MANOEL FRANCISCO BIANCARDI

"ESTUDO DA ORGANOGÊNESE PROSTÁTICA NORMAL E OS EFEITOS DA EXPOSIÇÃO ANDROGÊNICA INTRAUTERINA E PUBERAL SOBRE A MORFOFISIOLOGIA DA PRÓSTATA DE GERBILOS SENIS"

"STUDY OF NORMAL PROSTATE ORGANOGENESIS AND THE EFFECTS OF INTRAUTERINE AND PUBERTAL ANDROGENIC EXPOSURE ON THE MORPHOPHYSIOLOGY OF OLD GERBIL PROSTATE"

Campinas, 2014

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

MANOEL FRANCISCO BIANCARDI

"Estudo da Organogênese Prostática Normal e os Efeitos da Exposição Androgênica Intrauterina e Puberal Sobre a Morfofisiologia da Próstata de Gerbilos Senis"

Orientador: Dr. Sebastião Roberto Taboga

"Study of Normal Prostate Organogenesis and the Effects of Intrauterine and Pubertal Androgenic Exposure on the Morphophysiology of old Gerbil Prostate"

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Doctorate thesis presented to the State University of Campinas in fulfillment of the requirements for the degree of Doctor on Cellular and Structural Biology Postgraduate Programme, in the Biology Institute, in the area of Cellular Biology.

Este exemplar corresponde à versão final da tese defendida pelo aluno ***Manoel Francisco Biancardi*** e orientado pelo Dr. Sebastião Roberto Taboga.

Assinatura do Orientador

Campinas, 2014

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

Biancardi, Manoel Francisco, 1981-
B47e Estudo da organogênese prostática normal e os efeitos da exposição androgênica intrauterina e puberal sobre a morfofisiologia da próstata de gerbilos senis / Manoel Francisco Biancardi. – Campinas, SP : [s.n.], 2014.

Orientador: Sebastião Roberto Taboga.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Próstata. 2. Gerbilos. 3. Disruptores endócrinos. 4. Testosterona. 5. Organogênese. I. Taboga, Sebastião Roberto. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Study of normal prostate organogenesis and the effects of intrauterine and pubertal androgenic exposure on the morphophysiology of old gerbil prostate
Palavras-chave em inglês:
Prostate
Gerbils
Endocrine disruptors
Testosterone
Organogenesis
Área de concentração: Biologia Celular
Titulação: Doutor em Biologia Celular e Estrutural
Banca examinadora:
Sebastião Roberto Taboga [Orientador]
Hernandes Faustino de Carvalho
Jaqueline de Carvalho Rinaldi
Renato Simões Cordeiro
Luis Antonio Justulin Junior
Data de defesa: 25-02-2014
Programa de Pós-Graduação: Biologia Celular e Estrutural

Campinas, 25 de fevereiro de 2014.

BANCA EXAMINADORA

Dr. Sebastião Roberto Taboga (Orientador) _____ Assinatura

Dr. Hernandes Faustino de Carvalho _____ Assinatura

Dra. Jaqueline de Carvalho Rinaldi _____ Assinatura

Dr. Renato Simões Cordeiro _____ Assinatura

Dr. Luis Antonio Justulin Junior _____ Assinatura

Dra. Silvia Borges Pimentel de Oliveira _____ Assinatura

Dra.Taize Machado Augusto _____ Assinatura

Dr. Ricardo Alexandre Fochi _____ Assinatura

Pesquisas recentes tem mostrado que a predisposição às desordens prostáticas tem origem nos momentos iniciais da vida. Portanto, o entendimento detalhado da organogênese prostática e dos mecanismos envolvidos na predisposição à estas desordens são de fundamental importância para se compreender a etiologia das patologias prostáticas. Assim, o objetivo deste trabalho foi avaliar a organogênese prostática normal e a influência da exposição à testosterona durante a vida intrauterina e puberal sobre a próstata de fêmeas e machos senis do gerbilo da Mongólia. Para isto, o presente trabalho foi dividido em duas etapas. Na primeira, grupos de fêmeas grávidas de gerbilo receberam injeções subcutâneas de testosterona (500 μg/dose) 4 dias antes do parto (grupos TG e TG + T) e/ou durante a vida pós-natal (grupos C + T e TG + T). Os filhotes destas mães envelheceram até serem mortos com 1 ano de idade. Na segunda etapa, foi realizado um estudo normativo da organogênese do lobo ventral da próstata de machos. Para isto, fetos machos de 20 a 24 dias de gestação (E20 - E24), além de recém nascidos de 1 dia de idade (P1), foram empregados no estudo. Todos os fragmentos teciduais foram dissecados e fixados em metacarn ou paraformaldeído 4%, sendo processados para inclusão em parafina. Posteriormente, as seções teciduais foram submetidas à análises biométricas, morfológicas, estereológicas, imuno-histoquímicas, de imunofluorescência e de reconstrução tridimensional. Os resultados mostraram que somente os machos velhos do grupo TG + T foram acometidos por hiperplasia adenomatosa associada à inflamação, embora nos animais dos outros grupos experimentais também foram observados focos hiperplásicos na próstata. Já as fêmeas expostas à testosterona na fase pré-natal (grupos TG e TG + T) apresentaram uma maior heterogeneidade de alterações em decorrência do tratamento, que envolveram desde malformações do sistema reprodutor até a formação de tecido ectópico ao redor da vagina, além da presença de lesões prostáticas também caracterizadas por focos hiperplásicos adenomatosos. Estes resultados mostram que a exposição anormal à testosterona afeta a organogênese prostática tanto em machos quanto em fêmeas, alterando os processos normais do desenvolvimento e aumentando a suscetibilidade ao desenvolvimento de doenças ao longo do envelhecimento. Em relação ao estudo da organogênese prostática, foi observado que os brotos ventrais emergem do epitélio uretral

entre o 20º e o 21º dia de vida pré-natal, atingindo o pé mesenquimal ventral (VMP) e iniciando o processo de morfogênese de ramificação no primeiro dia de vida pós-natal. Os achados também demonstraram que a camada de musculatura lisa parece ter um papel central neste processo, a qual pode atuar como uma barreira física à expansão dos brotos em direção ao VMP. Em relação aos receptores nucleares, o receptor de andrógeno apresentou uma expressiva imunomarcação no mesênquima periuretral, enquanto que células com marcação para receptor alfa de estrógeno foram identificadas tanto nos brotos como no mesênquima periuretral. Por fim, os resultados demonstram que a organogênese prostática, além de envolver uma série de eventos finamente regulados, é um processo extremamente sensível e determinante para a saúde da glândula ao longo da vida.

Palavras chave: Próstata, gerbilo, disruptores endócrinos, testosterona, organogênese.

Recent researches have been shown that the predisposition to prostatic disorders has its origin during moments of early life. Therefore, the detailed understanding of prostate organogenesis and the mechanisms underlying in predisposing to these disorders are extremely important in order to highlight the etiology of prostatic pathologies. Thus, the aim of this work was to evaluate the normal prostate organogenesis and the influence of testosterone exposure during prenatal and pubertal life on the prostate of male and female Mongolian gerbil. To this, the present work was divided into two phases. First, groups of pregnant female received subcutaneous injections of testosterone (500 µg/dosage) four days before the parturition (groups TG and TG + T) and/or during postnatal life (groups C + T and TG + T). The offspring of these mothers aged, being killed with one-year-old. In a second moment, we realized a normative study of the organogenesis of male ventral prostate lobe. To this, male fetus with 20 and 24 (E20 - E24) days of gestation, besides newborn with one-day-old (P1), were employed in this study. All tissue fragments were dissected and fixed in methacarn or 4% paraformaldehyde, being processed for paraffin embedding. Following, tissue sections were submitted to biometrical, morphological, stereological, immunohistochemical, immunofluorescence and three dimensional reconstruction analyses. The results showed that only the old males of TG + T group were affected by adenomatous hyperplasia associated with inflammation, although in other male experimental groups we also observed hyperplastic foci in the prostate. The female exposed to testosterone during prenatal phase (TG and TG + groups) have demonstrated a higher heterogeneity of alterations due treatment, being characterized by reproductive system malformations, formation of ectopic prostatic tissue surrounding vaginal wall, besides the presence of prostatic lesions characterized by adenomatous hyperplasic foci. These findings show that abnormal prenatal exposure to testosterone affect the prostate morphogenesis in both male and female, disrupting normal developmental processes and increasing the susceptibility to the development of prostatic diseases during aging. Regarding the prostate organogenesis study, it was observed that the first ventral buds emerge from the ventral urethral epithelium between the 20^{th} and 21^{th} day of prenatal life, reaching the ventral mesenchymal pad (VMP) and initiating the branching process at the first day of postnatal

life. We also noted that the smooth muscle layer seems to have a central play during this process, which may act as physical barrier to the buds heading to VMP. Regarding the nuclear receptors, the androgen presented a high immunomarking at the periurethral mesenchyme, whereas cells with positive marking for estrogen receptor alpha were identified either in the periurethral mesenchyme or in the buds. Finally, the results demonstrate that the prostate organogenesis, besides involving a series of events finely regulated, is an extremely sensitive and crucial to the health of the gland throughout the life.

Keywords: Prostate, gerbil, endocrine disruptors, testosterone, organogenesis.

Sumário

Dedico minha tese a todos os professores, pesquisadores e pessoas do mundo inteiro que lutam por um mundo melhor, mais humano e mais esclarecido.

A minha mãe, Fatima, por todo amor incondicional, amizade, cumplicidade e dedicação para que eu me tornasse a pessoa que sou.

Ao meu orientador Prof. Dr. Sebastião Roberto Taboga pela sua inestimável amizade e por confiar no meu trabalho como pesquisador, tornando possível a minha realização profissional.

Ao meu irmão, João Fernando, por todo amor e amizade desde sempre.

Ao meu amor, Fernanda, pela sua atenção, amizade e por toda sua dedicação em todas as etapas do desenvolvimento desta tese.

A minha avó, Carmem, e a minha tia, Aparecida, por todo carinho e cuidados ao longo de minha vida.

Ao meu tio, Antonio Cabrera, por todo carinho e amizade.

Ao Leontil Sérgio dos Santos, Alcina Alcantara dos Santos, Aline Danielle Alcantara dos Santos e Alexandre Alcantara dos Santos pelos ótimos momentos de convivência e amizade que sempre passamos juntos.

Aos meus amigos, Marcão, Jean, Christian, Teófilo, Verena, Ana Cláudia e Allan, pela amizade e por todos os riquíssimos momentos que passamos juntos.

