural studies demonstrate that hormonal fluctuations that occur during the estrous cycle in gerbils (*Meriones unguiculatus*) drastically alter the morphophysiology of the female prostate (Fochi *et al.*, 2008). These changes include glandular growth and increased secretory activity of the prostate during proestrus (P) and estrus (E), and glandular regression and decrease in secretory activity during diestrus I (DI) and II (DII). These repetitive cycles of glandular development and regression cause extensive reconstruction and remodeling of prostate stroma throughout the

reproductive life of the female gerbil. Thus, the objective of this study was to evaluate the effect that hormonal fluctuations due to the reproductive cycle have on the stromal components of the adult gerbil female prostate, with special attention to histological localization and activity of MMP2 and MMP9.

MATERIAL AND METHODS

Experimental Design

Forty adult female gerbils (*Meriones unguiculatus*), with 90 days of age and regular 4-days estrous cycle were used in this study. Five adult males were used as controls in the biochemical experiments. The animals were maintained in the animal facility of the Biology Department of the Institute of Biosciences, Letters and Sciences campus of São José do Rio Preto - SP, under adequate light and temperature conditions, with food and water *ad libitum,* according to internal rules of the Committee on Ethics and Animal Welfare (Protocol# 14/07 – CEEA, UNESP).

The cycling procedure was performed by analysis of vaginal smear, always at 10:00AM on the day of sacrifice. The females in the stages of P, E, DI and DII (n=10) according to Nishino & Totsukawa (1996), were sacrificed by inhalation of CO_2, followed by decapitation with guillotine. The prostates (removed along with the urethra and vagina) were fixed or frozen according to the methodology for each analysis. The prostates of male gerbils were dissected in ventral (VP), dorsolateral (DLP) and dorsal lobes (DP), and frozen in liquid nitrogen.

Hormonal dosage

Blood samples were collected from each animal right after decapitation, placed in test tubes of 4 ml with separation gel, centrifuged at 3000 rpm, and serum levels of estradiol, progesterone

and testosterone were measured. The hormonal dosages were performed by using chemiluminescence method in automated Vitros-ECi (Johnson & Johnson, Orthoclinical Diagnostics Division, Rochester, NY). The detection level was 0.1-3814 pg/ml for estradiol, 0.1-150 ng/ml for testosterone, and 0.1-100 ng/ml for progesterone.

Structural Analysis

The female prostatic complexes were fixed by immersion in 4% paraformaldehyde in phosphate buffer for 24 hours. The tissues were dehydrated in crescent series of ethanol, cleared in xylene and embedded in paraffin (Histosec – MERK). Sections of 5µm were produced on a rotary microtome (Leica RM2155, Nussloch, Germany). Histological sections were stained with the picrosirius-hematoxylin (Bancroft & Gamble, 2002) to mark up the collagen fibers, and with hematoxylin-eosin for general analysis. The prostate tissue was examined under an Olympus BX60 light microscope (Olympus, Hamburg, Germany) and images were digitized by the Software Image-Pro Plus version 4.5 for Microsoft Windows™.

Immunohistochemical analysis

Histological sections of the female prostatic complex were subjected to immunohistochemistry for detection of matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9), as previously described to male prostate (Delella *et al.*, 2010; Justulin-Jr *et al.*, 2010a; b). Briefly, after heat mediated antigen retrieval with citrate buffer, primary antibodies for MMP-2 and MMP-9 (sc6838 and sc6840 respectively, Santa Cruz Biotechnology, Inc. – Santa Cruz, CA/USA) were applied in a dilution of 1:20, overnight at 4°C. The slides were rinsed with TBS and incubated with secondary antibody labeled with biotin for 2 hours, followed by incubation with avidin-biotin peroxidase for 45 minutes (Santa Cruz Biotechnology, Inc. – Santa Cruz, CA/USA). The reaction

was revealed with diaminobenzidine (DAB) and the sections were counterstained with Harris' hematoxylin.

Transmission Electron Microscopy (TEM)

Fragments of 0.5 mm from the female prostate gland at different stages of the estrous cycle were fixed in 3% glutaraldehyde diluted in Milloning buffer pH 7.3 containing 0.25% tannic acid for 24 hours. The material was rinsed in buffer and post-fixed in 1% osmium tetroxide for 2 hours. Subsequently, the fragments were dehydrated in acetone and embedded in Araldite resin (Cotta-Pereira *et al.*, 1976). Copper grids containing the ultrathin sections (50nm) were contrasted with 2% uranyl acetate solution (20 minutes), washed in bi-distilled water, dipped in a solution of lead citrate at 2% (6 minutes) and washed again. The observations were performed in a Zeiss EM-910 transmission electron microscope.

Biochemical Analysis

The biochemical evaluation of the activity of MMP-2 and MMP-9 in the prostates of gerbil males and females during the estrous cycle were performed using frozen samples of 5 animals for each group. In males, the prostate was separated into its three lobes: ventral (VP), dorsal (DP) and dorsolateral (DLP). In females, the prostate was completely isolated from the urethra and adipose tissue associated. The study was conducted using the zymography technique. The prostates were homogenized in extraction buffer (30mg tissue/100µl solution) containing 50 mM Tris-HCl pH 7.4, NaCl 0.2 M, Triton X-100 0.1% and 0.1% protease inhibitor cocktail (P-8849-CO-Sigma, St Louis, MO, USA). The homogenate was incubated for 2 hours at 4° C to increase the efficiency of extraction. After incubation, the homogenate was centrifuged at 14000 rpm and the supernatant was maintained at -80° C. The protein concentration in each sample was determined according to

Bradford's method (Bradford, 1976). Aliquots of the extract were subjected to electrophoresis under nonreducing conditions (100V at 4° C) in 8% polyacrylamide gel containing 0.1% gelatin (denatured collagen). After electrophoresis, the gels were washed in Triton X-100 2.5% and in 50mM Tris HCl pH 8.4. Subsequently, the gels were incubated for 12 hours at 37° C in the same buffer containing 5 mM $CaCl_2$ and 1μM of $ZnCl_2$. After incubation, the gels were stained with Brilliant Blue Comasie 0.25%. The bands obtained in zymography were scanned and analyzed by densitometry. The gelatinolytic activity of MMPs was analyzed obtaining the integrated optical density (IOD) of bands using a computer software (Image Master VDS, 3.0) coupled to the Image Master VDS apparatus. The values were plotted in histogram showing the ratio of the IOD groups of estrous cycle and IOD of male prostates.

RESULTS

Serum Hormone Levels

Table I shows the hormone levels of estradiol, progesterone and testosterone in gerbil adult females during the estrous cycle. Serum levels of estradiol remained constant throughout the estrous cycle (33 ± 7.4 to 38.5 ± 9 pg/ml), with a peak concentration only in phase E (49.6 ± 15 pg/ml). Progesterone showed a peak concentration during DII (19.1 ± 10.7 ng/ml) and remained constant in the other phases of the cycle (10.1 ± 4.5 to 12.8 ± 4.1 ng/ml). Testosterone levels remained constant throughout the estrous cycle (0.3 ± 0.1 to 0.5 ± 0.1 ng/ml).

Morphological Analysis

Figure 1 shows the collagen fibers distribution pattern in the female prostate during the estrous cycle. In phase P, the stroma showed a large amount of collagen fibers arranged between the

smooth muscle and the base of the alveolar epithelium (Fig. 1a-b). In the phases of E (Figs. 1c-d) and DI (Figs. 1e-f) the alveolar lumen became wider and collagen fibers were distributed as delicate fibrils interspersed with the smooth muscle cells. In the DII phase, prostatic alveoli were reduced and stroma became very dense, presenting a large amount of collagens fibers either in the epithelium basis or in deeper portions of stroma (Fig. 1g-h).

Ultrastrucutual Analysis

In P, smooth muscle cells are uniform and closely associated to bundles of collagen fibers (Fig. 2a). In E, the smooth muscle cells assume a synthetic and spiny aspect, typical of contracting cells (Fig. 2b). At this stage, bundles of collagen fibrils and elastic system fibers can be observed in the spaces formed among the smooth muscle cells extensions (Fig. 2c). In DI, the smooth muscle cells appear less developed and interspersed by irregular bundles of collagen fibrils and elastic fibers (Fig. 2d), although in DII, the perialveolar stroma is densely filled by bundles of collagen fibrils interspersed by smooth muscle cells (Fig. 2e). In this phase, the extensions of smooth muscle cells show numerous caveolae, typical of intense contractile activity, and its projections appeared associated with elastic fibers (Fig. 2f).

Immunohistochemical Analysis of MMP2 e MMP9

Tissue immunoreaction for MMP-2 was intense in the cytoplasm of epithelial cells and in perialveolar stroma of female prostates in the phases P, E, DI and DII (Figs. 3a-i) Conversely, MMP-9 reaction was more intense in phase P, and especially in phase E (Figs. 3j-q). In E, epithelial cells and fibroblasts of the deep portions of stroma showed very strong reaction for MMP-9. Smooth muscle cells did not present this metalloproteinase in E (Figs. 3l, m) and in the phases of DI

and DII the immunoreactivity of MMP-9 was weaker and restricted to the apical cytoplasm of secretory epithelial cells and fibroblasts (Figs. 3f-q).

By analyzing the vaginal tissue closely associated to the prostate (Fig. 4) it could be noticed that MMP-2 occurs especially in the epithelium and in the vaginal muscle layer. This same pattern was also observed in phases P, DI and DII. However, in E the immunoreaction for MMP-2 was more pronounced and occurred in all layers of the vaginal wall (Fig. 4b). In addition, MMP-9 was observed in the lining epithelial cells and diffusely in the fibroblasts of the lamina propria and in the vagina muscle layer of gerbil female prostates in the phases P, DI and DII (Figs. 4e, g, h). In E phase, MMP-9 immunoreaction was found to be more intense and in all tissues that constitute the vaginal wall (Fig. 4f).

Zymography

The representative zymography gel of the female and male gerbil prostates shows the typical clear bands of MMP-2 (pro, intermediate and active enzyme) and MMP-9 (pro and active enzyme) (Fig. 5a). All forms of MMP-2 and MMP-9 showed higher gelatinase activity in the prostate of females than in the three prostatic lobes of the male (Figs. 5b-f).

In the female prostate, all forms of MMP-2 and MMP-9 presented strong activity in P and, especially, in E (Figs. 5b-f). Phase DI was the one with the lower gelatinase activity both for MMP-2 and MMP-9 (Figs. 5b-f). In DII, pro and active MMP-9 and pro-MMP-2 showed a recovery in its activity, whereas intermediate and active MMP-2 maintained a lower activity according to IOD analysis.

DISCUSSION

Several studies have reported the tissue structure and composition of the female prostate in rodents (Gross & Didio, 1987; Flamini *et al.*, 2002; Santos *et al.*, 2006; 2008; Fochi *et al.*, 2008; Custódio *et al.*, 2008) and in humans (Sloboda *et al.*, 1998; Zaviačič *et al.*, 2001). However, this is the first study detailing the stroma profile and the histological localization and activity of MMPs in the female prostate.

The morphophysiological pattern of the prostate glands analyzed here is consistent with data previously published (Fochi *et al.*, 2008), where the gerbil female prostate shows a well developed and active secretory phenotype in the phases P and E, and a glandular involution in DI and DII. Our serological data showed that the major hormonal alterations of the reproductive cycle in the female gerbil occur during E, with a peak of estradiol, and in DII, with a peak of progesterone. These hormonal variations coincide with major stromal alterations observed in this study, indicating the importance of estradiol and progesterone in the regulation of components that promote stromal remodeling during the estrous cycle in gerbils. However, data from serum levels of estradiol and progesterone described by Fochi *et al.* (2008) are opposite to those obtained by this research. New groups of animals and the adoption of a new clinical analysis laboratory can explain these divergent results. Importantly, the hormonal results presented here were confirmed by three repetitions and are consistent with hormonal pattern showed by females of other rodent species (Edwards, 1977; Westwood, 2008).

The ultrastructural analysis of the prostatic stroma during the estrous cycle revealed that smooth muscle cells alternate their function from a highly synthetic state in phases E and P to a contractile state in phases DI and, especially, DII. In DII, the epithelial involution is accompanied by a reduction in the glandular lumen and an intense stromal rearrangement. In this respect, the contractility of smooth muscle cells in this phase seem to participate in the remodeling of collagen fibers in the compact bundles that were observed around the prostatic alveoli.

However, the main finding of our study was about the pattern of MMPs in the gerbil female prostate during estrous cycle. The results obtained with the techniques of immunocytochemistry and zymography demonstrated that MMP-2 and MMP-9 have a similar immunolocalization and activity during estrous cycle. The E was the phase of greater immunoreaction and activity of MMP-2 and MMP-9. On the other hand, in DI and DII, the tissue localization and activity of MMP-2 and MMP-9 was very weak. These results suggest that MMPs activity in female prostate follows a similar pattern described for uterine endometrium, in which estrogen has a stimulatory action and progesterone has an inhibitory role (Mönckedieck *et al.*, 2009; Russo *et al.*, 2009). This finding is very important, since the vast majority of studies about MMPs expression and activity indicate the testosterone as key hormone in the regulation of these peptidases in male prostate (Wilson *et al.*, 1991; Felisbino *et al.*, 2007; Limaye *et al.*, 2008, Justulin *et al.*, 2010b). Furthermore, analysis of the vaginal tissue adjacent to the urethra and prostate showed that even though at the same stage of estrus cycle, the female prostate and the vagina have distinct MMPs immunoreaction.

Several previous studies have demonstrated that the rodent female prostate is homologous to the ventral lobe of male rodent prostate (Gross & Didio, 1987; Zaviačič *et al.*, 2000; Santos *et al.*, 2003). However, one important feature observed in this study was that adult gerbil female prostate, in all phases of the estrous cycle, presented activities of MMP2 and MMP9 up to 12 times higher than any adult male prostatic lobes. These differences possibly reflect the repetitive cycles of development and involution that the gerbil female prostate undergoes every 4-5 days. These modifications require constant involvement of MMPs in the degradation and reconstruction of the extracellular matrix components, such as basement membranes and collagen system fibers.

Studies with senile gerbils demonstrated that the female prostate presents signs of aging and spontaneous neoplastic foci earlier than males of the same age (Custódio *et al.*, 2008). In addition, studies involving the expression and activity of MMPs in rodents prostate cancer have demonstrated that the increased activity of these enzymes is related to the high indexes of invasiveness and indifferentiation of the tumor cells (Lokeshwar, 1999). In this manner, the high and constant

activity of MMP2 and MMP9 in the female prostate of gerbil may be associated to the precocity of cellular aging and with the appearance of spontaneous lesions in this gland.

Thus we can conclude that MMP-2 and MMP-9 exhibit a pattern of histological localization and activity in the gerbil female prostate during the estrous cycle similar to observed in uterine endometrium, actively participating in the tissue remodeling during glandular growth that occurs in proestrus and estrus phases.

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Table 1. Serum levels of estradiol, progesterone and testosterone in adult gerbil females during the phases of the estrous cycle (n=5 samples/group; mean ± SE).

	Proestrus	**Estrus**	**Diestrus I**	**Diestrus II**
Estradiol ***(pg/ml)***	38.5 ± 9.0	49.6 ± 15	33.0 ± 7.4	34.5 ± 9.9
Progesterone ***(ng/ml)***	10.1 ± 4.5	10.6 ± 4.6	12.8 ± 4.1	19.1 ± 10.7
Testosterone ***(ng/ml)***	0.3 ± 0.1	0.5 ± 0.1	0.41 ± 0.1	0.3 ± 0.1

Figure Legends

Figure 1. Histological sections of the gerbil female prostate stained with picrosirius-hematoxylin. (a-b) Phase P: dense stroma with compact bundles of collagen fibers (arrows) at the base of the epithelium (Ep). Smooth muscle cells (smc). (c-d) Phase E. (e-f) Phase DI: alveolar expansion leads to cellular redistribution of collagen fibers (arrows) in thin fibrils arranged around the prostatic alveoli. Lumen (L). (g-h) Phase DII: Reduced prostatic alveoli are presented surrounded by compact layers of collagen fibers (arrows).

Figure 2. Ultrastructure of the stroma of the gerbil female prostate in all phases of the estrous cycle. (a) Phase P: regular smooth muscle cells (SMC) interspersed with collagen fibers (CO). Bar = 2μm. (b-c) Phase E: Smooth muscle cell (SMC) with contractile aspect associated to collagen (CO) and elastic fibers (arrows). Bars: b = 2 μm, c = 700nm. (d) Phase DI: smooth muscle cells (SMC) presenting numerous extensions in association with the extracellular matrix elements. Elastic fibers (arrows). Bar = 2μm. (e-f) Phase DII: Dense layers of collagen fibers (CO) interspersed with smooth muscle cells (SMC). The extensions of the smooth muscle cells exhibit numerous caveolae (arrowhead), and are associated with elastic fibers (arrows). Basal lamina (BL). Bars: e = 2μm and f = 700nm.

Figure 3. Immunoreaction of MMP-2 and MMP-9 in the gerbil female prostate during the estrous cycle. Reaction to MMP-2 is observed in the cytoplasm of secretory epithelial cells (Ep) and fibroblasts (arrows) in deep regions of the prostatic stroma of females in phases P, E, DI and DII. The immunolocalization of MMP-9 is more strong in the epithelial cells (Ep) and in fibroblasts (arrows) of the prostates in the phases P and, especially, E. In DI and DII this reaction was more evident in the fibroblasts placed next to the smooth muscle cells (arrows).

Figure 4. Immunolocalization of MMP-2 and MMP-9 in the female gerbil vagina, adjacent to the prostatic tissue, during the estrous cycle. MMP-2 is observed in the epithelial (Ep) and muscle (M) layer of the vagina of females in phases P, DI and DII. In phase E the immunoreaction of MMP-2 is very intense in all layers of the vaginal wall. Lamina propria (L). MMP-9 is observed in the luminal epithelium (Ep) and diffusely in the lamina propria (L) and smooth muscle layer (M) at stages P, DI and DII. In E, the immunoreaction for MMP-9 is very intense in the epithelium (Ep) and the muscular layer (M).

Figure 5. Representative zymography gel showing the typical clear bands of MMP-2: proenzyme (72 kDa), intermediate (64 kDa) and active enzyme (57 kDa); and MMP-9: proenzyme (92 kDa) and active enzyme (81 kDa). In the gel and in the quantification graphs are represented the male prostate lobes: ventral (VP), dorsal (DP) and dorsolateral (DLP), and female prostate in all phases of the estrous cycle: proestrus (P), estrus (E), diestrus I (DI) and diestrus II (DII). Quantification of gelatinase activity of MMPs is expressed as IOD (integrated optical density). The male prostatic lobes are represented in black and the female prostate in the different estrous cycle phases is shown in gray.

Figure 1

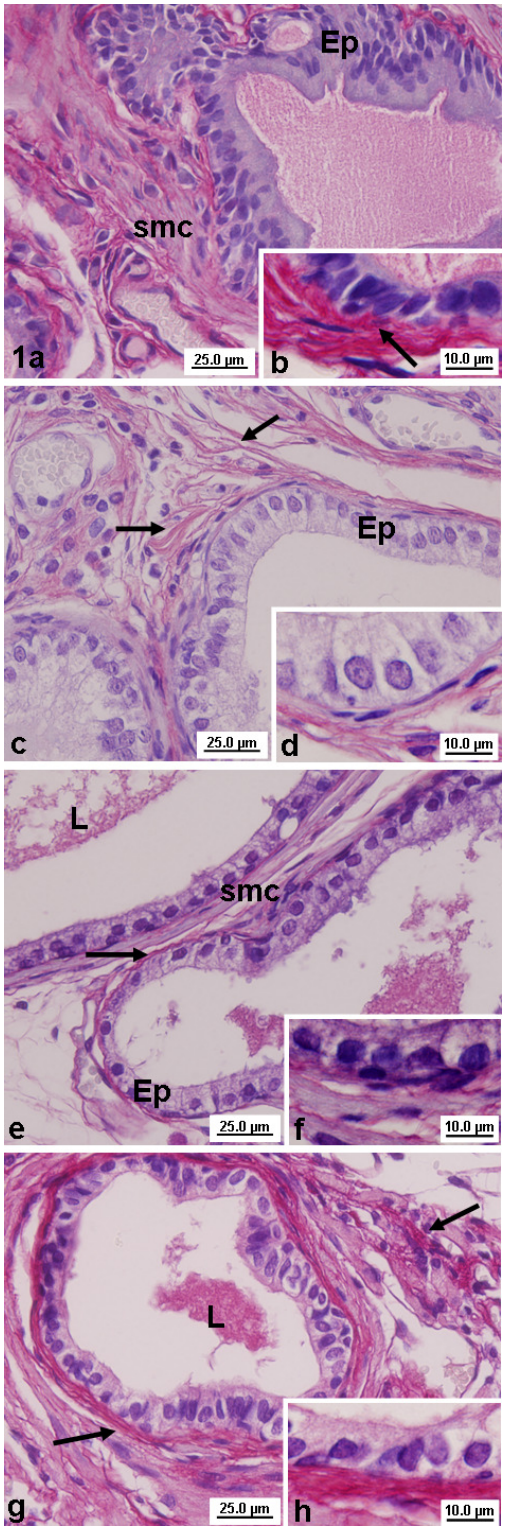
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Figure 2

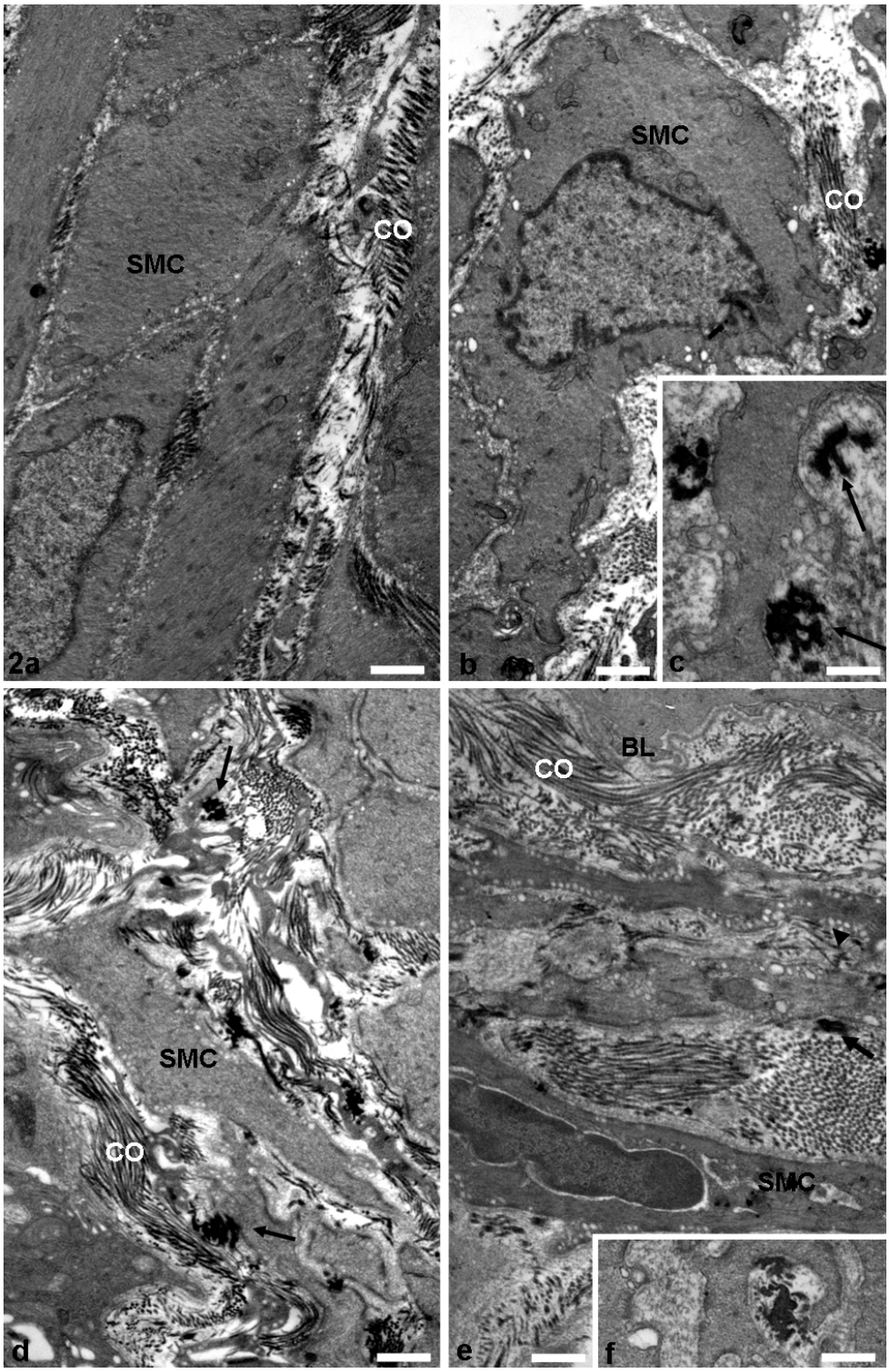

Figure 3

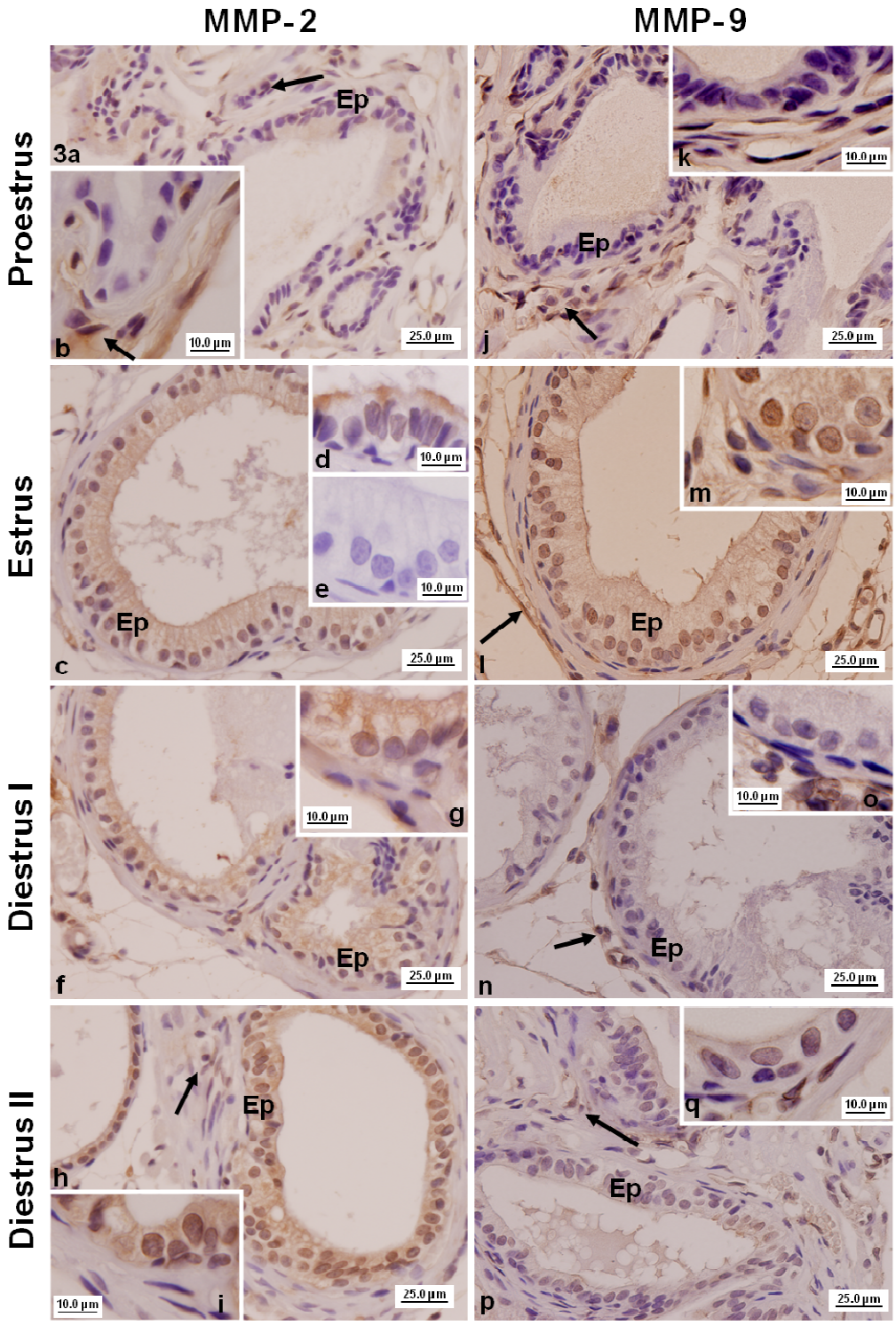
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Figure 4