Agradeço à todos os meus colegas de laboratório, Sérgio Marcelino, Lara, Cristiane, Ana Maria, Renato, Silvana, Ana Paula, Bruno, Cássia, Patrícia Vilamaior, Ricardo, Luiz, Maê, Diego, Mariana Pulégio, Mariana Marcielo, Mariana Zanateli, Patrícia, Filipe, Bianca, Carol, Mateus e Cintia que, de uma forma ou de outra, sempre estiveram dispostos a ajudar no que fosse preciso.

Agradeço à todos os professores e funcionários em geral da Universidade Estadual Paulista, UNESP/Ibilce, onde me graduei e realizei toda parte experimental do meu trabalho de doutorado.

Ao Prof. Dr. Paulo César Ghedini, da Universidade Federal de Goiás, pela amizade e pela atenção no aprendizado de novas técnicas moleculares que serão futuramente empregadas em novos estudos.

Ao Prof. Dr. Athanase Billis, da Universidade Estadual de Campinas, pelo fundamental auxílio com as avaliações histopatológicas do meu material.

Aos professores do departamento de Morfologia da Universidade Federal de Goiás pelos bons momentos de convivência e pelas trocas de experiências.

Ao amigo e técnico do laboratório, Luis Roberto, pela amizade e pela inestimável ajuda e eficiência em todos os momentos necessários.

À Líliam Panagio, secretária do Programa de Pós-Graduação em Biologia Celular e Estrutural, pela amizade, dedicação e admirável profissionalismo.

Aos professores que fizeram parte da minha qualificação de doutorado, Profª. Drª. Luciana Bolsoni Lourenço Morandini, Prof. Dr. Renato Simões Cordeiro e Profª. Drª. Jaqueline de Carvalho Rinaldi, por toda atenção e contribuição destinados a este trabalho de doutorado.

Aos professores que fizeram parte da minha banca de doutorado, Prof. Dr. Sebastião Roberto Taboga, Profª. Drª. Jaqueline de Carvalho Rinaldi, Prof. Dr. Hernandes Faustino de Carvalho, Prof. Dr. Renato Simões Cordeiro, Prof. Dr. Luis Antonio Justulin Junior, Profª. Drª. Silvia Borges Pimentel de Oliveira, Profª. Drª. Taize Machado Augusto e Prof. Dr. Ricardo Alexandre Fochi, por toda atenção e dedicação destinados a este trabalho de doutorado.

Aos professores do departamento de Biologia Celular e Estrutural de Campinas que foram fundamentais para o aperfeiçoamento dos meus conhecimentos neste campo tão vasto da biologia.

À FAPESP e à CAPES/PROEX pelos incentivos através de bolsas e auxílios de pesquisa.

Ao Programa de Pós-Graduação em Biologia Celular e Estrutural pela inestimável oportunidade de realização deste trabalho de doutorado.

À todos aqueles que foram essenciais para que tudo isso pudesse acontecer, mas que no momento cometo a injustiça de não me recordar.

À vida.

Como a essência - ao contrário dos fenômenos - não se manifesta diretamente, e desde que o fundamento oculto das coisas deve ser descoberto mediante uma atividade peculiar, tem de existir a ciência e a filosofia. Se a aparência fenomênica e a essência das coisas coincidissem diretamente, a ciência e a filosofia seriam inúteis.

Karel Kosik, Dialética do Concreto

III.1 Organogênese Prostática

A organogênese prostática, tanto em roedores como em humanos, tem início durante a vida pré-natal. Embora existam diferenças intrínsecas, este evento se apresenta muito conservado entre as diferentes espécies. Em ratos, por exemplo, o completo desenvolvimento prostático envolve cinco estágios sequenciais, que são determinação, iniciação e brotamento, morfogênese de ramificação, diferenciação e maturação (Fig. 1) (Prins e Putz, 2008). A formação de uma próstata adulta normal depende da exata ocorrência destes estágios, de forma que sensíveis interferências nestes eventos podem alterar o desenvolvimento prostático, afetando a formação normal da glândula tanto em termos de estabelecimento da arquitetura glandular como na predisposição para o desenvolvimento de lesões prostáticas (Timms, 2008; Schaeffer et al., 2008; Biancardi et al., 2012).

Uma das principais exigências para o início da organogênese prostática é a presença da testosterona secretada pelos testículos fetais. Em embriões de camundongos, por exemplo, a diferenciação das gônadas ocorre no 13º dia do desenvolvimento embrionário (E13), quando os testículos tornam-se morfologicamente distintos dando início a produção de testosterona (Staack et al., 2003). A presença de andrógenos leva ao início da organogênese prostática, que ocorre a partir do seio urogenital (UGS), uma estrutura embrionária indiferenciada de origem endodérmica, formada por uma parte epitelial (UGE) e por uma parte mesenquimal (UGM). O UGS é encontrado tanto em machos quanto em fêmeas de mamíferos em um estágio indiferenciado aproximadamente no 13º dia do desenvolvimento embrionário e na 7ª semana de gestação em humanos (Wilhelm e Koopman, 2006).

O tecido mesenquimal (UGM) que envolve o epitélio do UGS é formado por três zonas distintas. A mais adjacente ao UGE é chamada de mesênquima periuretral, permanecendo como tecido mesenquimal durante os estágios pré-natais do desenvolvimento. Paralelo a este mesênquima periuretral, situa-se outra zona que se diferenciará em musculatura lisa dando origem ao músculo detrusor da uretra. A terceira

zona do UGM é denominada de pé mesenquimal ventral (VMP), sendo caracterizado por ser um tecido mesenquimal condensado (Thomson et al., 2002) que dará origem ao futuro estroma prostático.

Estudos tem sugerido que o VMP é a parte mesenquimal do UGS responsável pela indução prostática durante o período de desenvolvimento embrionário (Timms et al., 1995; Thomson et al., 2002). No entanto, os mecanismos de indução deste mesênquima, e mesmo do UGM como um todo, ainda não são completamente compreendidos (Cunha, 2008; Thomson et al., 2002).

Durante a organogênese prostática, as células mesenquimais do VMP expressam receptores androgênicos (AR) (Thomson et al., 2002). Embora tenha sido relatada a expressão destes receptores no UGE, tem-se sugerido que o AR epitelial é dispensável para o início da indução do desenvolvimento prostático (Schaeffer et al., 2008). Entretanto, sabe-se que o desenvolvimento da próstata a partir do VMP se dá a partir de interações epitélio-mesenquimais, as quais ocorrem em resposta à ativação dos receptores de esteroides. As células mesenquimais do VMP que expressam AR, por exemplo, sob estímulo androgênico, produzem moléculas de ação parácrina que irão agir no UGE, levando ao início da formação dos brotos prostáticos. Alguns destes reguladores parácrinos, como os fatores de crescimento de fibroblastos (Fgf-7 e Fgf-10) e o fator de crescimento semelhante à insulina (IGF1) já foram identificados (Thomson et al., 2002).

Além destes, existem muitos outros genes morforegulatórios que são expressos durante a organogênese prostática, como, por exemplo, Nkx3.1, Bmp-4, Bmp-7, FOXA1, Hoxb13, Hoxd-13, Hoxa-13, Wnt 2, Wnt 5a, Shh, além de outros (Fig. 1) (Prins e Putz, 2008). Entretanto, ainda não se conhece os principais mecanismos pelos quais o AR controla, direta ou indiretamente, a expressão destes genes reguladores do desenvolvimento prostático (Thomson, 2008).

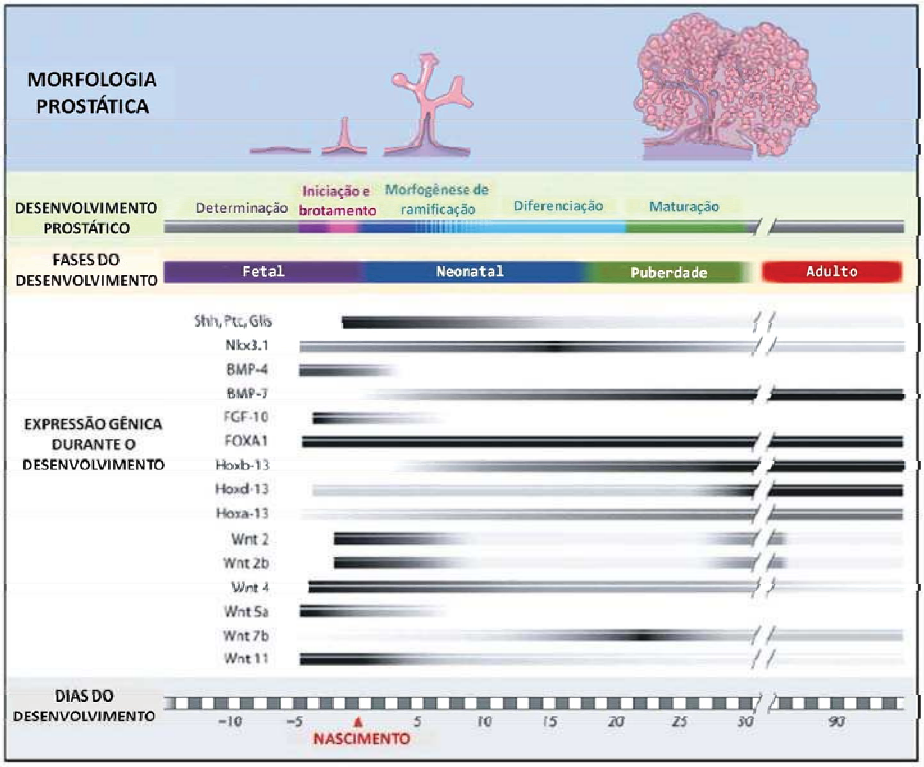


Figura 1. *Desenho esquemático mostrando a expressão de genes morforegulatórios ao longo dos estágios do desenvolvimento da próstata ventral de ratos. Os dias da vida fetal e pós-natal estão representados inferiormente. Observe que a diferenciação celular inicia-se ao final da morfogênese de ramificação, como indicado pelo segmento em azul claro. Os padrões temporais da expressão dos genes morforegulatórios estão representados em barras preto-cinza-branco, as quais mostram os níveis de expressão gênica determinados por reação em cadeia da polimerase (PCR) em tempo real. Adaptado de Prins e Putz (2008).*

Além do AR, estudos tem mostrado que os receptores de estrógenos (ER) também exercem papéis essenciais durante a organogênese prostática, pois os mesmos são diferencialmente expressos durante os estágios do desenvolvimento prostático (McPherson et al., 2008). Como a maioria destes genes estão envolvidos, direta ou indiretamente, com o crescimento, a diferenciação e a manutenção desta glândula, é de suma importância o conhecimento sobre os mecanismos de ação destes fatores, tendo em vista possíveis terapias para o câncer de próstata.