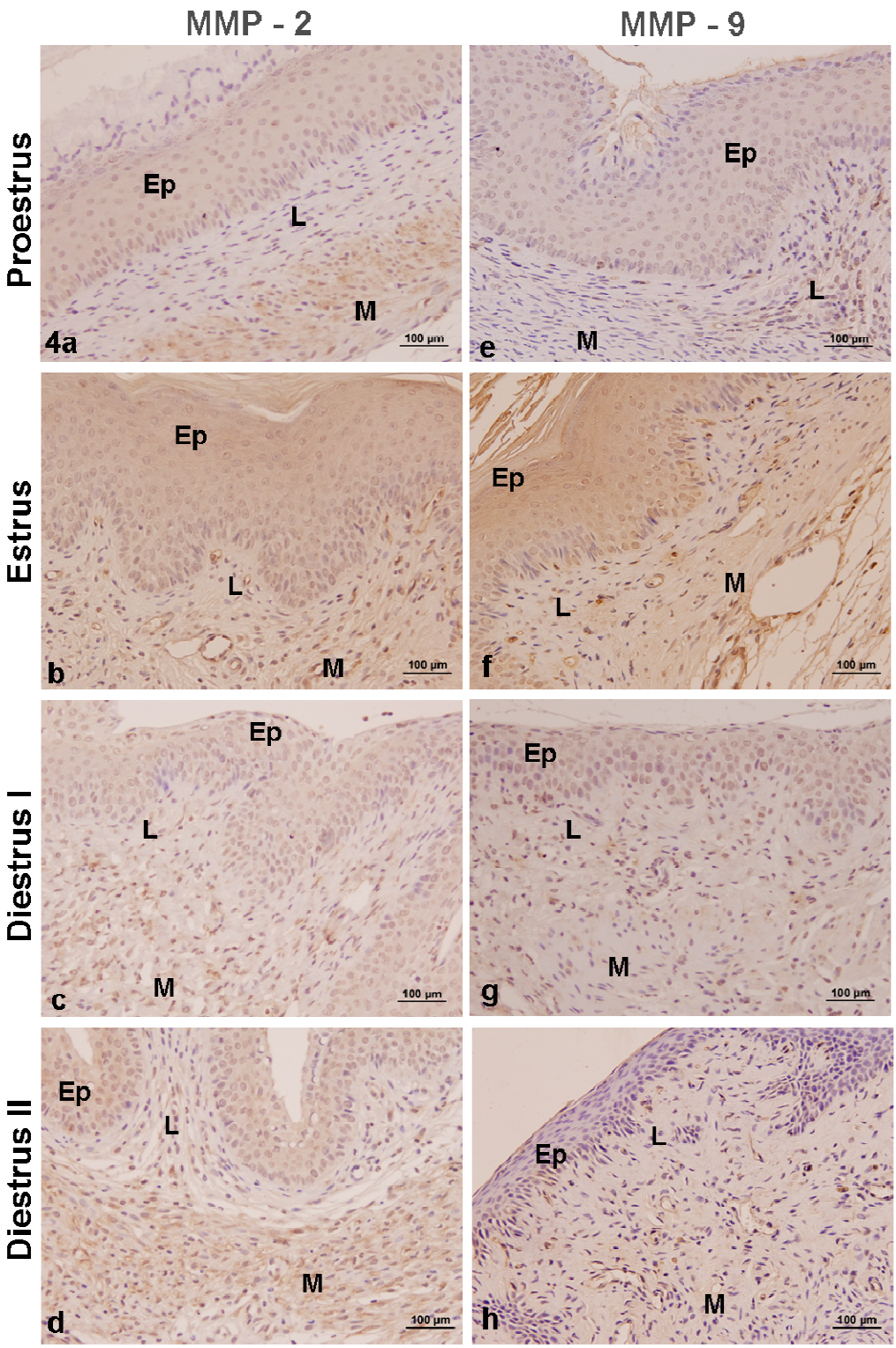
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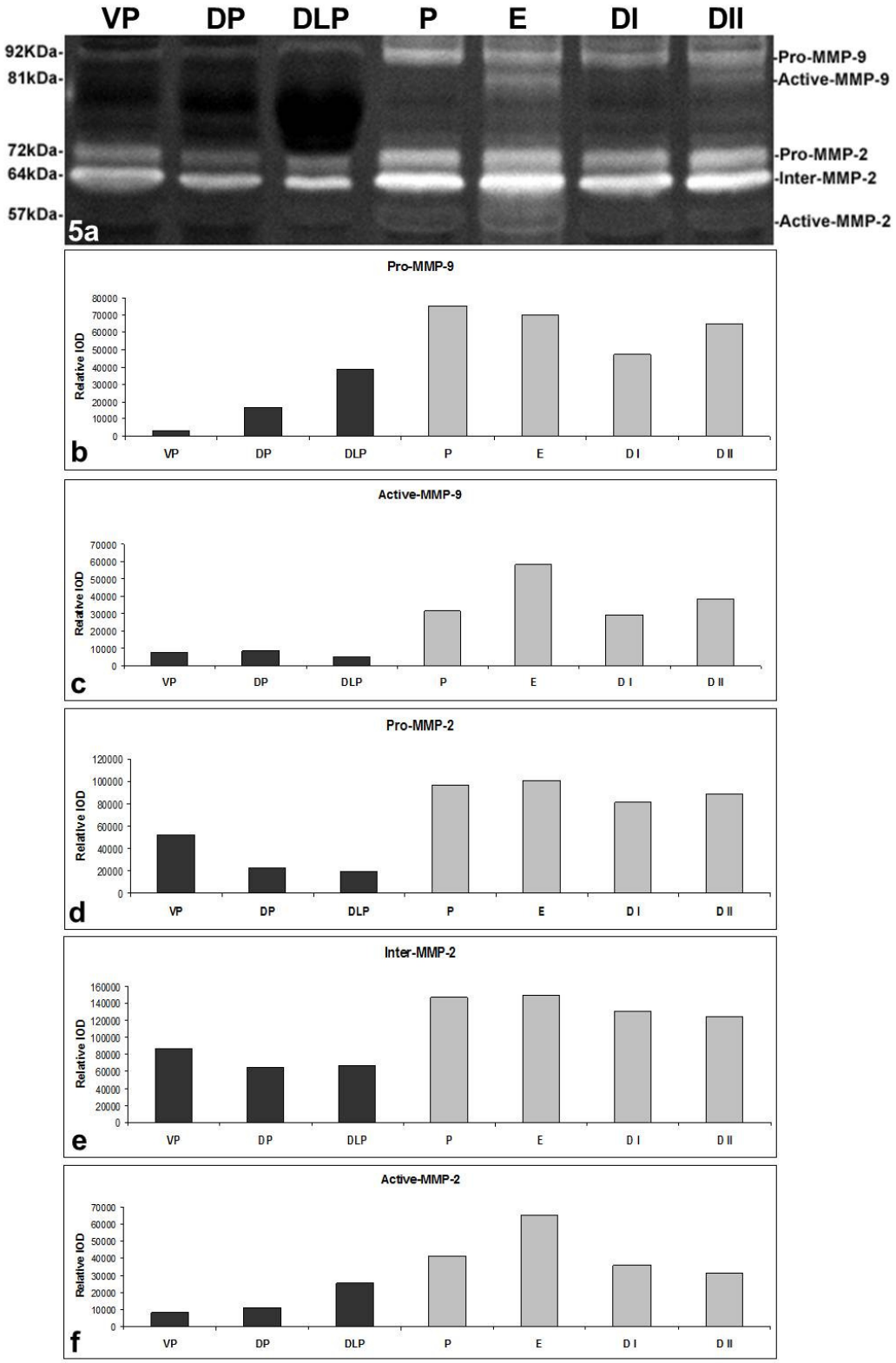
Figure 5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-Pro-MMP-2
-Inter-MMP-2
-Active-MMP-2
5a
Pro-MMP-9
Relative IOD
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Active-MMP-2
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ARTIGO II

MMP-2 AND TIMP-2 IN MALE AND FEMALE PROSTATES OF MONGOLIAN GERBIL: EFFECTS OF HORMONAL MANIPULATION

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MMP-2 AND TIMP-2 IN MALE AND FEMALE PROSTATES OF MONGOLIAN GERBIL: EFFECTS OF HORMONAL MANIPULATION

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Running Title: MMP and TIMP in male and female gerbil prostate.

Key-words: MMP, TIMP, female prostate, ventral prostate, testosterone.

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ABSTRACT

This paper investigated the presence of MMPs and TIMPs in the prostates of male and female gerbils and evaluated the effects of testosterone on the expression of these enzymes. Ventral prostates from male gerbils that were either intact or had been castrated for 7 or 21 days, along with prostates from female gerbils that were either intact or had been treated with testosterone for 7 or 21 days, were submitted to histological, stereological and immunohistochemical analyses. Stereology of prostatic components showed significant alterations of tissue compartments in the ventral male prostate after castration, especially after 21 days, with a significant increase in stroma. Administration of testosterone led to disorganization in the female prostate, with a significant increase in collagen fibers and smooth muscle cells after 21 days, along with the development of epithelial lesions such as PINs. MMP-2 increased after 21 days of castration in males; however, the TIMP-2 immunoreaction for this group was weak or absent. In females, the expression of MMP-2 appeared to decrease after 7 days of treatment with testosterone, but after 21 days, both epithelium and stroma showed a stronger reaction for MMP-2 than the controls. The expression of TIMP-2 in the treated females was similar to its expression in the castrated males. We conclude that the distribution of MMPs and TIMPs in both male and female prostates is altered by androgen manipulation, but the mechanism of stromal regulation appears to be distinct between genders because both the lack of T in castrated males and the excess levels of T in treated females lead to the same effect.

INTRODUCTION

The prostate is an accessory gland located in the genital tract of mammals that, together with the seminal vesicle, produces the bulk of the seminal fluid (Hayward et al., 1996). Some female species also have a prostate gland in their reproductive system, but its function remains unclear. However, strong evidence suggests that female prostate may have function in the transport of male gametes (Zaviacic, 1999).

In both males and females, the prostate is composed of a glandular epithelium immersed in a fibromuscular stroma that is rich in extracellular matrix (ECM) elements such as collagen fibers (Vilamaior et al., 2000), proteoglycans (Kofoed et al., 1990), laminin (Carvalho and Line, 1996), fibronectin and elastin (Carvalho et al., 1997). In addition to a structural role, the ECM and related molecules have been recognized as key regulatory components during the morphogenesis and cell differentiation of many branched organs (Timpl, 1989; Bruni-Cardoso et al., 2008), and they play important roles in cellular processes such as migration, adhesion, proliferation, differentiation and apoptosis (Hay, 1991; Alberts et al., 2008).

The ECM constantly undergoes changes in response to cellular and hormonal stimuli during many physiological situations such as morphogenesis (Werb and Chin, 1998) and cyclic female reproductive organ activity (Green and Lund, 2005; Zhang and Nothnick, 2005). Furthermore, during pathological conditions such as the inflammatory response (Arroyo and Iruela-Arispe, 2010), cardiac and vascular diseases (Dobaczewski et al., 2010) and prostate tumor progression (Tuxorn et al., 2001; Stewart et al., 2004; Niu and Xia, 2009), the ECM is extensively remodeled. Thus, tight regulation of ECM degradation and resynthesis is essential for the maintenance of tissue homeostasis.

The matrix metalloproteinases (MMPs) are metal-dependent endopeptidases responsible for the first step in ECM degradation, and they have a wide range of substrates. These enzymes are secreted as latent zymogen molecules called proMMPs that must be proteolytically processed to

become active. In addition, the activity of MMPs can be inhibited by a group of four glycoproteins that are known as tissue inhibitors of metalloproteinases (TIMPs) (Matrisitan, 1990; Basset et al., 1997; Hullboy et al., 1997; Stamenkovic, 2000; Wilson et al., 2002; Visse and Nagase, 2003).

MMP-2 and MMP-9, also known as gelatinases, are key members of the MMP family, and they preferentially degrade basement membrane components, especially type IV collagen and laminin. The expression of gelatinases has been associated with many pathological conditions, particularly cancer metastasis and angiogenesis. As they play a pivotal role in normal physiological processes and pathological stages, an imbalance between TIMPs and MMPs appears to participate in tumor progression and the metastatic process in several tissues and organs (Wilson et al., 2002; Zhang et al., 2004; Delella et al., 2007). In addition, it is known that the expression of MMPs and TIMPs is regulated by hormones, especially androgens (Hulboy et al., 1997; Wilson et al., 2002; Felisbino et al., 2007).

Due to its suitability for studies of prostate development and pathogenesis in males and the high frequency of prostate occurrence in females, the Mongolian gerbil (*Meriones unguiculatus*) is a rodent that has been widely used in studies of the reproductive tract (Pinheiro et al., 2003; Corradi et al., 2004; Oliveira et al., 2007; Góes et al., 2007; Rochel et al., 2007; Campos et al., 2008; Scarano et al., 2008), especially for studies related to the female prostate (Custódio et al., 2004; Santos et al., 2006; Santos et al., 2007; Custódio et al., 2008; Fochi et al., 2008; Santos et al., 2008).

The above-mentioned studies have demonstrated morphological similarities between the ventral lobe of the male prostate and the female prostate, but more detailed studies, especially concerning stromal components, are necessary. Thus, this investigation presents a characterization of MMP-2 and TIMP-2 distribution in both the female prostate and the ventral lobe of the male prostate of the Mongolian gerbil. In addition, the effect of hormonal manipulation on the prostatic stroma was also assessed.

MATERIAL and METHODS

Animals and experiments

Male and female adult gerbils (*Meriones unguiculatus*, Gerbilinae: Muridae) weighing between 70 and 90 g (15-18 weeks old) were maintained under controlled conditions of luminosity (12 h light: 12 h dark) and temperature (25ºC) with water and ration *ad libitum.* The experiments were performed in accordance with the Ethical Principles for Animal Experimentation – Brazilian College of Animal Experimentation.

Ten males were submitted to bilateral orchiectomy under anesthesia with ketamine (800 µl/kg) and xylazine (200 µl/kg), and they were sacrificed at 7 or 21 days after surgery (Castrated groups, n=5). To determine the estrous cycle phase in gerbil females, daily vaginal smears were collected at 10:00 AM for 12 consecutive days. Ten females in the *proestrus* phase received intradermal injections of Testosterone Cypionate (5 mg/kg, diluted in corn oil, 0.1 ml per dose) every other day and were sacrificed 7 or 21 days after the beginning of the treatment (Testosterone-treated groups, n=5). Both males and females without experimental manipulation constituted the Control groups (n=5).

Histological processing and stereological analysis

The prostates of the female gerbils and the prostatic ventral lobes of the males were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and were embedded in Paraplast (Sigma-Aldrich Co, USA). Histological sections (5 µm) were stained with Gömori's Trichrome for stereological analysis to evaluate the relative proportion (relative volume) of each prostatic tissue component, including the epithelium, lumen, smooth muscle cells (smc), collagen fibers and non-muscular stroma, as described by Huttunen and collaborators (1981). A total of 30 microscopic fields (200x magnification) from at least three animals per group were randomly digitalized and analyzed using Weibel's

multipurpose grid with 130 points and 60 test lines (Weibel, 1963). In summary, the relative values were determined by counting the number of intersections of the grid that overlapped each tissue compartment and dividing them by the total number of points. Absolute volumes could not be determined because it was not possible to isolate the female prostate from the adhering tissue to determine its accurate weight.

Immunohistochemistry

For MMP-2 and TIMP-2 detection, paraplast sections (5 µm) from all experimental groups were pretreated with the citric acid monohydrate antigen retrieval method. Next, they were immunostained with mouse monoclonal antibodies against MMP-2 (sc-13595, Santa Cruz Biotechnology, Inc) and TIMP-2 (sc-21735, Santa Cruz Biotechnology, Inc) at a 1:100 dilution in TBS containing 3% bovine serum albumin (BSA) overnight at 4°C, according to the manufacturer's protocol. The primary antibody was detected using a peroxidase-conjugated Polymer (Novolink Polymer, Leica Microsystems, Inc., USA), and the peroxidase activity was revealed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co, USA). The sections were counterstained with Harris hematoxylin. Negative controls were obtained by omitting the primary antibody incubation step.

Hormonal dosage

Circulating serum testosterone and estradiol levels were determined using immunochemical assays. Serum was separated by centrifugation and stored at -20°C for subsequent assays. Measurements were performed in triplicate, using automated equipment (Vitros-ECi; Johnson & Johnson, orthoclinical Diagnostics Division, Amershan, UK) for detection by ultrasensitive chemiluminescence. The sensitivity was 0.1–150 ng/ml for testosterone and 0.1–3.814 pg/ml for

estradiol. For testosterone, the intra- and inter-assay variations were 1% and 1.1%, respectively, and for estradiol they were 2.1% and 1.5%.

Statistical analysis

The data were evaluated by analysis of mean ± standard deviation (SD). Statistical analyses were performed with Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA). One-way Analysis of Variance (ANOVA) was performed to determine possible differences among groups, and the Tukey HSD test was employed to determine the significance of the differences. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Serum steroid hormones

Serum steroid hormone levels are shown in Table 1. In males, serum testosterone levels drastically dropped after surgical castration, with values ranging from 1.5 ng/ml in the control group to below 0.2 ng/ml at day 7 and 21 of the experiment. The levels of estradiol showed a slight decrease only after 21 days of castration (11.3 pg/ml), which was not statistically significant. In females, exogenous testosterone administration resulted in sustained high levels of circulating testosterone, with values reaching as high as 14 ng/ml and 12 ng/ml after 7 and 21 days of treatment, respectively. These values were significantly higher than the levels in the control females in the proestrus phase (0.2 ng/ml). The circulating estradiol levels did not show significant alterations with T treatment.

Morphological and stereological analysis

The stereological data of the prostates of normal, castrated male and testosterone-treated female gerbils are shown in Table 2. The stereological assessment of the male ventral prostate tissue compartments revealed significant alterations for all evaluated components of the Castrated groups when compared to the Controls especially at 21 days after castration.

In the 7-day castrated group (Fig. 1B), the drop in the androgen levels led to a drastic reduction in the height of epithelial cells and consequently in their secretory activity. The volume occupied by the epithelial compartment showed a significant decrease in this group, from 14.5% in the intact animals to 10% in the 7-day castrated group.

After 21 days of castration (Fig. 1C, G), the extended decline in T levels promoted the involution of the whole organ, with both epithelial regression and stromal reorganization. Furthermore, the appearance of epithelial folds, connective tissue condensation and fibromuscular wall thickening was observed. The acini also suffered a reduction in luminal size, and an apparent increase in the amount of blood vessels with reduced diameter was observed. Stereology showed that epithelial volume significantly increased, and the morphology of epithelial cells changed from pavimentous, as found in the 7-day castration group, to cubic or columnar. In this group, the increase in epithelial volume density was counterbalanced by the reduction of the volume occupied by the lumen, which was reduced from 72% in control animals to 28% in the Castrated group, showing great involution of the whole organ after 21 days of androgen suppression (T). The results also demonstrated that the stromal compartment occupies approximately 13% of the ventral lobe in intact adult male gerbils, with 7% including the smc and 4% including collagen fibers (Table 2). These values were not significantly changed after 7 days of castration (6.8% smc and 3.4% collagen fibers), but they significantly increased in the 21-day Castrated group, rising to 22% smc and to 15.7% collagen fibers (Table 2, Fig. 1G). These results demonstrated great sensitivity of the stromal components to the absence of testosterone in the male ventral prostate,

because together they occupied approximately 40% of the total organ after 21 days of castration versus only 12% in the intact animals of the control group.

In the prostates of females in the proestrus phase, the acinar compartment (epithelium+lumen) composed the majority of the prostate volume (~60%) and remained relatively constant throughout the treatment with testosterone (T), as shown in Table 2. The remaining volume was shared by the smc, collagen fibers and other connective tissue components, which were, respectively, 16%, 9.6% and 6.7% of the total volume. Stereological evaluation showed a significant increase in the amount of collagen fibers after 7 and 21 days of T treatment compared to the control group (Table 2). In addition, the administration of T for 21 days led to the disorganization of the female prostate. Tissue components were disrupted, stromal remodeling occurred, epithelial lesions such as prostatic intraepithelial neoplasia (PIN) developed and microacini formed in the epithelium (Fig. 1F, H).

Immunohistochemical analysis

Immunohistochemical analysis of MMP-2 and TIMP-2 in the male ventral prostate

In the male ventral prostate, an immunohistochemical reaction to active MMP-2 occurred only in blood vessels in the intact animals of the control group, with no positive reaction in the epithelial cells. The immunoreaction was specific to blood plasma, (diffuse immunolabeling) because the reaction was negative in erythrocytes and other blood cells (Fig. 2A, B). In the 7-day castrated group, the immunoreaction for active MMP-2 occurred in the lumen secretions of only some acini and was located on the apical border of the epithelial cells. Marked immunolabeling was also observed in some stromal fibroblasts and in blood plasma (Fig. 2C, D). After 21 days of castration, the immunoreaction for MMP-2 was more intense than in the other male groups, with strong MMP-2 immunostaining in the blood

vessels and various stromal regions. Moreover, several MMP-2-positive fibroblasts were observed in regions with epithelial disruptions (Fig. 2E, F).

Alternatively, immunolabeling for the tissue inhibitor TIMP-2 was positive in the epithelial cells of the ventral prostate in the male control group, but the staining was non-homogeneous because not all of the secretory epithelial cells showed a positive immunoresponse. Additionally, among the positive cells, a marked difference in the immunolabeling intensity was observed (Fig. 3A, B). Furthermore, the majority of blood vessels showed a positive immunoreaction in blood plasma in this group. In the prostates of males castrated for 7 days, every evaluated acini showed a TIMP-2-positive reaction in the epithelial cells that varied from weak to strong. Even the pavimentous epithelial cells that were observed in this group were TIMP-2-positive, and in some regions, this enzyme was found in prostatic secretions and in blood plasma (Fig. 3E, F). After 21 days of castration, immunolabeling for TIMP-2 appeared to be preferentially plasmatic, with weak or absent reactions in other prostatic components (Fig. 3I, J).

Immunohistochemical analysis of MMP-2 and TIMP-2 in the female prostate

The immunolocalization of active MMP-2 in control female prostates was quite diverse, occurring in some epithelial cells, in the secretions and in the stromal compartment (Fig. 4A-C). In most acini, MMP-2 was present in the apical border of the luminal secretory cells (Fig. 4B); however, in the stroma, the immunoreaction was visualized in both the interior of blood vessels and in connective tissue fibroblasts (Fig. 4A, C). After 7 days of T administration (Fig. 4D-F), the expression of active MMP-2 appeared to decrease and was found only in some positive fibroblasts in the stroma (Fig. 4E) and in blood vessels (Fig 4.D, F); however, only a few positive secretory cells were observed in the epithelium, as shown in detail in Figure 4E. The prostatic secretion released into the lumen also presented a weak reaction to active MMP-2 in this group. The reaction for active MMP-2 in the 21-day T group (Fig. 4G-K)

appeared to be stronger than that in other groups, both in the epithelium (Fig. 4I) and in stroma (Fig. 4H). A strong reaction was observed in the basal membrane (Fig. 4H) and in the secretion discharged into the lumen (Fig. 4G, J).

TIMP-2 immunolocalization in the female control group was similar to that in the ventral prostates of control males, as shown by positive reactions in the cytoplasm of epithelial cells of all acini but with non-homogeneous distribution (Fig. 3C, D). In some regions, the reaction was strong, but in others regions, the secretory cells showed no reaction. TIMP-2 was also found in areas of non-muscular stroma, especially in blood vessels. After 7 or 21 days of T administration, the expression of TIMP-2 was weak, only occurring in the prostatic secretion and in the apical border of the secretory cells (Fig. 3G, H, K, L). Only a few epithelial cells demonstrated TIMP-2-positive staining in the epithelium. The stromal compartment and the blood plasma of the testosterone-treated female groups also showed a negative reaction to the TIMP-2 antibody.

DISCUSSION

This study demonstrates that both male and female gerbil prostates are sensitive to the circulating levels of androgens, which may cause important alterations in the prostate gland, especially regarding the ECM. However, males and females showed different responses to altered serum testosterone levels.

The experimental protocol employed in this study resulted in circulating levels of testosterone that were 7X lower in males and more than 50X higher in females than the levels measured in untreated animals. As the levels of serum estradiol showed no significant alterations in either males or females, we assume that the results obtained here are due to the effects caused by the excess of testosterone in females or its absence in males.

A female prostate has been reported in humans (Zaviacic, 1999; Wimpissinger et al., 2007) and in several rodent species (Price, 1939; Shehata, 1980; Gross and Didio, 1987; Flamini et al., 2002), and its morphology and biochemistry show similarity to the ventral lobe of the male prostate (Santos and Taboga, 2006). In addition, the prostate gland in females has been reported to be susceptible to benign and malignant pathologies similar to the conditions developed by the male gland (Uzoaru et al., 1992, Dodson et al., 1995, Sloboda et al. 1998; Zaviacic et al., 2000, Custodio et al., 2004; Wimpissinger et al., 2009). The occurrence of pathological conditions in the female prostate of humans and rodents that are similar to the conditions affecting men such as cancer, benign prostatic hyperplasia and prostatitis (Sloboda et al., 1998; Zaviacic, 1999; Chan et al., 2000; Pongtippan et al., 2004; Santos et al., 2006; Custódio et al., 2008; Wimpissinger et al., 2009), indicates that it is an organ highly dependent on hormonal action, in particular to testosterone levels, as demonstrated in this study. Excess T induced stromal alterations in the female prostates treated for 21 days, including an increase in the amount of collagen and smooth muscle cells along with overexpression of MMP-2. The epithelium was also impaired by androgen activity, resulting in the appearance of pathological epithelial lesions such as PINs and microacini.