Segundo evidências da literatura, a formação dos brotos prostáticos a partir do UGE de roedores se dá durante os últimos dias da vida embrionária. Em ratos, por exemplo, os primeiros brotos começam a aparecer entre o 17º e 18º dia do desenvolvimento

embrionário. Nesta fase, a musculatura lisa uretral localizada adjacente à zona periuretral do UGM apresenta-se como uma camada descontínua tanto em machos quanto em fêmeas.

Em ratos machos, devido ao ambiente rico em testosterona, esta camada de musculatura lisa permanece descontínua durante o desenvolvimento da próstata, permitindo o fluxo de fatores de ação parácrina do VMP para o epitélio, favorecendo, desta forma, a organogênese prostática completa. No entanto, nas fêmeas, aproximadamente no 20º dia embrionário, devido à ausência de testosterona, esta camada de musculatura lisa não permanece descontínua, fechando-se completamente e impedindo a ação dos fatores parácrinos a partir do VMP, levando a não expansão desta glândula (Thomson, 2008).

III.2 Aspectos morfofisiológicos da próstata masculina e feminina

O termo próstata tem origem na palavra grega 'prohistani', a qual faz referência à sua localização frontal em relação a bexiga urinária (Kirby, 1996). Consta na literatura, que o termo foi empregado em 335 a.C. por Herophilus de Alexandria (Kirby, 1996), um médico grego considerado um dos primeiros anatomistas da história. A principal função desta glândula é produzir uma secreção rica em glicoproteínas, enzimas e íons essenciais para a manutenção de um ambiente adequado à sobrevivência dos espermatozoides, condição determinante para a garantia do sucesso reprodutivo.

Em homens, a próstata apresenta-se como um órgão compactado, não sendo dividida em lobos como no caso de roedores e outros mamíferos (Fig. 2). A classificação mais atual separa, virtualmente, a próstata masculina em quatro zonas distintas, que são: zonas periférica, central e de transição, além do estroma fibromuscular anterior (McNeal, 1981). Já em roedores, salvo a diferença interespecífica, a próstata se apresenta organizada em lobos. Em ratos, por exemplo, a próstata é dividida em lobos ventral, lateral e dorsal, além da glândula coaguladora (próstata anterior) (Timms, 2008). Em gerbilos da Mongólia (*Meriones unguiculatus*), embora exista uma pequena diferença em termos de classificação dos lobos (que são: lobos ventral, dorsolateral, dorsal e glândula coaguladora) (Rochel et al., 2007), em termos gerais a morfologia e os aspectos fisiológicos desta glândula são muito semelhantes aos de outros roedores.

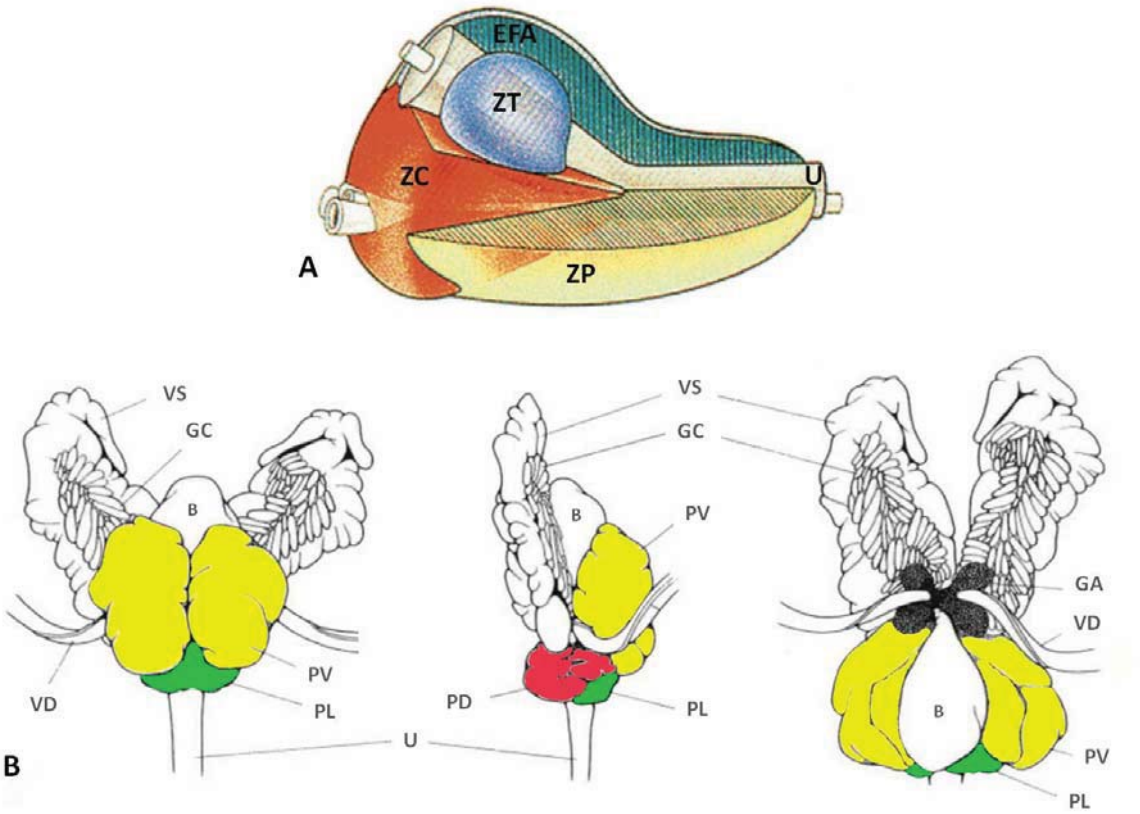


Figura 2. ***(A)*** *Representação tridimensional mostrando a estrutura compacta da próstata masculina humana e sua divisão em zonas. U (uretra), ZT (zona de transição), ZC (zona central), ZP (zona periférica), EFA (estroma fibromuscular anterior).* ***(B)*** *Representação esquemática do complexo prostático de ratos machos adultos mostrando a divisão da próstata em lobos e sua distribuição anatômica. U (uretra), B (bexiga), VS (vesícula seminal), VD (vaso deferente); PV (próstata ventral; em amarelo), PL (próstata lateral; em verde), PD (próstata dorsal; em vermelho), GC (glândula coaguladora), GA (glândula ampular). Figura adaptada de Timms (2008).*

Em relação à arquitetura tecidual, a próstata é caracterizada com base em três compartimentos distintos, que são epitélio, estroma e lúmen. O epitélio prostático, pode variar de simples cuboidal ou cilíndrico à pseudoestratificado, sendo constituído por células epiteliais secretoras, basais e neuroendócrinas. O compartimento estromal é composto, principalmente, de fibroblastos, células musculares lisas, células do sistema imune e àquelas que fazem parte de vasos e nervos, além dos componentes fibrilares (colágenos e fibras elásticas) e não fibrilares (proteoglicanos, glicosaminoglicanos, glicoproteínas e outros elementos da matriz extracelular como água e íons). Por fim, o lúmen é o

compartimento responsável por receber e armazenar as secreções provenientes das células epiteliais secretoras. Embora existam variações morfológicas intrínsecas entre os lobos, a arquitetura tecidual e a composição celular da próstata é muito semelhante entre as diferentes espécies.

Do ponto de vista hormonal, a manutenção da homeostasia prostática está sujeita ao controle de hormônios esteroides, principalmente andrógenos e estrógenos. Estes, por sua vez, são regulados pelo eixo hipotalâmico-hipofisário-gonadal.

Embora o grande volume de conhecimento nesta área tenha origem em estudos realizados com a próstata masculina, esta glândula também apresenta suas particularidades em fêmeas de algumas espécies. Em alguns roedores, por exemplo, uma glândula prostática (historicamente conhecida como glândula de Skene) homóloga à próstata ventro-lateral dos machos pode ser encontrada na base da bexiga e ao redor da uretra destes animais (Mahoney e Witschi, 1947; Price, 1963; Shehata, 1972, 1980; Santos et al., 2006; Biancardi et al., 2010). No entanto, ainda são desconhecidos os detalhes dos mecanismos envolvidos no desenvolvimento desta glândula em fêmeas de diferentes espécies, principalmente em mulheres. Segundo Thomson (2008), a formação da próstata em fêmeas de roedores pode estar relacionada a diversos fatores, como níveis aberrantes de testosterona, aumento da sensibilidade androgênica ou mesmo em decorrência de um programa intrínseco da organogênese.

No que diz respeito à localização anatômica, em fêmeas de roedores como ratos e gerbilos a próstata é lobulada e está situada na base da bexiga e ao lado da uretra (parauretral) (Mahoney e Witschi, 1947; Price, 1963; Shehata, 1972; 1980; Santos et al., 2006; Biancardi et al., 2010). Já em mulheres, diferentemente de roedores como ratos e camundongos, a próstata não apresenta lobulação, estando a mesma localizada na parede da uretra, o que é diferente do observado em homens, nos quais a esta glândula envolve a porção correspondente da uretra (Zaviačič, 1999). Em relação à composição da secreção prostática, estudos mais atuais têm mostrado que a secreção prostática de mulheres tem uma composição bioquímica muito semelhante àquela encontrada na secreção da próstata de homens (Wimpissinger, 2007; 2009). No entanto, novos estudos se fazem necessários no sentido se obter evidências mais detalhadas sobre a função que esta secreção prostática tem para a biologia reprodutiva das fêmeas.

Quanto à morfologia glandular, diversos estudos vem demonstrando que a próstata feminina de roedores tem os mesmos componentes teciduais encontrados nas glândulas de machos, com exceção de algumas diferenças intrínsecas de cada espécie (Shehata, 1972; 1975; 1980; Santos et al., 2006; Biancardi et al., 2010). Uma particularidade das próstatas de fêmeas de alguns roedores, como o gerbilo e o rato, é que esta glândula muito se assemelha, histologicamente, ao lobo prostático ventral dos machos da mesma espécie (Santos et al., 2006; Biancardi et al., 2010). No entanto, não se conhece os motivos desta particularidade e também o porque das fêmeas não desenvolverem, em condições normais, lobos prostáticos iguais aos lobos dorsais ou anteriores de machos.

Dessa forma, estes aspectos tem chamado bastante a atenção do nosso grupo de pesquisa em relação à estas questões. Além disso, características peculiares como a alta frequência (90%) da próstata em fêmeas do gerbilo, o que se assemelha ao observado em mulheres (Zaviačič, 1999), tem proporcionado o desenvolvimento de projetos de pesquisa que visam avaliar diferentes aspectos morfofisiológicos e moleculares desta glândula.

III.3 Características dos andrógenos e seus efeitos sobre a próstata

Os andrógenos são hormônios esteroides derivados a partir da oxidação do colesterol, um tipo de esterol com estrutura molecular contendo quatro anéis fundidos, sendo três com seis carbonos e um com cinco (Fig. 3).

A síntese destes andrógenos está sob controle do hormônio luteinizante (LH) e do hormônio folículo estimulante (FSH), os quais são requeridos para o desenvolvimento e a manutenção das funções testiculares, local de maior produção de andrógenos pelas células de Leydig (Nieschlag e Behre, 1998). No entanto, embora outros fatores também estejam envolvidos na manutenção da síntese de andrógenos, o LH é o hormônio mais importante, pois controla diretamente as funções das células de Leydig.

Dentre os andrógenos existentes, a testosterona (Fig. 3) é o principal hormônio sexual masculino produzido pelos testículos (~ 95%) (Nieschlag e Behre, 1998). A testosterona tem uma estrutura molecular contendo quatro anéis fundidos, sendo três com seis carbonos e um com cinco, além de um grupamento OH ligado ao carbono 1 do anel com cinco carbonos, aspecto que confere um caráter alcoólico à este andrógeno (Fig. 3).

Em homens adultos, cerca de 6-7 mg de testosterona é produzida pelos testículos diariamente. Embora a produção da testosterona esteja centrada nos testículos, outros locais como o córtex das adrenais também auxiliam na síntese deste andrógeno, porém em menor proporção (Nieschlag and Behre, 1998).

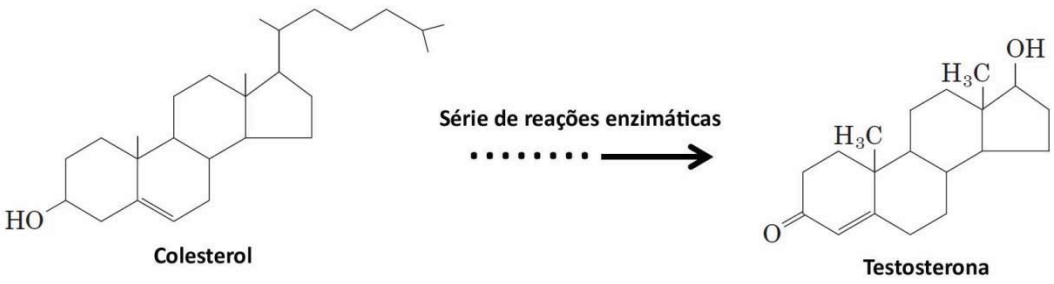


Figura 3. *Esquema simplificado mostrando a síntese da testosterona a partir do colesterol e as principais alterações, a nível de estrutura molecular, ocorridas no processo. Imagem adaptada de Nelson e Cox (2000).*

A testosterona, por sua vez, pode ser alterada sinteticamente gerando outros tipos de esteroides de interesse farmacológico. O cipionato de testosterona (Fig. 4), por exemplo, é uma andrógeno sintético obtido através da esterificação da testosterona. A esterificação confere à molécula uma maior solubilidade lipídica, retardando sua liberação na corrente sanguínea e, consequentemente, prolongando sua ação no organismo (Cunha et al., 2004).

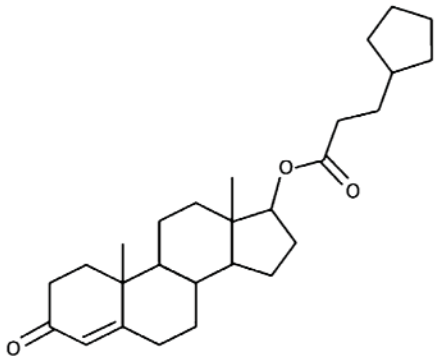


Figura 4. *Fórmula estrutural do cipionato de testosterona. Imagem adaptada de Meireles et al. (2013).*

A testosterona tem diferentes destinos dentro do organismo, sendo que dentre as diferentes possibilidades de metabolização, destacam-se duas vias: a sua conversão em diidrotestosterona (DHT) ou em estradiol. Ao entrar em tecidos alvo como a próstata, por exemplo, este andrógeno é convertido em DHT, reação catalisada pela enzima 5α-redutase (Wilson, 2011). A DHT é um andrógeno muito mais potente que a testosterona em termos de afinidade por receptores específicos de andrógenos. Esta peculiaridade faz com que a eficácia da testosterona seja aumentada significativamente, potencializando a ação androgênica sobre tecidos alvo como a próstata. Além deste destino, a testosterona também pode ser aromatizada em estradiol pela enzima aromatase (p450) (Fig. 5), um estrógeno responsável por mediar importantes funções na próstata (Wilson, 2011).

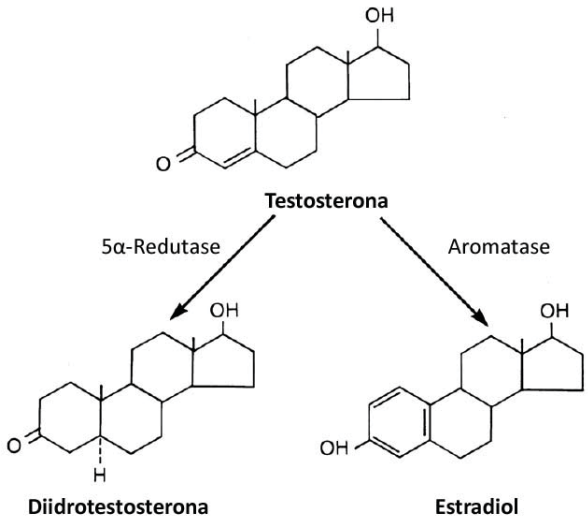


Figura 5. *Esquema simplificado mostrando a conversão da testosterona em outros hormônios esteroides. Imagem adaptada de Wilson (2011).*

Esteroides como a testosterona e a DHT agem na próstata via interação com receptores de andrógenos (AR). Os AR são fatores de transcrição que quando ativados no citoplasma pela ligação ao hormônio correspondente são translocados para o interior do núcleo, ativando a expressão de genes alvo. A expressão destes genes é espacialmente e temporalmente regulada, variando de acordo com as fases do desenvolvimento em que a glândula se encontra (Prins e Putz, 2008). Segundo Black e Paschal (2004), o AR é responsável por regular a expressão de mais de 100 genes na próstata, evidência que comprova a importância do controle androgênico sobre esta glândula.

Durante as fases iniciais do desenvolvimento prostático embrionário, o AR está expresso quase que exclusivamente no UGM (Takeda et al., 1985; Cunha, 2008). As células mesenquimais que expressam AR respondem ao estímulo androgênico produzindo fatores de ação parácrina que irão agir no UGE, induzindo a proliferação e a diferenciação das células epiteliais (Prins e Putz, 2008). Estas evidências mostram que os ARs das células mesenquimais estão diretamente envolvidos com as fases iniciais do desenvolvimento prostático (Cunha et al., 2003). Posteriormente, em fases mais adiantadas, os ARs começam a ser expressos nas células epiteliais, sendo responsáveis por fornecer as sinalizações para promover a diferenciação de células mesenquimais, como, por exemplo, as que irão se diferenciar em células musculares lisas (Prins e Putz, 2008).

Sabe-se que a testosterona é fundamental para a biologia prostática, sendo diretamente responsável pelo desenvolvimento, maturação e manutenção morfofuncional desta glândula (Wilson, 2011). Além disso, os andrógenos estão diretamente relacionados com o surgimento de doenças prostáticas como hiperplasias e o câncer de próstata. Embora exista uma rica literatura sobre assunto, muitos mecanismos de ação destes hormônios em patologias desta glândula ainda são desconhecidos (Nicholson e Ricke, 2011), particularmente no que diz respeito à exposição androgênica durante a vida intrauterina.

III.4 Características dos disruptores endócrinos

Químicos de disrupção endócrina (EDCs) são substâncias químicas exógenas que podem mimetizar hormônios esteroides, alterando o sistema endócrino de forma a afetar diretamente o metabolismo de hormônios no organismo (Bigsby et al., 1999; Toppari, 2008). Tendo em vista o seu potencial hormonal, estas substâncias podem agir diretamente sobre órgãos do trato reprodutivo, tanto de machos quanto de fêmeas, principalmente por competir com esteroides endógenos por sítios de ligação em receptores específicos nas células-alvo.

Algumas destas substâncias agem como disruptores androgênicos e outras como estrogênicos. Muitos destes EDCs já foram estudados e relatados na literatura. Alguns deles, como o disruptor estrogênico dietilestilbestrol (DES), são responsáveis por causar diversas alterações em mulheres expostas a estas substâncias durante a vida pré-natal (Prins

et al., 2008). Estas mulheres apresentaram displasia epitelial na vagina superior além de um alto risco em desenvolver adenocarcinoma no colo do útero e da vagina durante a vida adulta (Soder, 2005).

Embora a literatura científica relacionada aos disruptores estrogênicos seja vasta, muito pouco se conhece sobre os efeitos de disruptores com potencial androgênico. Estudos realizados por nosso grupo de pesquisa mostraram que o emprego de testosterona sintética pode ser um bom modelo de enfoque experimental para se avaliar os efeitos destas substâncias sobre a próstata (Perez et al., 2011; Biancardi et al., 2012).

O acetato de trembolona, por exemplo, que é um disruptor com potencial androgênico, vem sendo muito utilizado para promover o rápido crescimento do gado (Hotchkiss et al., 2007a; b), além de ser usado como esteroide anabolizante por humanos. Outros disruptores, entretanto, são provenientes de pesticidas e outras substâncias químicas, sendo responsáveis por contaminar o meio ambiente (Orlando et al., 2004). Tendo em vista que o período relacionado ao desenvolvimento prostático é extremamente sensível a pequenas interferências, torna-se cada vez mais preocupante os efeitos que estes disruptores androgênicos podem ter sobre a organogênese prostática, visto que esta glândula carrega um potencial muito grande para desenvolver doenças na vida adulta e senil.

Como a próstata é altamente dependente de hormônios esteroides como andrógenos e estrógenos, é de fundamental importância se entender os possíveis mecanismos de ação destas substâncias nesta glândula. Durante o período embrionário, em especial, a ação destas substâncias pode ser responsável por causar um *imprint* irreversível em alguns tecidos que constituem o seio urogenital (UGS), responsável pela formação de órgãos do sistema reprodutor tanto nos machos quanto nas fêmeas. Este *imprint* pode ser mantido ao longo do desenvolvimento, podendo manifestar-se mais tardiamente em algum momento da vida e favorecer o desenvolvimento de doenças.

III.5 Aspectos do comportamento reprodutivo no gerbilo da Mongólia (*Meriones unguiculatus*).