The suppression of androgens in the male leads to a process of regression known as prostate involution, which is mainly characterized by epithelial atrophy and a progressive decrease in the volume of the gland (Lee, 1996). This process of involution that occurs in the gerbil is similar to the process described for the mouse (Sugimura et al., 1986; Vilamaior et al., 2000; Antoniolli et al., 2004), which is characterized by a decrease in acinar size and epithelial atrophy, and these changes become clearer due to apoptosis (Rittmaster et al., 1995; Kurita et al., 2001; Campos et al., 2010) that occurs two weeks after castration. The stroma and epithelium have a direct interaction with each other, the so-called epithelial-stromal interaction; therefore, the stroma undergoes remodeling after castration, as shown by increases in both muscle cells and collagen fibers and a change in the composition of the basement

membrane (Vilamaior et al., 2000; Cunha et al., 2004). According to some authors, alterations in the composition of the basement membrane and other components of connective tissue may influence cellular processes such as proliferation and differentiation in the prostate, and these changes may have fundamental importance in the etiology and progression of pathological processes (Tuxhorn et al., 2001). This hypothesis would explain the appearance of pathological epithelial alterations, as observed in the 21-day castration group. Moreover, a drastic reorganization of collagen fibers and dedifferentiation of smooth muscle cells of the prostatic stroma have been previously detected in the ventral lobe of rats after surgical castration (Carvalho and Line, 1996, Carvalho et al., 1997) or chemical castration using finasteride (Corradi et al., 2004); the authors suggest that stromal remodeling following androgen ablation involves transformation of the contractile phenotype of smooth muscle cells to a secretory phenotype, indicating that these smooth muscle cells may be key players in stromal remodeling. However, the results found in this study suggest that several factors in addition to smooth muscle cells may act in the process of prostatic regression, including the regulators of the extracellular matrix MMP-2 and TIMP-2.

The stereology results obtained in this study show a significant increase in the volume of collagen fibers in the ventral male prostate after 21 days of castration, although there was an apparent increase in the expression of MMP-2 in this group. Thus, these data show that the collagen degradation promoted by MMP-2 does not necessarily reduce the fiber content in the ventral male prostate, but it does cause a break in its structure, allowing tissue reorganization in this group when the prostate undergoes structural regression and the ECM is remodeled.

Metalloproteinases are secreted as latent molecules called proMMPs that must be proteolytically processed to become active. Some MMPs can become activated on the cell surface by complexing with metalloproteinases linked to the cell membrane (the MT-MMPs), which can accumulate in the boundary and express local gelatinolytic activity (Hullboy et al., 1997; Quaranta, 2000;

Stamenkovic, 2000, Wilson et al., 2002; Murphy and Nagase, 2008; Belhocine et al, 2010). According to Itoh et al. (2008), pro-MMP-2 is activated at the cell surface by active MMP-14 in cooperation with TIMP-2. In some of the studied groups, MMP-2 and TIMP-2 expression occurred close to the apical membrane of the epithelial cells on the luminal side, and this expression may be related to the process of enzymatic metalloproteinase activation.

Our results demonstrate the presence of MMP-2 and TIMP-2 in blood plasma, with an increase in their expression after 21 days of castration. According to some authors, white blood cells produce MMPs when they are involved in inflammatory reactions, and tissue remodeling is considered to be an inflammatory reaction that attracts inflammatory cells. It has been shown in the literature that castration induces an increase in the number of stromal and intraepithelial macrophages and mast cells, which also express MMP-2 (Franck-Lissbrant et al., 1998; Kwong et al., 1999; Elkington et al., 2009; Justulin Jr. et al., 2010). According to Justulin Jr. et al. (2010), stromal cells such as macrophages and mast cells also contribute to the entire content of prostate MMPs and may also participate in the regulation of tissue remodeling during prostate atrophy.

The prostatic secretions of both the male and female controls of this study had a positive immunoreaction for MMPs and TIMPs, indicating the presence of these proteases in the fluid produced by the prostate secretory epithelial cells. Studies have shown that, in addition to acting as components of the extracellular matrix, MMPs and TIMPs have also been found in seminal plasma, and their presence in prostatic secretion has been demonstrated (Yin et al., 1990; Wilson et al., 1993; Delella et al., 2007; Belhocine et al., 2010). Belhocine and collaborators (2010) suggested that MMP-2 and MMP-9, found in the prostate of *Meriones libycus*, facilitate the process of secretion in the tubules and help to maintain the flow of secretion.

Recently, zymographic analysis of Wistar rat prostates showed that MMP-2 and MMP-9 activity increases after castration, and it is mainly the active forms of these enzymes that increase (Justulin et al., 2010). Accordingly, our results demonstrated that after 21 days of treatment, both castrated males and females treated with T showed more intense active MMP-2 immunoreactivity when compared to their controls. However, the tissue distribution of MMP-2 in the 21-day castrated group changed. MMP-2 was primarily located in stromal cells and in blood vessels rather than on the epithelium surface, and this distribution contributes to tissue remodeling. As expected, the opposite occurred with the TIMP-2 reaction, which was weak and sometimes absent in both male and female prostates.

This finding demonstrates that T promotes an alteration in the MMP/TIMP balance; however, it acts antagonistically in both organisms because an excess of T appears to promote an increase in the expression of MMP-2 in female prostates. This increase assists the process of remodeling and causes pathological changes, whereas a drop in T levels exerts this effect on the ventral prostate lobe of male gerbils; this mechanism remains to be elucidated.

Thus, MMP-2 and TIMP-2 are key factors that contribute to the ability of both female and male gerbil prostates to respond to the alterations promoted by androgenic changes. This response involves ECM degradation and stromal remodeling. Further biochemical studies are extremely important because they could elucidate the behavior of these proteases in male and female diseases.

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Table 1: Serum levels of testosterone (T) and estradiol (E) of male gerbils intact or castrated for 7 or 21 days and of female gerbils in proestrus phase or treated with testosterone for 7 or 21 days (n = 10 samples/group). Values are means ± SE. Asterisks represent significant differences relative to the control group.

	MALES			FEMALES		
	C	**7**	**21**	**C**	**7**	**21**
T (ng/ml)	1.5 ± 0.2	0.2 ± 0.03*	0.2 ± 0.04*	0.22 ± 0.04	14.2 ± 0.5*	12.2 ± 1.2*
E (pg/ml)	15.3 ± 1.7	14.8 ± 2.3	11.3 ± 1.2	23.9 ± 3.1	17.5 ± 2.9	24.1 ± 5.8

*Tukey HSD Test, $p \leq 0.05$.

Table 2: Descriptive prostatic stereology statistics of male and female adult gerbils in different hormonal situations. Values (%) are means ± SD. The asterisks (*) indicate statistically significant differences relative to controls.

	MALE VENTRAL PROSTATE			FEMALE PROSTATE		
	Control	**Castration (7 d)**	**Castration (21 d)**	**Control**	**Testosterone (7 d)**	**Testosterone (21 d)**
Epithelium	14.5 ± 5.7	10 ± 6.05*	28.5 ± 8.2*	26.2 ± 11	26.7 ± 4.5	27.7 ± 10.7
Lumen	72.6 ± 9.8	78.3 ± 9.7	28.3 ± 15*	41.1 ± 19	27.2 ± 8.3*	36.3 ± 22.7
Collagen fibers	4.4 ± 2.2	3.4 ± 2.64	15.7 ± 7.3*	9.6 ± 4.5	16.3 ± 4.2*	15.5 ± 10*
Smooth muscle cells	7 ± 3.9	6.8 ± 2.8	21.9 ± 5.5*	16.2 ± 8.1	22 ± 5.6	13.9 ± 12
Other	1.4 ± 1.7	1.5 ± 1.42	5.5 ± 2.9*	6.7 ± 6.6	7.6 ± 3.3	6.5 ± 5.5

*Tukey HSD Test, $p \leq 0.05$.

FIGURE LEGENDS

Figure 1: Histology of the ventral prostate of male gerbils and the prostate of female gerbils stained with Gömori's Trichrome. Male ventral prostate controls (A) castrated for 7 (B) or 21 days (C, G). Female prostate controls (D) and females treated with testosterone for 7 (E) or 21 days (F, H). In G, detail of a ventral male prostate after 21 days of castration showing epithelial in-folding with subepithelial accumulation of collagen fibers stained in green. In H, a female prostate treated with testosterone for 21 days showing microacini (arrows) and epithelial proliferative lesions (*). ep = epithelium, L = lumen. Bars: A-F = 50 µm, G = 10 µm, H = 20 µm.

Figure 2: Immunohistochemical reaction for active MMP-2 in male gerbil ventral prostates. Controls (A, B) and castrated for 7 (C, D) or 21 days (E, F). The immunoreaction was more intense after 21 days of castration, with strong immunostaining in the blood vessels and various MMP-2-positive regions throughout the stroma. S = secretion; arrow heads = MMP-2-positive blood vessels; arrows = positive stromal cells. Bars: A, C, E = 20 µm; B, D, F = 10 µm.

Figure 3: Immunohistochemical reaction for TIMP-2 in male gerbil ventral prostates and female gerbil prostates. Ventral prostates from control males (A, B) and from males castrated for 7 (E, F) or 21 days (I, J). Prostates from intact females (C, D) and from females treated with testosterone for 7 (G, H) or 21 days (K, L). In L and K, epithelial alterations in female prostates resulting from 21-day T treatment; microacini filled with immunostained secretions (*) and prostatic intraepithelial neoplasias (PINs), respectively. The inset in A shows a negative control for TIMP-2 immunohistochemical reaction. ep = epithelium, L = lumen; S = secretion; PIN = prostatic intraepithelial neoplasia; asterisk = microacinus with TIMP-2 positive secretion; arrows = TIMP-2-positive stromal cells; full arrowhead = TIMP-2-positive blood vessels; empty arrowhead = TIMP-2-positive epithelial cells. Bars: A, C, E, G, I, K = 20 µm, B, D, F, H, J, L = 10 µm.

Figure 4: Immunohistochemical reaction for active MMP-2 in the female gerbil prostate. Female prostates from intact animals (A-C) and from animals treated with T for 7 (D-F) or 21 days (G-K). In E, the inset shows positive epithelial cells among negative cells in the group treated with T for 7 days. In J and K, respectively, microacini filled with immunolabeled secretion (*) and prostatic intraepithelial neoplasias (PIN), both epithelial alterations present in the 21-day group showing MMP-2-positive reaction. ep = epithelium, S = secretion; PIN = prostatic intraepithelial neoplasia; asterisk (*) = microacinus; arrows = positive stromal cells; arrowheads = positive blood vessels. Bars: A, D, F, G, H, K = 10 µm; B, C, E, I, J = 20 µm