Em termos taxonômicos, o gerbilo da Mongólia (*Meriones unguiculatus*) pertence a família Muridae e a subfamília Gerbillinae. Embora existam outras espécies do mesmo

gênero, muitas pesquisas na área biomédica tem empregado o *Meriones unguiculatus* como modelo experimental. No entanto, o gerbilo é uma espécie de roedor que apresenta algumas peculiaridades no que tange o seu comportamento reprodutivo. A fêmea desta espécie apresenta um ciclo estral que varia de 4 a 6 dias (Barfield e Beeman, 1968) e um período gestacional entre 24 e 26 dias (Adams e Norris, 1973). O ciclo estral compreende 4 fases diferentes (proestro, estro I, estro II, metaestro e diestro) (Almeida et al., 2001), que podem ser observadas através da técnica de espalhamento vaginal. Dentre estas fases, o estro (cio) é a fase na qual a fêmea se torna receptiva ao macho, sendo caracterizada pela presença, predominantemente, de células corneificadas anucleadas (Almeida et al., 2001; Nishino e Totsukawa, 1996).

Em relação ao comportamento reprodutivo, o gerbilo (*Meriones unguiculatus*) apresenta algumas diferenças em relação a outros roedores. Em ratos, por exemplo, a determinação do dia da cópula pode ser facilmente obtida pela presença do tampão (plug) vaginal que se forma após a cópula, ou mesmo pela visualização de espermatozoides no espalhamento vaginal. Assim, para ratos, a união de uma fêmea no cio (fase de estro) com um macho sexualmente maduro, normalmente resulta em cruzamento, o que proporciona uma garantida e relativa facilidade de aplicação desta técnica em estudos que dependem desta abordagem metodológica.

No entanto, quando se trata de gerbilos, a metodologia aplicada para se determinar o dia da cópula entre a fêmea e o macho apresenta algumas diferenças particulares. Diferentemente do que ocorre com ratos, a formação dos casais de gerbilos não é garantia de que haverá cruzamento, mesmo quando as fêmeas estão na fase de estro (cio) e os machos se encontram sexualmente maduros.

Alguns trabalhos da literatura (Adams e Norris, 1973; Wu, 1974; Norris e Adams, 1981) já haviam reportado estas particularidades no comportamento reprodutivo destes roedores, principalmente no que diz respeito à regularidade do ciclo estral como fator determinante para o sucesso do cruzamento. Segundo Norris e Adams (1972), em um estudo feito com 224 fêmeas de gerbilos, o tempo médio, a contar da data da montagem dos casais até o nascimento da primeira cria, foi de 3 meses, podendo variar de 24 à 250 dias. Estudos recentes feitos em nosso laboratório mostraram que as fêmeas de gerbilo tem níveis sorológicos elevados de testosterona em todas as fases do ciclo estral (Fochi et al.,

2008), que é uma característica fisiológica que tem sido associada ao comportamento dominante das fêmeas desta espécie. Estes achados sugerem que o comportamento dominante pode ser o motivo das fêmeas desta espécie não serem regulares quanto ao comportamento de cópula, tendo em vista que elas seriam altamente seletivas quanto à escolha sexual do parceiro.

Outro aspecto diferencial do gerbilo é que o tampão vaginal que se forma após a cópula fica situado internamente, próximo à bifurcação do útero, o que impede a visualização externa desta estrutura (Norris e Adams, 1981). Portanto, estas particularidades do comportamento reprodutivo do gerbilo exigem metodologias diferenciadas para a detecção exata do dia do acasalamento.

Para tal, existe outra abordagem para se determinar de forma mais eficiente e regular o dia exato do cruzamento nesta espécie. De acordo com Norris e Adams (1981), a fêmea do gerbilo se torna altamente receptiva ao macho dentro de um período de 24 horas após o parto, favorecendo o cruzamento. Um estudo feito com 235 fêmeas maduras de gerbilo (*Meriones unguiculatus*) mostrou que o parto ocorre, majoritariamente, no final da tarde e início da noite, sendo seguido por uma onda de calor que caracteriza um período de alta fertilidade por parte da fêmea. Espalhamentos vaginais feitos no mesmo dia de nascimento dos filhotes e no dia seguinte ao nascimento revelaram a presença de espermatozoides, confirmando o cruzamento. Segundo Adams e Norris (1973), para que a fêmea não atrase a gestação em decorrência da sobreposição das ninhadas, a retirada da cria logo após o nascimento não interfere na duração da gestação da próxima geração. Desta forma, este tipo de abordagem pode ser empregado de forma eficiente nos estudos que necessitam de precisão sobre o conhecimento do dia exato de acasalamento.

III.6 Justificativas e hipótese

Em um trabalho de 1995, William A. Gardner, professor do departamento de patologia da Universidade do sul do Alabama, formulou a seguinte hipótese:

"*As origens das doenças prostáticas, incluindo o carcinoma, podem ser encontradas durante a vida intrauterina em decorrência de influências sobre o desenvolvimento da próstata*."

Neste mesmo trabalho, Gardner (1995) expõe aspectos fundamentais sobre a importância dos momentos iniciais do desenvolvimento e sua implicação com o surgimento de doenças na vida adulta. Influências hormonais maternas, dieta, dentre outros, podem influenciar diretamente o destino da próstata em termos de predisposição para o desenvolvimento de lesões (Gardner, 1995). Este trabalho mostra que há muito tempo atrás já podiam ser encontradas evidências sobre a origem pré-natal de certas doenças da próstata.

Mais recentemente, uma série de publicações (Singh e Handelsman, 1999; Prins et al., 2008; Biancardi et al., 2012) tem demonstrado que os momentos iniciais do desenvolvimento prostático são muitos sensíveis ao desequilíbrio hormonal causado por diferentes agentes hormonais de origem exógena ou endógena. Desta forma, alterações sobre a homeostasia do desenvolvimento podem levar a um quadro irreversível no qual a glândula apresentará uma maior suscetibilidade para o desenvolvimento de lesões durante a vida adulta ou senil.

Além disso, um importante estudo feito em camundongos mostrou que o programa de expressão gênica induzido por andrógenos, e que é ativado durante o desenvolvimento inicial da próstata, relembra o programa que é ativado durante a instalação do câncer de próstata humano (Schaeffer et al., 2008). Estas evidências abrem novas possibilidades para a introdução de novos modelos experimentais tratáveis para se investigar o papel destes genes envolvidos com o câncer de próstata (Schaeffer et al., 2008).

Tendo em vista os aspectos apresentados até aqui, o presente estudo se baseou em uma questão principal: como criar situações que simulassem possíveis alterações hormonais durante o desenvolvimento da próstata do gerbilo da Mongólia? Sabendo que a testosterona é um dos principais hormônios esteroides que agem durante o desenvolvimento da glândula, nossa hipótese se baseou na afirmação de que a exposição anormal à testosterona durante o desenvolvimento pode ser um dos fatores responsáveis pelo aumento da predisposição à doenças prostáticas ao longo da vida.

O presente trabalho teve como objetivos:

Avaliar os efeitos da exposição intrauterina e puberal ao cipionato de testosterona sobre a morfofisiologia da próstata de machos e fêmeas de gerbilos senis.

Avaliar o padrão da organogênese do lobo prostático ventral de machos do gerbilo da Mongólia (*Meriones unguiculatus*) durante a vida pré-natal até o primeiro dia de nascimento.

O presente trabalho deu origem a três artigos científicos, os quais estão apresentados a seguir:

Artigo 1. Prenatal exposure to testosterone masculinizes female gerbil and promotes the development of lesions in the prostate (Skene's gland).

Artigo 2. Effects of prenatal and pubertal testosterone exposure on the ventral prostatic lobe of old male gerbils.

Artigo 3. Budding process during the organogenesis of the ventral prostate lobe in Mongolian gerbil.

V.1 Prenatal exposure to testosterone masculinizes the female gerbil and promotes the development of lesions in the prostate (Skene's gland)

Manoel F. Biancardi[1], Ana P. S. Perez[1], Cássia Regina Suzuki Caires[2], Rejane M. Góes[2], Patrícia S. L.Vilamaior[2], Fernanda C. A. Santos[3], Sebastião R. Taboga[1,2]

Abridged title: Prostate disruption by testosterone exposure

[1]*Department of Structural and Functional Biology, State University of Campinas, Av. Bertrand Russel s/n, Campinas, São Paulo, 13084864, Brazil.*
[2]*Univ. Estadual Paulista – UNESP*, *Department of Biology, Laboratory of Microscopy and Microanalysis, Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo, 15054000, Brazil.*
[3]*Department of Morphology, Federal University of Goiás,* Samambaia II, *Goiânia, Goiás, 74001970, Brazil.*

Corresponding author.
Dr. Sebastião R. Taboga: *Department of Biology, Laboratory of Microscopy and Microanalysis, São Paulo State University, 2265 Cristóvão Colombo Street, Jardim Nazareth, São José do Rio Preto, São Paulo, 15054-000, Brazil. E-mail address: taboga@ibilce.unesp.br.* Tel: +55 17 32212386; Fax: +55 17 32212390.

Author Contributions

The work presented here was carried out as collaboration. All of the authors (MFB, APSP, CRSC, RMG, PSLV, FCAS, and SRT) participated in the design, interpretation of the results, and review of the manuscript. MFB and APSP performed the experiments. MFB wrote the manuscript. APSP, CRSC, RMG, PSLV, FCAS, and SRT equally contributed to the supervision of this work.

Abstract

Androgenic imbalance may disrupt prostate development, leading to morphological alterations in adulthood and predisposing this gland to develop diseases during aging. However, little is known about the endocrine disruption of the prostate that is caused by androgenic compounds, especially in female experimental models. Therefore, this study aimed to evaluate the prostates of aged female gerbils exposed to testosterone at certain periods in intrauterine and postnatal life, to determine whether exposure at a particular age increases susceptibility to prostatic lesions in these animals. To this end, we employed morphological, stereological, immunohistochemical, and immunofluorescence analyses. We observed that females exposed to testosterone during intrauterine life were masculinized, showing increased anogenital distance, absence of the vaginal opening, and ectopic development of prostatic tissue. We also observed several areas of adenomatous hyperplasia, generally associated with inflammatory foci, mainly located in the ectopic prostatic tissue around the vaginal wall. In conclusion, the results showed that abnormal prenatal exposure to testosterone severely affect the reproductive systems of female animals by disrupting normal prostate morphogenesis and increasing the susceptibility to the development of prostatic diseases during aging.

Additional Keywords: endocrine-disrupting chemicals, androgens

1. Introduction

Prostate morphogenesis is a process highly dependent on androgenic action during both the prenatal and postnatal periods. Steroids such as testosterone, the principal androgen secreted by the fetal and adult testes, play a central role during prostate formation (Wilson 2011). Early events in prostate development are very sensitive to testosterone imbalance, since this androgen, acting through its receptor (AR), is responsible for the expression of more than a hundred genes (Black and Pascal 2004). The first manifestation of the presence of ARs appears during the prenatal period, when testosterone produced by the Leydig cells acts in the periurethral mesenchyme of the urogenital sinus (UGS) (Takeda *et al.* 1985), leading to a cascade of paracrine signaling from the mesenchymal cells to the epithelial compartment.