Figure 1

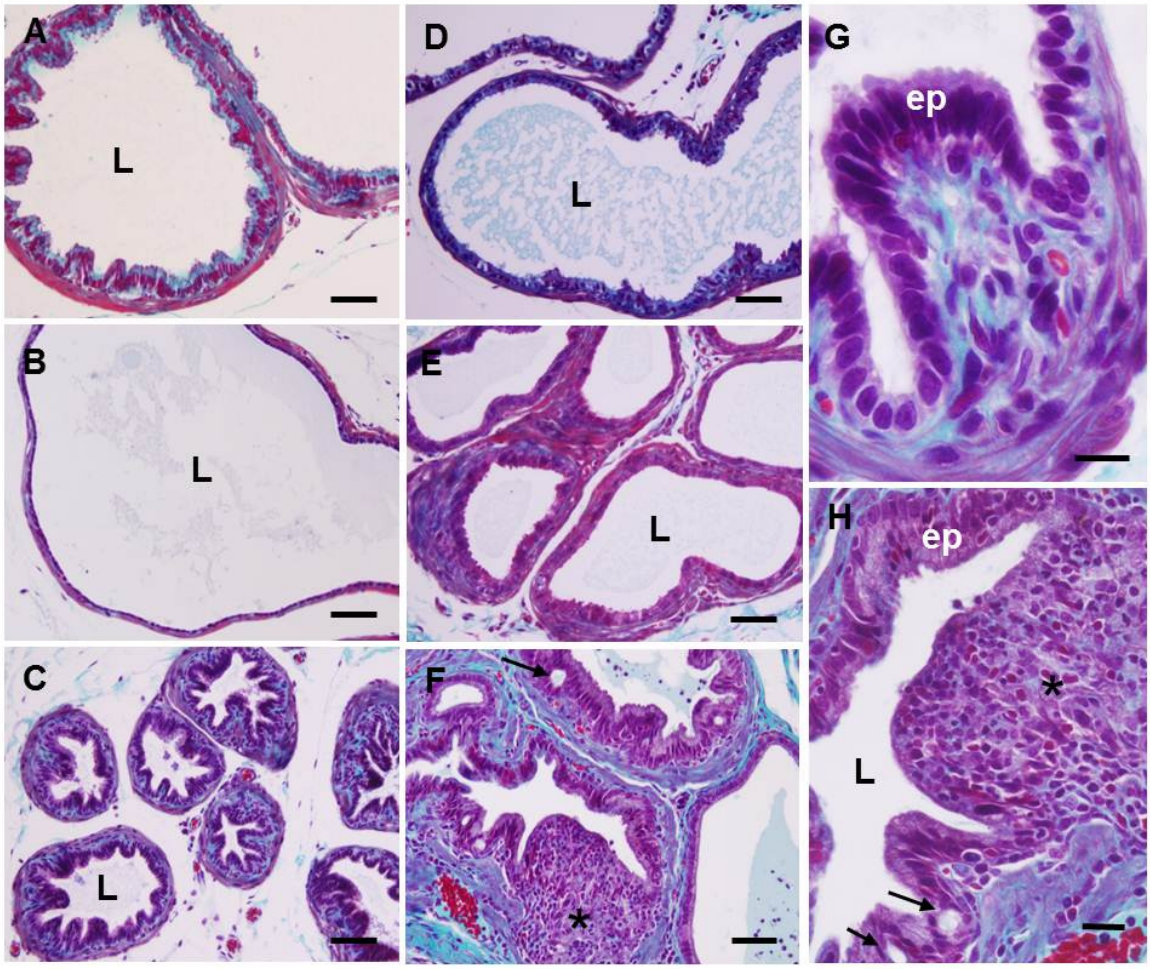


Figure 2

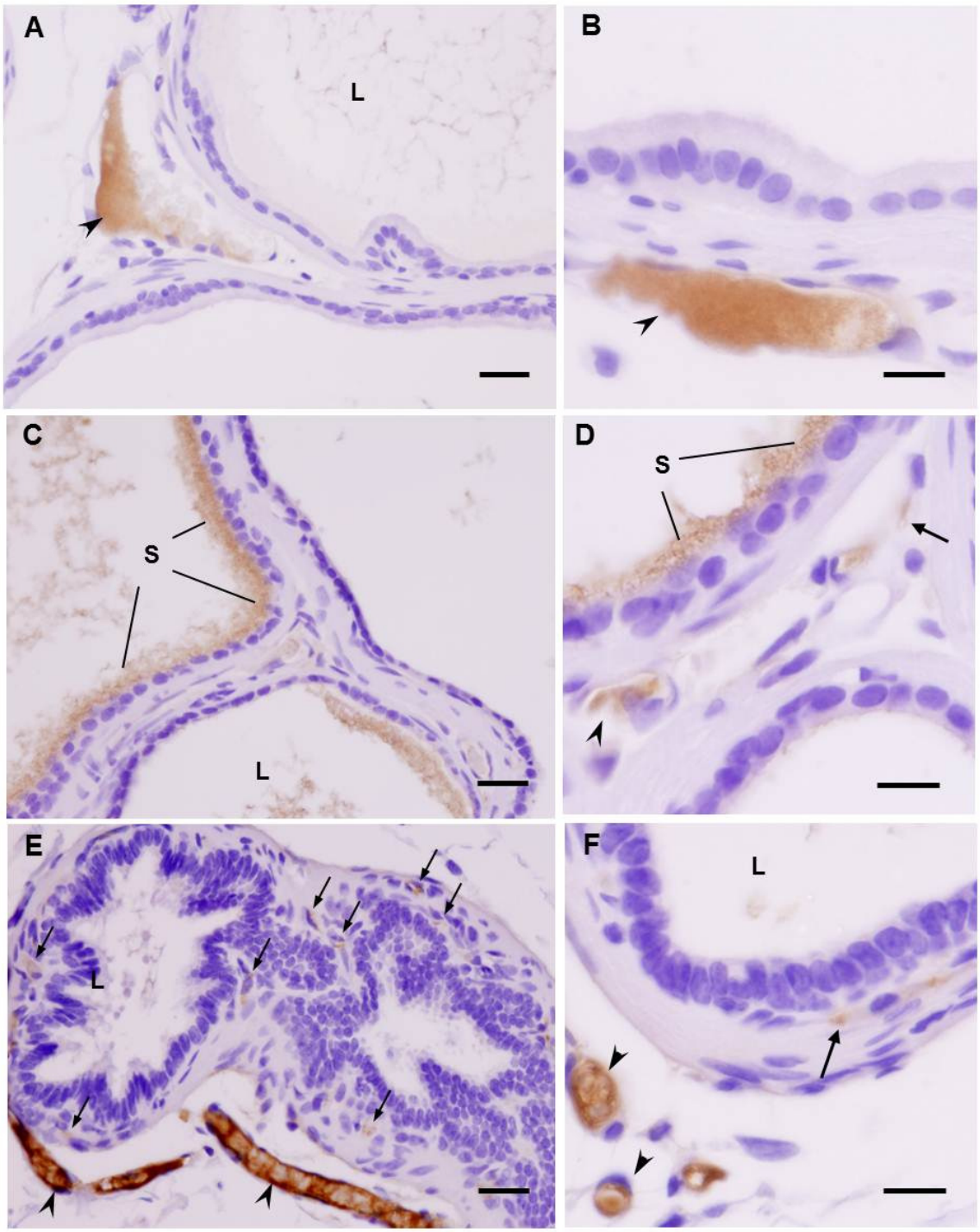


Figure 3

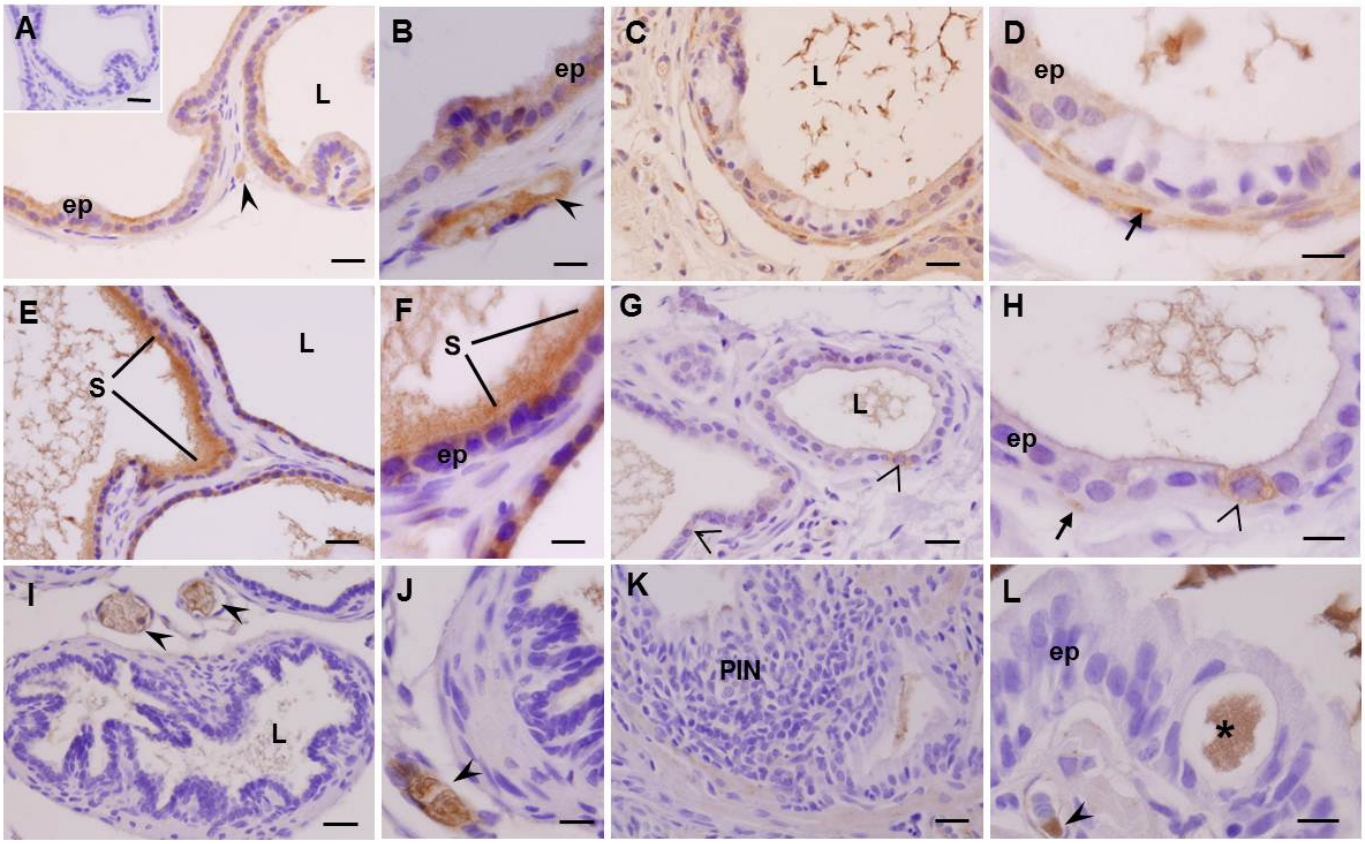
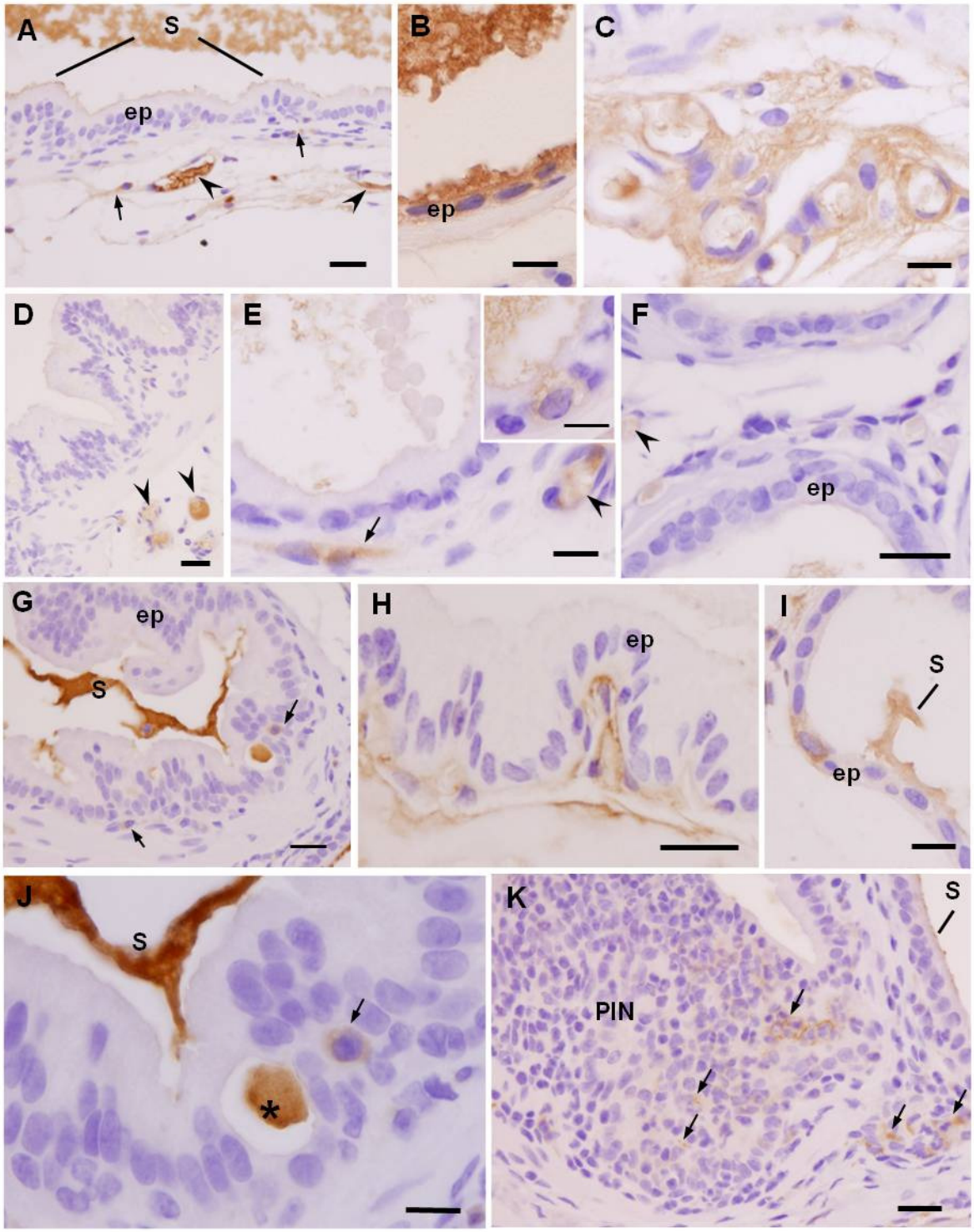


Figure 4



ARTIGO III

IMMUNOLOCALIZATION OF ESTROGEN RECEPTOR ALPHA AND BETA IN THE PROSTATE OF MALE AND FEMALE GERBILS SUBMITTED TO HORMONAL MANIPULATION

Artigo em vias de submissão à revista

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IMMUNOLOCALIZATION OF ESTROGEN RECEPTOR ALPHA AND BETA IN THE PROSTATE OF MALE AND FEMALE GERBILS SUBMITTED TO HORMONAL MANIPULATION

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Key-words

ERα, ERβ, Castration, Testosterone, DHEA, Female Prostate, Ventral Prostate, Gerbil.

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ABSTRACT

Prostate development and homeostasis are regulated by the joint action of androgens and estrogens and an imbalance in the testosterone/estrogen ratio may have pathological consequences in both male and female prostates. Estrogen action is mediated by estrogen receptors alpha and beta (ERα and ERβ), each acting at a particular time of prostate development and with different responses to hormonal stimulation. The present study aimed to describe and compare ERα and ERβ expression in male and female gerbil prostates, and to evaluate the effect of testosterone and DHEA on these receptors. Immunohistochemistry for ERα and ERβ was carried out with the prostates of intact or castrated (7 and 21 days) male gerbils, as well as intact females treated with testosterone or DHEA for 7 and 21 days. The quantitative analysis showed that in the prostate stroma there are more ERα-positive cells in females than in males, while the hormone treatment experiments showed a decreased expression of ERα in females after 21 days of treatment with testosterone. ERβ expression was similar between intact male and female prostates, but after castration of males or the application of testosterone or DHEA to females it showed a specific response for each group. In addition, the hypertrophic epithelial lesions found in the experimental groups were ERα-positive and ERβ-negative, reinforcing the studies that correlate ERα with development and progression of prostatic diseases and ERβ as a target for prevention and treatment of prostate cancer.

INTRODUCTION

Androgens are responsible for differentiation and development of prostate as well as for support and maintenance of prostatic tissue in the adult (Thomson, 2001; Prins and Putz, 2008). Furthermore, androgens regulate the development and progression of age-related diseases in men, including benign prostatic hyperplasia and prostate cancer (Dehm and Tindall, 2007; Richter *et al.*, 2007; Balk and Knudsen, 2008). Although androgens are the major hormones acting in the prostate, several studies describe the importance of estrogens in the development and homeostasis of normal prostatic tissue (Chen *et al.*, 2008; McPherson *et al.*, 2008; Scarano *et al.,* 2008; Ellem and Risbridger, 2009). However, excess of estrogen may cause adverse responses in prostate epithelium, such as proliferation, inflammation and cancer, and absence of estrogen may lead to prostatic hypertrophy and hyperplasia (Chen *et al.*, 2008, Scarano *et al.*, 2008; Ellem and Risbridger, 2009).

Estrogen action is mediated by two types of nuclear receptors, estrogen receptors alpha and beta (ERα and ERβ), each acting at a particular time of prostate development and with different responses to hormonal stimulation (Chen *et al.*, 2008; Morani *et al.*, 2008). ERα has been reported as responsible for the development of the prostate gland before puberty while ERβ seems to be more important during later periods of prostate development, such as puberty and adulthood, acting together with other factors in regulating the differentiation and functional activity of adult tissue (Imamov *et al.*, 2004; Omoto *et al.*, 2005, McPherson *et al.*, 2008).

The gerbil is a rodent whose prostate has been the subject of several studies in recent years, especially regarding the effects of hormonal manipulations on the prostatic structural organization (Corradi *et al.*, 2004; Garcia *et al.*, 2007; Oliveira *et al.*, 2007; Rochel *et al.*, 2007). Furthermore, the female prostate in the gerbil has been widely investigated in view of its susceptibility to diseases similar to those affecting males (Santos *et al.*, 2003; Custodio *et al.*, 2004; Santos *et al.*, 2006; Custodio *et al.*, 2010).

The present study aimed to analyze the occurrence of estrogen receptors alpha and beta in the female prostate and in the ventral prostate lobe of male adult gerbils, and assess the influence of hormonal manipulation on the expression of these nuclear receptors.

MATERIALS AND METHODS

Animals and experimental design

Adult (4 months old) male and female gerbils were randomly divided into control and treated groups (n = 5). The control group consisted of intact animals, free from hormonal manipulation, and in the case of females, in the proestrus phase of the estrous cycle.

The treated males were anesthetized with ketamine (800 µl/kg) and xylazine (200µl/kg) and underwent bilateral orchiectomy, being sacrificed 7 and 21 days after castration. Treated females received every other day, intramuscular injections of testosterone cypionate (T, 1 mg/kg diluted in corn oil) or dehydroepiandrosterone (DHEA, 1mg/kg diluted in saline). Initial applications occurred with reference to the estrous cycle, always at the proestrus phase, and sacrifice took place 7 and 21 days after beginning of treatment.

Histological processing

To collect the prostatic complex, the animals were anesthetized by CO_2 inhalation and sacrificed by decapitation. The female prostates and the ventral lobe of male prostates were fixed by immersion in Methacarn (Methanol/Chloroform/Acetic acid - 6:3:1) for 3 h at 4°C. Tissues were dehydrated in an ascending series of ethanol, cleared in xylene, embedded in Paraplast (Merck, Darmstadt, Germany) and then subjected to immunohistochemical analysis and immunofluorescence following protocols described below.

Immunohistochemistry and immunofluorescence

Histological sections (5µm) from all experimental groups were subjected to immunocytochemical reaction for ERα and ERβ. The antibodies used and their specifications are described in Table 1. The deparaffinized and rehydrated histological sections underwent antigen retrieval with citrate buffer pH 6.0 at 100°C. Peroxidase endogenous blocking occurred in 3% H_2O_2 diluted in 50% methanol and the blocking of nonspecific proteins was performed using bovine serum albumin (BSA), diluted to 3% in PBS. The primary antibodies were used in variable dilutions (table 1) and the slides were incubated overnight at 4°C. For regular immunohistochemistry, the slides were rinsed in

PBS, and incubated with biotinylated secondary antibodies and with Vectastain ABC kit (VECTOR Laboratories Ltd., UK). The reaction was revealed with diaminobenzidine (DAB) and sections were counterstained with Harris Hematoxylin. For immunofluorescence, the slides were rinsed in PBS, the sections were incubated with secondary antibody conjugated with Cy3 fluorescent particle, followed by nuclear staining with DAPI (blue). Negative controls were obtained omitting the primary antibody incubation step.

Table 1 – Primary and secondary antibodies and its specifications.

	Antibody	Specifications	Dilution	Manufacturer
Primary	ER-α (MC-20)	Rabbit anti-human polyclonal antibody (sc-7207)	1:200	**Santa Cruz Biotech. (Santa Cruz, CA/USA)**
	ER-β (503)	Chicken anti-human polyclonal antibody	1:100	**Karolisnka Institute Personal Production**
Secondary	(IF) Cy3	Donkey anti-rabbit (red) (A-31572)	1:200	**Invitrogen (Carlsbad, CA/USA)**
	(IHC) Anti-rabbit	Biotin goat anti-rabbit IgG (65-6140)	1:200	**Invitrogen (Carlsbad, CA/USA)**
	(IHC) Anti-chicken	Biotin rabbit anti-chicken IgG (61-3140)	1:200	**Invitrogen (Carlsbad, CA/USA)**

Quantitative and statistical analysis

The quantification of ERα-positive cells in the stroma of both male and female prostates was performed using 30 microscopic fields (400X magnification) randomly selected from each experimental group. The immunoreactivity rate* was calculated as the number of ERα-positive stromal cells divided by the total number of stromal cells, expressed as percentage and at least 1000 stromal cells were counted for each group.

**Immunoreactivity rate = (ERα+ stromal cells / Total of stromal cells) x 100*

Data was evaluated by analysis of mean ± standard deviation. Statistical analysis was performed with the software Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). One-Way Analysis of Variance (ANOVA) was performed to determine the possible differences between groups and Tukey HSD test was used to determine the significance of differences found. A p value ≤ 0.05 was considered statistically significant.

RESULTS

ERα

According to the quantitative analysis, the number of ERα-positive cells in the prostate of adult females averaged 56.5% of total stromal cells (Fig. 1A), significantly higher than in the male gerbil ventral prostate, in which only 13% of stromal cells stained positively for this receptor (Fig. 1B). Orchiectomy induced T ablation caused a slight increase in the number of ERα-positive cells in males, but these data were not statistically significant. On the other hand, treatment with T in females led to a significant decrease in the number of ERα-positive cells after 21 days of treatment, with 27% of stromal cells ERα-positive while treatment with DHEA caused no significant alteration in the number of ERα-positive stromal cells either after 7 or 21 days of treatment (Fig. 1A).

Either in male or in females, the staining for ERα occurs in the prostatic stromal cells, preferentially in the nuclei of fibroblasts and smooth muscle cells, while secretory epithelial cells are ERα-negative (Figure 2). Hormonal injections in female gerbils promoted morphologic alterations in the prostate, causing pathological lesions such as epithelial hyperplasia in all experimental groups, and anomalous proliferation after 21 days of treatment with DHEA (*data not shown*). The proliferative epithelium observed after 21 days of treatment with T showed positive reaction for ERα (Fig. 2 F, inset).

ERβ

Immunohistochemical analysis showed that estrogen receptor beta (ERβ) is consistently and homogeneously distributed in the ventral lobe of adult male gerbil controls. Although the intensity of the reaction was weak, both stromal cells and the vast majority of epithelial cells of all acini were positive for ERβ (figure 3).

T ablation influenced the expression of ERβ in the male prostate, and in most acini of the 7-day castration group ERβ staining was either undetectable or extremely weak. Surprisingly, however, after 21 days of castration, ERβ staining in the epithelial cells was intense, but only in some cells of each acini, as shown in Figure 3 (I), with half the acini being ERβ-positive and half ERβ-negative. Castration also promoted the appearance of epithelial proliferative lesions in the ventral lobe of some animals in the 7 and 21 days groups, but the cells presented in the lesions did not show positive reaction for ERβ (Fig. 3, detail in H and I).

Regarding the female prostate controls in the proestrus phase, immunohistochemistry showed that ERβ is present in the nucleus of most epithelial cells, many with strong intensity (Figure 3 - A, D). However, after treatment with T the reaction was weaker, even absent in some cases (Fig. 3 B, C), indicating that excess of T may have caused changes in the expression of this receptor. The females treated with DHEA, both 7 and 21 days of treatment, presented very weak ERβ reaction, although homogeneously in the secretory epithelial cells, with a stronger reaction in the basal cells, illustrated in Figure 3 (E, F). Some stromal cells in this group were also ERβ-positive.

DISCUSSION

A female prostate has been reported in several rodent species and its morphology and biochemistry shows similarities with the ventral lobe of the male prostate (Santos and Taboga, 2006). Furthermore, the prostate gland in females has been described as an organ susceptible to benign and malignant pathologies similar to those developed by the male gland (Uzoaru *et al.*, 1992; Dodson *et al.*, 1995; Sloboda *et al.* 1998; Zaviacic et al., 2000; Custodio *et al.* 2004; Custodio *et al.* 2010).

In this study we compared the expression of ERα in the stroma of gerbil male and female prostates and the quantitative analysis revealed that female prostate has more ERα-positive stromal cells than does the male prostate. In addition, the treatment with T for 21 days in the females lead to a significant decrease in the expression of these receptors in the prostate stroma, but the number of ERα-positive cells remained higher than in male untreated controls. On the other hand, castration of male gerbils had no effect on the number of ERα-positive stromal cells, even after 21 days of T absence. Thus, according to our results, excess of testosterone down regulated ERα expression in the stromal cells of gerbil female prostate, whereas removal of T (castration) did not affect ERα expression in male prostates.

It has been demonstrated that nuclear receptors regulate the action of androgens and estrogens, influencing the function of the prostate of males and females. Evidence for the involvement of estrogen receptors in the pathogenesis of cancer has grown and several studies associate ER, especially ERα, with the development and progression of benign and malignant diseases of the prostate. It is known that ERα has a proliferative action, and when activated it can trigger various adverse effects on the prostate of rodents, such as anomalous epithelial proliferation, inflammation and development of premalignant lesions (Richter *et al.*, 2007, Chen *et al.*, 2008; Ellem and Risbridger, 2009). On the other

hand, ERβ is related to quiescence and acts on epithelial-stromal cell signaling, as well as on the regulation of the antiproliferative effects responsible for modulating the hypertrophic action of androgens in prostatic epithelium (Weihua *et al.*, 2002; Imamov *et al.* 2004; Omoto *et al.*, 2005, McPherson *et al.*, 2008; Ellem and Risbridger, 2009). Several studies about prostatic ERβ demonstrate a silencing of this receptor as the cancer progresses, suggesting a protective role of this receptor against proliferative disorders and indicating the possible use of ERβ as a target for prevention and treatment of prostate cancer (Imamov *et al.*, 2004; Morani *et al.*, 2008; Ellem and Risbridger, 2009). In our study we observed that the proliferative epithelium found in experimental groups of T and DHEA administration were ERα-positive and ERβ-negative, reinforcing the involvement of ERα with proliferation and pathological disorders.

The action of estrogens on the prostate is very complex, with different effects via ERα and ERβ, respectively. In this manner, not only androgen/estrogen ratio influences the development and homeostasis of the prostate, but also the balance between the expression of ERα and ERβ is crucial to determine the response of the prostate to estrogen. The occurrence of pathological conditions in the female prostate of rodents, similar to those developed by men, such as carcinoma, benign prostatic hyperplasia and prostatitis, indicates that it is an organ highly dependent on hormone action, and a detailed assessment of the location and expression of its nuclear receptors may help to understand the mechanisms that lead to development of these diseases.

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FIGURE LEGENDS

Figure 1: Immunoreactivity rate of ERα-positive stromal cells of gerbil prostates. In **A**, prostate of female controls (proestrus phase) and treated with T for 7 and 21 day and treated with DHEA for 7 and 21 days. In **B**, ventral prostate of male gerbil controls and castrated for 7 and 21 days. Percentage of stromal ERα-positive cells is higher in females than in males and significantly decreases after long term treatment with T. Asterisk(*)= Significantly different from control group.

Figure 2: Immunofluorescence for ERα in histological sections of control female prostate at the proestrus phase (A-C) and immunohistochemistry reaction for ERα in histological sections of adult male and female gerbil prostate, controls and under hormonal manipulation (D-L). Cell nuclei stained in brown indicates positive reaction to ERα. **A**- DAPI nuclear staining, **B** – ERα-positive cells in stroma of control female prostate and **C** – overlay of both images A and B. In **D** and **G**, control female prostate with ERα-positive stromal cells in brown. In **E** and **F**, prostate of females treated with T for 7 and 21 days, respectively. In **H** and **I**, prostate of females treated with DHEA for 7 and 21 days, respectively. The arrows indicate positive reaction for ERα in epithelial cells. **J**, **K** and **L** – male ventral prostates of control and 7 and 21 days castrated gerbils, respectively. Bars = 20µm.

Figure 3: Immunohistochemical reaction for ERβ in histological sections of male and female gerbil prostates, controls and under hormonal manipulation. **A** and **D** - Prostate of female controls in the proestrus phase. Cell nuclei stained in brown indicates positive reaction to ERβ. In D, positive epithelial cells beside negative cells (blue nucleus). In **B** and **C**, prostate of female treated with T for 7 and 21 days respectively. In **E** and **F**, prostate of female treated with DHEA for 7 and 21 days respectively. The arrows indicate positive reaction in the epithelial basal cells. In **G**, **H** and **I**, ventral prostate of male gerbil controls and castrated for 7 and 21 days, respectively. Detail in H shows ERβ-negative epithelial proliferative lesion.