The UGS is an undifferentiated embryonic structure with an endodermic origin (Staack *et al.* 2003). It is found in male and female mammals of several species during an undifferentiated stage of embryonic life; in humans, this stage occurs during the 7th week of gestation (Wilhelm and Koopman 2006). The UGS is in charge of prostate development, a process that begins with determination of the cells, followed by initiation and budding, branching morphogenesis, differentiation, and maturation of the gland (Prins and Putz 2008). Together, these events are responsible for normal prostate morphogenesis. The process of prostate formation encompasses complex cascades of signaling (Meeks and Schaeffer 2011; Thomson 2008; Timms 2008; Wilson 2011) that are precisely regulated spatially and temporally, so that certain kinds of interference with this homeostasis may be detrimental to normal prostate organogenesis, predisposing the gland to develop lesions with aging (Biancardi *et al.* 2012; Timms and Hofkamp 2011; Schaeffer *et al.* 2008).

Lately, a growing body of evidence has shown a close relation between abnormal processes in prostate development and susceptibility to the development of prostate disorders during aging (Biancardi *et al.* 2012; Perez *et al.* 2011; Schaeffer *et al.* 2008; Timms and Hofkamp 2011). Taken together, this evidence has drawn researchers' attention to the initial stages of prostate development, considering that this period of life has a profound effect on the formation of this gland. Furthermore, a better understanding of these events may be helpful since the early events of prostate organogenesis are very similar to events seen in disease development in adult or aged patients (Schaeffer *et al.* 2008; Timms and Hofkamp 2011).

Although several studies have shown the influence of endocrine-disrupting chemicals (EDCs) with estrogenic potential on the reproductive system (Perez *et al.* 2011; Prins *et al.* 2008; Söder 2005; Timms *et al.* 2005), little is known about the roles of EDCs with regard to androgenic

potential and its mechanisms of action on the reproductive tract, and specifically on the prostate (Biancardi *et al.* 2012; Perez *et al.* 2012). Besides, several conditions such as polycystic ovary syndrome (PCOS), adrenal hyperplasia, and exposure to certain drugs may increase the serological concentrations of androgenic compounds, which may be harmful during critical periods of prenatal development. This changed hormonal environment may cause irreversible interference during prostate development, increasing the likelihood that the individual will develop prostatic lesions as an adult or aged person (Biancardi *et al.* 2012; Schaeffer *et al.* 2008).

Female gerbils (*Meriones unguiculatus*) have been employed as an experimental model with increasing frequency lately, mainly because most of them develop a functional prostate (Skene's gland) (Santos *et al.* 2006; Santos and Taboga 2006) similar to that found in humans (Zaviačič 1999). In addition, these females furnish valuable data in experiments concerning drug administration (Biancardi *et al.* 2012; Perez *et al.* 2011; Zanatelli *et al*. 2013). These factors have alerted us to the usefulness of the female gerbil as a model for the early stages of prostate development, since new evidence regarding these stages may be very helpful to our understanding of the complex events that regulate both normal and abnormal prostate growth. Based on this evidence, and considering that normal prostatic morphogenesis is highly dependent on precise androgenic regulation, our hypothesis was that abnormal exposure to testosterone would affect prostate development in females, predisposing them to developing lesions in old age. Thus the aim of this study was to evaluate the interferential effects of exogenous testosterone exposure during the prenatal and pubertal periods on the morphophysiology of the prostate in aged female gerbils.

2. Materials and Methods

2.1 Animals and experimental design

The animals were provided by the São Paulo State University (UNESP) (São José do Rio Preto), maintained in polyethylene cages under controlled conditions of light and temperature, and provided with filtered water and rodent food *ad libitum*. Animal handling and experiments were performed according to the ethical guidelines of the São Paulo State University (UNESP) (ethical committee number 021/09 CEUA) and in keeping with the Guide for Care and Use of Laboratory Animals. During all experiments we provided filtered water in glass bottles to avoid exposing the animals to endocrine-disrupting chemicals such as bisphenol A from plastic bottles.

We selected 20 adult (3–4 months old) female and 20 adult male gerbils (*Meriones unguiculatus*, Muridae: Gerbillinae) for mating. We paired each male with one female at random to

form independent families. We assigned five couples to each group. The pregnant females of these couples underwent different manipulations and their offspring formed the experimental groups as follows: C (control) group: offspring from non-treated pregnant females; C + T (testosterone during puberty) group: offspring from non-manipulated pregnant females (littermates of the C group) treated with subcutaneous injections of 100 µg of T (testosterone cypionate: Deposteron, EMS, Hortolândia, São Paulo, Brazil) diluted in 100 µl of mineral oil during the 6th, 7th, and 8th weeks of life; TG (testosterone during gestation) group: offspring from mothers exposed to subcutaneous injections of 500 µg of T during gestation; and TG + T (testosterone during gestation plus puberty) group: offspring from mothers exposed to subcutaneous injections of 500 µg of T during gestation that were also exposed to subcutaneous injections of 100 µg of T during the 6th, 7th, and 8th weeks of life. Of the pups exposed to prenatal testosterone, only those born four days after exposure were employed in this study. All animals utilized in this study were killed at one year of age. The protocol of T treatment was adapted from that of Wolf *et al*. (2002). The overall experiment design, including details of T treatment and the age at which the animals were killed, is shown in Figure 1.

All animals were killed by CO_2 inhalation followed by decapitation. According to the method of Nishino and Totsukawa (1996), we killed the females in the C and C + T groups at the proestrus phase. The females in the TG and TG + T groups never cycled due to their lack of a vaginal opening. Body, prostatic complex (PrC - urethra, vagina, and prostate structures), ovaries, and adrenals were weighed. These fragments were dissected out using a Leica stereoscopic microscope (Leica, Wetzlar, Germany) to remove adipose tissues and isolate the urethral segment plus the associated prostatic tissue. The anogenital distance (AGD) was measured with a digital caliper rule. For the characterization and confirmation of the female prostate lesions we consulted a pathologist, that helped us with the pathological diagnoses of the prostate disorders.

2.2 Light microscopy

PrC from female gerbils were fixed by immersion in 4% paraformaldehyde (buffered in 0.1 M phosphate, pH 7.2) or in methacarn (proportions: methanol 60%, chloroform 30% and acetic acid 10%) for three hours. After fixation, the tissue were washed in water, dehydrated in ethanol, clarified in xylene and embedded in paraffin (Histosec, Merck, Darmstadt, Germany). All tissue fragments employed in this study were serially sectioned into 5 µm slices with an automatic rotator microtome (Leica RM2155, Nussloch, Germany). The sections were stained with hematoxylin-eosin (HE) and picrosirius for general morphological analysis. Prostatic reticular fibers and elastic fibers were identified, respectively, by the Gömöri's reticulin and resorcin-fuchsin techniques. The

secretory status was checked by Periodic Acid-Shiff (PAS) reaction. The specimens were analyzed with an Olympus BX60 light microscope (Olympus, Japan), and the images were digitalized using DP-BSW software v3.1 (Olympus, Japan) and a virtual slide system BX 61VS (Olympus, Tokyo, Japan).

2.3 Stereology

The stereological analyses were carried out using Weibel's multipurpose graticulate with 130 points and 10 test lines (Weibel 1963) to compare the relative proportion (relative volume) of each component of prostatic tissue (epithelium, lumen and muscle and non-muscle stroma), as described by Huttunen *et al.* (1981). We chose thirty microscopic fields at random from each experimental group (6 fields per animal; n = 5). Briefly, we determined the relative values by counting the coincident points in the test grid and dividing them by the total number of points. Stereological analysis was performed using Image-Pro Plus software v6.1 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA).

2.4 Immunohistochemistry

Tissue sections were subjected to immunohistochemistry for the detection of androgen receptor (AR), as described in protocols applied to the prostate (adapted from Cordeiro *et al*. 2008), estrogen receptor-alpha (ERα), and p63 protein. Primary antibodies reactive to AR (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER-α (rabbit polyclonal IgG, MC-20, sc-542, Santa Cruz Biotechnology), and p63 (mouse monoclonal IgG_{2a}, 4A4, sc-8431, Santa Cruz Biotechnology) were employed at a dilution of 1:100. Polymers (Post Primary Block and Polymer, Novocastra™, RE7260-K, Newcastle Upon Tyne, UK; DAKO Envision™ + Dual link system-HRP, K4061; DAKO, North America, Inc., Carpinteria, California, USA) were used as secondary antibodies, according to the procedures described by the manufacturers. The sections were stained with diaminobenzidine and counterstained with Harris's hematoxylin. The histological sections were analyzed using an Olympus BX60 light microscope (Olympus, Japan).

2.5 Immunofluorescence

Tissue sections were subjected to immunofluorescence for the detection of smooth muscle α-actin by means of incubation with mouse monoclonal IgG_{2a} (sc-32251, IA4, Santa Cruz Biotechnology) at a dilution of 1:100 overnight. The next morning, fluorochrome-conjugated specific second antibodies (anti-mouse, sc-2010, IgG-FITC, Santa Cruz Biotechnology) were used as secondary antibodies over two hours at room temperature. DAPI (4',6-diamidino-2-phenylindol) was employed to allow the visualization of the cells' nuclei. The histological sections were analyzed with a Zeiss Imager M2 fluorescence microscope (Zeiss, Göttingen, Germany), and Laser Scanning microscope (LSM 710, Zeiss, Jena, Germany).

2.6 Statistical analyses

The hypothesis tests employed to determine statistical significance were the Kruskal-Wallis test for non-parametric distributions and ANOVA for parametric distributions. Further localization of the statistically significant differences between experimental groups was performed using Student-Newman-Keuls's test for non-parametric distributions and Tukey's test for parametric distributions. The data were analyzed using Statistica 6.0 (StarSoft, Inc., Tulsa, OK, USA) and BioEstat 5.0 (*free statistical program*) software. The level of significance was set at 5% ($P \leq 0.05$). Values are presented as mean ± standard error of mean (SEM).

3. Results

3.1 Biometry

Both the TG group and the TG + T group had enlarged PrCs. The TG group exhibited a mean PrC weight significantly different from those of the C and C + T groups, whereas the TG + T group exhibited a mean PrC weight significantly different from that of the C group only (Table 1). In addition, total body weight and the anogenital distance (AGD) were significantly increased in the TG and TG + T groups when compared with the C and C + T groups. The others variables studied were not significantly different between the experimental groups.