FIGURE 1

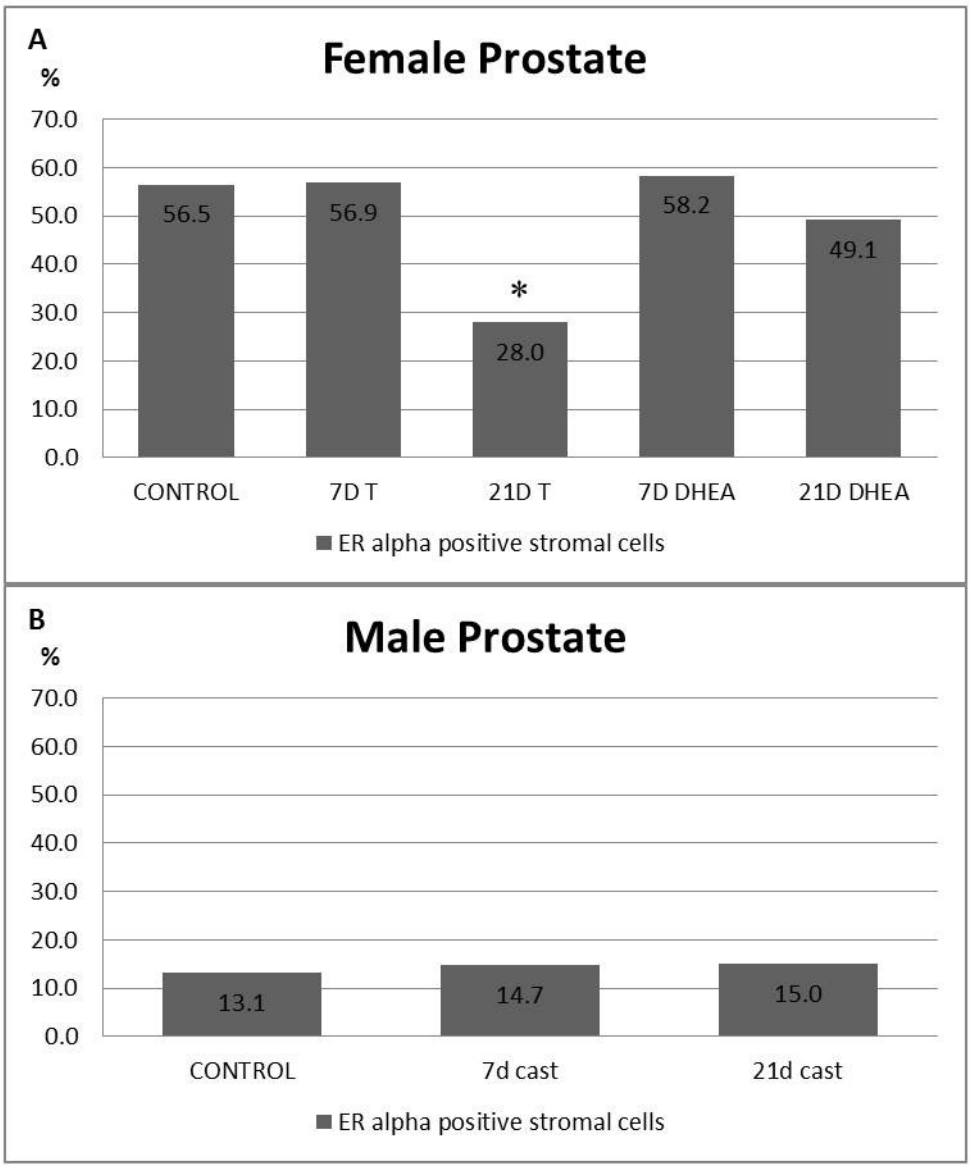

A
%
Female Prostate
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
56.5
56.9
28.0
*
58.2
49.1
CONTROL
7D T
21D T
7D DHEA
21D DHEA
ER alpha positive stromal cells
B
%
Male Prostate
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
13.1
14.7
15.0
CONTROL
7d cast
21d cast
ER alpha positive stromal cells

FIGURE 2

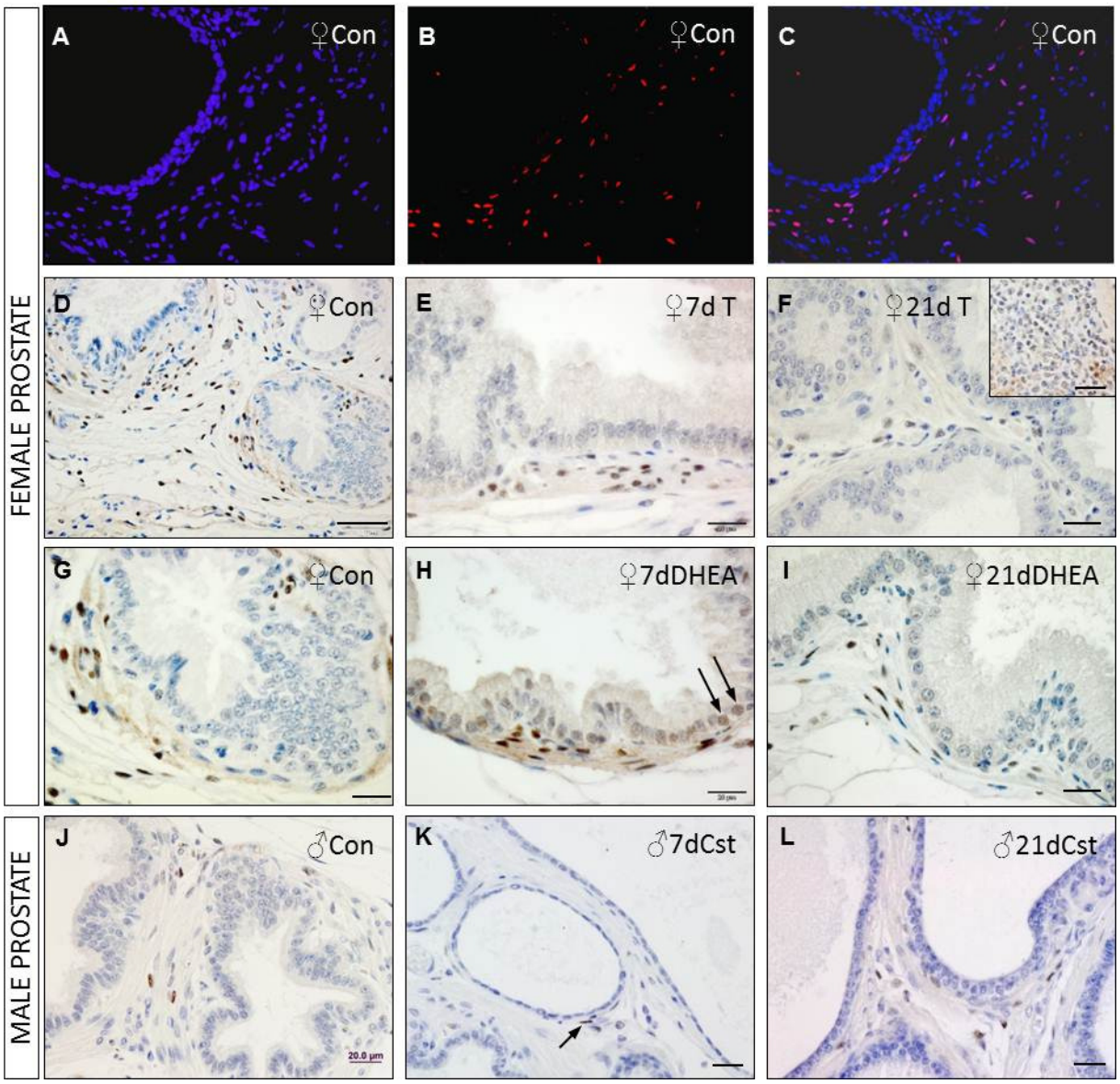
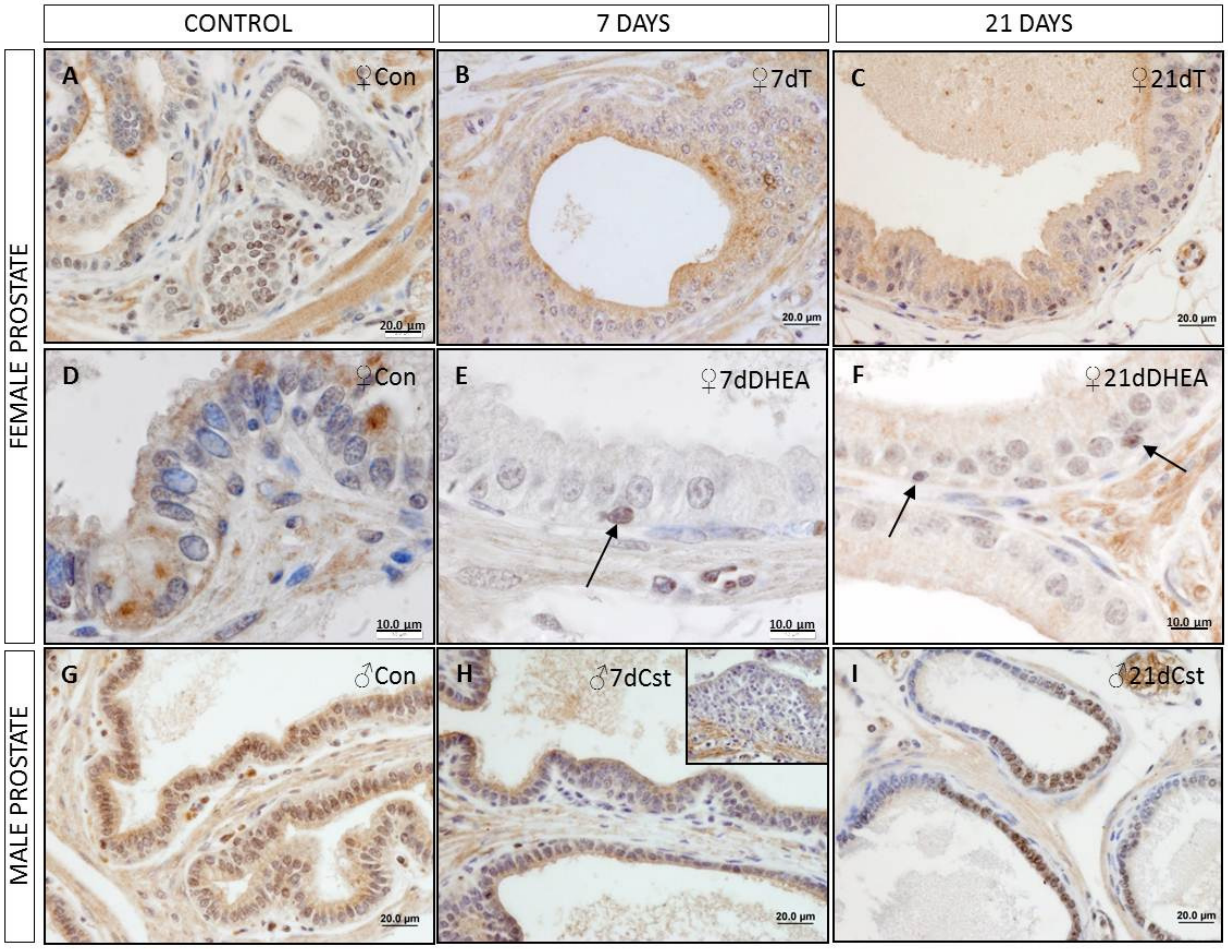

FEMALE PROSTATE
MALE PROSTATE
A
♀Con
B
♀Con
C
♀Con
D
♀Con
E
♀7d T
F
♀21d T
G
♀Con
H
♀7dDHEA
I
♀21dDHEA
J
♂Con
20.0 µm
K
♂7dCst
L
♂21dCst

FIGURE 3


CONTROL
7 DAYS
21 DAYS
FEMALE PROSTATE
MALE PROSTATE
A
♀Con
B
♀7dT
C
♀21dT
D
♀Con
E
♀7dDHEA
F
♀21dDHEA
G
♂Con
H
♂7dCst
I
♂21dCst
20.0 µm
10.0 µm

CONCLUSÕES

Os resultados obtidos através deste estudo vêm reforçar o conceito de que o equilíbrio estrógeno/testosterona é essencial para a homeostase prostática, e que tanto andrógenos quanto estrógenos atuam sobre as células epiteliais, através dos receptores nucleares, e exercem seus efeitos sobre o compartimento estromal, regulando componentes como as metaloproteinases de matriz. No caso da próstata feminina, essa interação é ainda mais complexa pois envolve as alterações cíclicas do ciclo estral. Algumas conclusões específicas, no entanto, podem ser ressaltadas:

- ✓ A próstata feminina de *Meriones unguiculatus* apresenta uma maior atividade gelatinolítica para todas as MMPs estudadas em comparação à todos os lobos prostáticos do gerbilo macho, provavelmente por se tratar de um órgão que sofre constante remodelação tecidual provocada pelas oscilações hormonais provenientes do ciclo estral.

- ✓ As oscilações hormonais que ocorrem durante o ciclo estral influenciam a expressão das MMPs 2 e 9 na próstata feminina do gerbilo, sendo a fase Estro a de maior atividade destas enzimas e as fases Diestro I e II as de menor atividade.

- ✓ A expressão prostática de MMPs e TIMPs é regulada por andrógenos, porém de forma distinta entre machos e fêmeas. Tanto a falta de T nos machos como o excesso de T nas fêmeas promovem remodelação tecidual na próstata, fazendo com que a MMP-2, deixe de ser expressa pelas células epiteliais e passe a ser sintetizada principalmente pelas células estromais para auxiliar no processo de reestruturação do estroma.

- ✓ A próstata feminina de gerbilos apresenta duas vezes mais ERα do que o lobo ventral de gerbilos machos, sendo predominantemente expressas pelas células estromais em ambos os tecidos.

- ✓ O excesso de testosterona regula de forma negativa a expressão de ERα na próstata feminina de gerbilos, diminuindo significativamente o número de células estromais positivas, porém a ausência de T não influencia a expressão deste receptor na próstata ventral masculina.

- ✓ Tanto o excesso quanto a ausência de andrógenos influenciam negativamente a ocorrência do ERβ prostático em gerbilos, uma vez que as células epiteliais apresentaram marcação fraca ou ausente para este receptor após os experimentos de castração nos machos ou de injeção hormonal em fêmeas;

- ✓ O epitélio hipertrófico visualizado na próstata feminina após o tratamento com T e DHEA apresentou expressão de ERα, mas não de ERβ, reforçando o envolvimento do ERα nas desordens proliferativas e patológicas.

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CERTIFICATE

We certify that the protocol nº 014/07 about "*Androgenic modulation of MMP-2 and -9, TIMP-2 and serinoproteases in the male and female gerbil's prostate*" agree with ETHICAL PRINCIPLES IN ANIMAL RESEARCH adopted by Brazilian College of Animal Experimentation (COBEA) and was approved "Ad referendum" in that date by the BIOSCIENCE INSTITUTE/UNESP ETHICAL COMMITTEE FOR ANIMAL RESEARCH (CEEA).

Botucatu, march 30, 2007.

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