3.2 Stereology

The epithelial relative volume was decreased in the prostates of C + T females (Fig. 2A). The luminal relative volume, on the other hand, was decreased in the prostates of the TG and TG + T groups, although the statistical analysis revealed that only the TG group was statistically significantly different from the others (Fig. 2B). The muscle relative volume was expressively increased in the prostates of TG and TG + T females compared with C and C + T (Fig. 2C). We did not observe any statistically significant difference in non-muscle stromal compartments between the groups (Fig. 2D).

3.3 General anatomical aspects and morphology of the PrC

In addition to the absence of a vaginal opening (data not shown) and an increase in the AGD, we also observed hydrometrocolpos in the PrC of the TG and TG + T groups. In such cases, the PrC contained a large vaginal cavity filled with an inflammatory secretion inside the uterus and vagina (Figs. 3C, D).

The prostates of the C and C + T groups showed normal anatomical localization, being located in a lateral position relative to the urethra (Figs. 3A, B). In TG and TG + T females, on the other hand, in addition to the normal paraurethral glands, the urogenital tract exhibited ectopic formation of prostate tissue surrounding the vaginal wall (Figs. 3C, D). We could observe a distinct difference between the glands located in the normal paraurethral positions and the ectopic glands located around the vaginal wall (Figs. 3A–D). The prostates of all experimental groups showed glands with large paraurethral acini, flattened epithelia and normal stromal compartments (Figs. 3A - D). In TG and TG + T, however, the ectopic glands surrounding the vaginal wall had distinct histological aspects (Figs. 3C, D). These glands presented ectopic acini, normally composed by a multilayered epithelium, and an unusually thick stromal compartment (Figs. 3C, D). Moreover, we observed the formation of abnormal prostate tissue above the urethra in females from the TG and TG + T groups (Figs. 3C, D).

3.4 Morphological aspects of the prostate and the association with lesions

Prostates in the C and C + T groups showed normal histological aspects, being characterized by acini composed of a simple columnar epithelium, rich in secretions, and surrounded by a stromal compartment composed mainly of fibroblasts, smooth muscle cells and

fibers such as collagen and elastic (Figs. 4A–H). In addition, we also found some foci of prostatic disorders such as hyperplasia in the glands of the C and C + T groups (Figs. 4C, G). On the other hand, in addition to the normal paraurethral glands, the urogenital tracts of the TG and TG + T groups presented ectopic glands severely affected by prostatic lesions characterized by adenomatous hyperplasia (Figs. 4I–O). These glands showed abnormal acinar architecture, normally with a invasive aspect, atypical cell nuclei, and generally associated with several inflammatory foci (Figs. 4I–O). Along with these aspects, prostates in the TG and TG + T groups presented increased stromal compartments and greatly increased fibromuscle compartments (Figs. 3C, D; 4I, M). All experimental groups contained prostates with normal secretory function, as determined according to the PAS reaction (Fig. 4D, H, L, O).

The C and C + T groups showed normal reticular, general collagen, and elastic fiber components of the stromal compartments (Figs. 5A, B, E, F, I, J). The TG and TG + T groups, on the other hand, showed abnormal stromal compartments, mainly in the form of glands with ectopic localization around the vaginal wall. Gömöri's reticulin technique showed an intense stromal reshuffling of collagen III around the affected acini, with some regions lacking these elements, mainly in invasive acini (Figs. 5C, D). The general collagen, in addition to an intense architectural reshuffling, had undergone a considerable increase in the stromal compartment (Figs. 5G, H). We also observed an abnormal increase in the thickness of the elastic components surrounding the acini in the TG and TG + T prostates (Figs. 5K, L) in comparison with prostates in the C and C + T groups (Figs. 5I, J).

3.5 Immunohistochemical analyses

AR identification was similar for all experimental groups, showing up as an intense mark in both the epithelial and stromal compartments (Figs. 6A–D). AR immunolocalization was strong in the nucleus for both epithelial and stromal cells (Fig. 6A–D). Some foci of prostatic lesions appeared as multilayered epithelia in which most cells positively expressed AR (Figs. 6C, D).

Regarding ERα immunolocalization, we observed these receptors to have similar localizations in the prostatic stromal compartment in all experimental groups (Figs. 6E–H). We did, however, observe an abnormal immunolocalization of p63 proteins (Figs. 6I–L) in some regions of the prostate in the TG and TG + T groups, mainly in the acini that had developed ectopically around the vaginal wall (Figs. 6K, L). Between the abnormalities, we observed a changed pattern of disposal in the basal layer (Figs. 6K, L), making it possible for p63-positive cells to be localized inside lesions (Figs. 6K).

3.6 Immunofluorescence analyses

Immunofluorescense analyses for smooth muscle α-actin confirmed what we had observed through cytochemical approaches (Figs. 7A-H). Besides the increase in the smooth muscle layer (Figs. 7E, H), the glands in the TG and TG + T groups exhibit invasive acini with an interrupted smooth muscle layer in some proliferative regions affected by lesions (Figs. 7E, H).

4. Discussion

This study showed that prenatal exposure to exogenous testosterone may severely disturb the normal process of urogenital development in female gerbils, leading to permanent masculinization, hydrometrocolpos and the formation of ectopic prostate tissue around the vaginal wall. In addition, the ectopic tissue found in females of the TG and TG + T groups presented several foci of inflammation which were generally associated with proliferative areas generally characterized by adenomatous hyperplasia. These findings show that the prenatal period of urogenital development is a critical period for the female prostate morphogenesis in gerbils, in which development is highly sensitive to external androgenic interferences. Additionally, the study showed that gerbil urogenital development in puberty is less sensitive to external interferences of androgenic stimulation, demonstrating that the potential for the prostate to develop differently in response to external factors is lost with aging.

Although a previous study by Welsh *et al.* (2008) has already demonstrated the appearance of prostates in female rats prenatally exposed to testosterone, the present study, which is a continuation of previous work by our research group (Biancardi *et al.* 2012; Perez *et al.* 2012), focused on the effects of in utero exposure to testosterone on the prostate gland. In this sense, the present work, in addition to showing the development of ectopic prostate tissue in female gerbils exposed to testosterone in utero, has analyzed other important aspects such as the manifestation of adenomatous hyperplasia, a novel aspect that has not been demonstrated in other studies employing similar methodologies.

Through biometrical analyses, our data showed that the TG and TG + T experimental groups were significantly different from the others only in PrC weight, relative weight and AGD. The increase in prostatic complex weight in the reproductive tracts of the TG and TG + T groups occurred mainly due to the development of hydrometrocolpos, an anomaly characterized by the retention of secretions inside the uterus and vagina (Wolf *et al.* 2002). The formation of new prostatic tissue surrounding the vaginal wall also contributed to the increase in prostatic complex

weight in TG and TG + T females. Although prostatic complex weight was not significantly different between TG + T and C + T, there was an expressive increase in this value in TG + T females, whereby the value came close to that observed in the TG group. This fact was confirmed by comparing the relative weight between the groups, which showed a statistically significant increase in the TG and TG + T groups in comparison with C and C + T.

The tendency toward hydrometrocolpos in female TG and TG + T gerbils is indicative of the effects of exogenous testosterone upon urogenital development. According to the literature, hydrometrocolpos is a rare condition characterized by the retention of liquid inside the vagina and uterus due to congenital vaginal obstruction (Khan *et al*. 2007). Our data showed that exogenous testosterone impaired the vaginal opening process, which led to the retention of secretions inside the vagina and uterus, leading to a chronic inflammatory response. It is likely that this inflammation reached the prostatic tissue around the vaginal wall, inducing proliferation and contributing to the development of adenomatous hyperplasia of the prostate.

Regarding the AGD, some previous studies employing female rats have shown that prenatal exposure to testosterone promotes increase of the AGD (Hotchkiss *et al.* 2007a, 2007b; Wolf *et al.* 2002), which is a parameter that indicates masculinization in the female. Indeed, the employment of AGD could provide a noninvasive method in order to predict neonatal and adult reproductive disorders (Welsh *et al*. 2008), being a useful technique to access suspected effects of the masculinization during gestation. Although prenatal exposure to testosterone did promote an increase in AGD in the TG and TG + T groups, we did not observe any variation in this parameter in the C + T group compared to the C group, which suggests that the prenatal period is more sensitive to the masculinizing effects of testosterone than puberty is.

Regarding the time-window for masculinization in gerbils we are unable to precise the events of this programming since there is a lack of information in the literature for the gerbil (*Meriones unguiculatus*). Thus, for the present study we adapted the treatment protocol based on previous studies reported in literature on the time-window for masculinization described for rats (Hotchkiss *et al*. 2007a, 2007b; Wolf *et al*. 2002; Welsh *et al*. 2008).

Our stereological analyses showed that females exposed to testosterone during the prenatal period (TG and TG + T) exhibited the most significant alterations of the prostate, mainly related to the lumen and stromal compartments. These animals typically presented glands with reduced lumina and increased smooth muscle layers. In fact, these characteristics were predominant in the ectopic tissues (dorsolateral prostate) around the vaginal wall. These results were very similar to those of previous studies by our research group, which showed the same tendency toward luminal

reduction and increased smooth muscle in the prostate stromal compartment of adult female gerbils exposed to testosterone cypionate during the prenatal period (Biancardi *et al.* 2012).

Recent studies have shown that one of the events that occurs during benign prostatic hyperplasia (BPH) is the epithelial-mesenchymal transition (EMT), in which epithelial cells differentiate into mesenchymal-like cells and further in myofibroblasts and smooth muscle cells, resulting in a considerable increase of the area of the glandular stroma (Alonso-Magdalena *et al.* 2008). Given this, the results of our study suggest that the expressive increase in the muscle stroma in the prostates of TG and TG + T gerbils may have occurred due the activation of the same EMT pathways. These findings may contribute to the discovery of new roles played by androgens during the differentiation of smooth muscle cells and their relation to the development of BPH during aging. Furthermore, they support the hypothesis that prostate development is determinant of future prostate health when it comes to lesions.

Another question regards the role of adjacent mesenchymal tissues with the potential to induce prostate developmental processes. Some studies have demonstrated the role of the ventral mesenchymal pad (VMP) during prostate development in male rodents (Thomson *et al.* 2002; Thomson 2008; Timms 1994, 1995, 2008). Yet little is known about the role of other mesenchymal tissues responsible for the development of the lateral, dorsal and anterior prostate in the male. In the female, there is a lack of data showing the role of mesenchymal tissue and its relation with prostate development. In female gerbils, the development of a prostate suggests the presence of paraurethral mesenchymal tissues with the potential to induce yearly prostatic development as well as to maintain this tissue in the condition of the stromal compartment. The present study, however, suggests that other mesenchymal tissues may be present in the UGS of the female gerbil, allowing the development of dorsolateral prostate-like glands around the vaginal wall. These observations show that the development of a female prostate is highly influenced by androgen activity.

Morphological analyses revealed that females in the C and C + T groups presented hyperplasic glands. Previous studies made by our research group have already related this phenomenon to the development of spontaneous prostatic lesions in aged female gerbils (Custódio *et al.* 2010). Custódio *et al.* (2010) also showed that aged female gerbils have a propensity to develop prostatic lesions such as hyperplasia and prostatic intraepithelial neoplasia (PIN).

Regarding the stromal components, we observed an intense reshuffling of the collagen and elastic fibers in TG and TG + T prostates. In both groups, the general collagen was intensely changed, mainly in regions affected by lesions; this phenomenon is characteristic of a reactive stroma. In addition, we observed an expressive increase in the elastic components, as evidenced by the thickness of the elastic fibers. The reasons why most of the stromal alterations occurred in

glands surrounding the vaginal wall (dorsolateral prostate), despite the paraurethral glands, are still unknown to our research group, although their proximity to the inflamed vagina could possibly be a cause. Alternatively, during organogenesis, an impairment of the signaling by the adjacent mesenchymes could cause an imprint predisposing the future gland to develop lesions with aging. These hypotheses need to be more thoroughly explored in future studies.

In our immunohistochemical analyses, AR immunomarking showed a close similarity between all the experimental groups, presenting epithelial and stromal localizations, suggesting that the gland is functional and active in terms of AR signaling. In addition, ERα showed a predominantly stromal localization which was very similar between all the experimental groups. However, we observed a disrupted pattern of p63 localization in several areas in the prostates of TG and TG + T gerbils, as some p63-positive cells occurred inside the multilayered epithelial compartment, mainly in altered glands. This evidence confirms other published findings which have shown the progressive loss of the basal layer in advanced prostatic lesions such as high-grade prostatic intraepithelial neoplasia (HGPIN) (Grisanzio and Signoretti 2008).

Immunofluorescence techniques for smooth muscle α-actin confirmed the increase of muscle stroma in the prostates of TG and TG + T, as shown through stereological analyses. In addition, several prostatic areas affected by lesions in these two groups showed an absent smooth muscle layer in regions of intense proliferation. This suggests an intense stromal reshuffling of the smooth muscle cells during epithelial-stromal interactions in the development of prostatic lesions.

Altogether, our new evidence reinforces other findings indicating the risk associated with androgenic exposure in the prenatal period, especially for females. This study also shows that testosterone exposure in utero may be involved in predisposing the gland toward the development of prostatic diseases with aging in this rodent model.

Recent studies have led to the development of a new hypothesis on prostate cancer development. According to De Marzo *et al.* (2008), exposure to environmental factors such as infectious agents and carcinogenic substances in the diet, along with hormonal imbalances, may lead to injury of the prostate and to the development of chronic inflammation, events that may lead to the development of proliferative inflammatory atrophy (PIA). These lesions then have the potential to become malignant lesions over time and eventually turn into prostate cancer (De Marzo *et al.* 2008).

Indeed, especially when considered together with the report by De Marzo *et al.* (2008), our data suggest a straightforward relation between inflammatory events and the onset of prostatic lesions, predominantly in the TG and TG + T groups. Our findings suggest that chronic inflammation status may arise due to the retention of a huge volume of inflammatory cells inside

the vaginal cavity (hydrometrocolpos), which affects the prostate which is located around the vaginal wall.

According to Schaeffer *et al.* (2008), the mechanisms involved in early prostate developmental processes are quite similar to those that control prostate cancer development. The program of mechanisms that control prostate cancer development is determined through the regulation of genes related to the acid phophatase pathway, Wnt, as well as others related to angiogenesis, apoptosis, migration and cell proliferation. Moreover, it has been suggested that the origin of prostate cancer may occur during the initial phases of prostate development, a process which may be potentiated by an abnormal hormonal environment, increasing the propensity for the development of prostatic lesions throughout life (Schaeffer *et al.* 2008).

Certain events such as the aromatization of testosterone into estradiol by aromatase or its conversion into dihydrotestosterone by 5α-reductase require detailed evaluation in future studies, as they may furnish straightforward answers concerning the mechanisms by which exogenous testosterone is metabolized during critical events of prostate organogenesis.

Taken together, the evidence in the literature demonstrates that exposure of female rodents to androgenic substances during the prenatal period may be very harmful to the normal processes of urogenital tract development (Hotchkiss *et al*. 2007a, 2007b; Wolf *et al.* 2002), especially for the prostate gland (Biancardi *et al*. 2012; Perez *et al.* 2012), an organ with a high potential for developing malignant lesions, such as cancer, throughout life. Therefore, several conditions, including PCOS, adrenal hyperplasia, and exposure to EDCs, or even to drugs with androgenic potential, may cause irreversible interferences during the developmental process and increase the likelihood of developing prostatic lesions during adulthood and aging. Thus it is extremely important to elucidate the mechanisms underlying these events and to identify ways to avoid exposure to harmful exogenous androgens, as well as to come up with new ways of diagnosing and treating female patients who have experienced these kinds of hormonal interference during their prenatal development.

Although the programming window for reproductive tract masculinization has not been established in gerbils, though it has in rats (Welsh *et al.* 2008), our research group has successfully used this rodent model in several new studies focusing on reproductive tract masculinization and prostate cancer development in order to improve the methodology for reproductive approaches to prostate cancer treatment.

In conclusion, the results of the present study along with a growing body of scientific literature on female prostate biology have highlighted several aspects related to the presence, function, development, physiology, and diseases of the prostate in the female organism. Besides,

our attempts to understand this gland more thoroughly have shown that the prostate in the female deserves more attention, considering its strong potential to respond to different treatments, as shown in the present work and others by our research group (Biancardi *et al*. 2012; Perez *et al.* 2011; Santos *et al.* 2006). Furthermore, the presence of this gland in females, as extensively demonstrated by Zaviačič (1999), alerts us to the possibility that women may be exposed to exogenous androgens by various means, mainly during the developmental period which is a very sensitive phase for prostate establishment. The evidence suggests a potential risk for pregnant women, as they may be exposed to exogenous androgens in various ways and even have their female fetuses affected by this exposure. Moreover, some direct evidence has recently emerged from clinical cases (Reis *et al.* 2011), demonstrating the exigency of more accurate diagnoses. These recent studies have reported important improvements in medical accuracy regarding diseases with possible origins from the female prostate (Skene's gland).

5. Acknowledgements

We are very grateful to Dr. Athanase Billis of the State University of Campinas (UNICAMP) for the histopathological diagnoses of the prostate lesions. We are also grateful to Luiz Roberto Falleiros-Jr. as well as other researchers at the Laboratory of Microscopy and Microanalysis for their technical assistance.

This paper was supported by a grant from the Brazilian agency FAPESP (São Paulo Research Foundation, Procs. Nr. 2009/16789-7; 2009/53990-2) and CNPq (Brazilian National Research and Development Council, Procs. Nr. 301596/2011-5).

6. Conflict of interest

The authors declare no conflict of interest to this work.

7. References

Alonso-Magdalena, P., Brossner, C., Reiner, A., Cheng, G., Sugiyama, N., Warner, M., and Gustafsson, J. A. (2008). A role for epithelial-mesenchymal transition in the etiology of benign prostatic hyperplasia. *Proc. Natl. Acad. Sci. USA* **106**, 2859–2863.

Biancardi, M. F., Perez, A. P. S., Góes, R. M., Santos, F. C. A., Vilamaior, P. S. L., and Taboga, S. R. (2012). Prenatal testosterone exposure as a model for the study of endocrine-disrupting chemicals on the gerbil prostate. *Exp. Biol. Med.* **237**, 1298-1309.

Black, B. E., and Paschal, B. M. (2004). Intranuclear organization and function of the androgen receptor. *Trends Endocrinol Metab.* **15(9)**, 411-417.

Cordeiro, R. S., Scarano, W. R., Campos, S. G. P., Santos, F. C. A., Vilamaior, P. S. L., Góes, R. M., and Taboga, S. R. (2008). Androgen receptor in the Mongolian gerbil ventral prostate: Evaluation during different phases of postnatal development and following androgen blockage. *Micron* **39**, 1312-1324.

Custódio, A. M. G., Santos, F. C., Campos, S. G., Vilamaior, P. S. L., Oliveira, S. M., Góes, R. M., and Taboga, S. R. (2010). Disorders related with ageing in the gerbil female prostate (Skene's paraurethral glands). *Int. J Exp. Pathol.* **91(2)**, 132-143.

De Marzo, A. M., Platz, E. A., Sutcliffe, S., Xu, J., Grönberg, H., Drake, C. G., Nakai, Y., Isaacs, W. B., and Nelson, W. G. (2007) Inflammation in prostate carcinogenesis. *Nat. Rev. Cancer* **7**, 256-269.

Grisanzio, C., and Signoretti, S. (2008). p63 in prostate biology and pathology. *J. Cell. Biochem.* **103**, 1354-1368.

Hotchkiss, A. K., Furr, J., Makynen, E. A., Ankley, G. T., and Gray, L. E. Jr. (2007a). In utero exposure to the environmental androgen trenbolone masculinizes female Sprague-Dawley rats. *Toxicol. Lett.* **174**, 31-41.

Hotchkiss, A. K., Lambright, C. S., Ostby, J. S., Parks-Saldutti, L., Vandenberg, J. G., and Gray, L. E. Jr. (2007b). Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicol. Sci.* **96(2)**, 335-345.

Huttunen, E., Romppanen, T., and Helminen, H. J. (1981). A histoquantitative study on the effects of castration on the rat ventral prostate lobe. *J. Anat.* **132(pt3)**, 357-370.

Khan, R. A., Ghani, I., and Wahab, S. (2007). Hydrometrocolpos due to persistent urogentital sinus mimicking neonatal ascites. *Iran. J. Pediatr.* **18(1)**, 67-70.

Meeks, J., and Schaeffer, E. M. (2011). Genetic regulation of prostate development. *J. Androl.* **32(3)**, 210-217.

Nishino, N., and Totsukawa, K. (1996). Study on the estrous cycle in the Mongolian gerbil (*Meriones unguiculatus*). *Exp. Anim.* **45(3)**, 283-288.

Perez, A. P. S., Biancardi, M. F., Góes, R. M., Santos, F. C. A., and Taboga, S. R. (2011). Exposure to ethinylestradiol during prenatal development and postnatal supplementation with

testosterone causes morphophysiological alterations in the prostate of male and female adult gerbils. Int. J. Exp. Pathol. **92(2)**, 121-130.

Perez, A. P. S., Biancardi, M. F., Vilamaior, P. S. L., Góes, R. M., Santos, F. C. A., and Taboga, S. R. (2012). Microscopic comparative study of the exposure effects of testosterone cypionate and ethinylestradiol during prenatal life on the prostatic tissue of adult gerbils. *Microsc. Res. Tech.* **75(8)**, 1084-1092.

Prins, G. S., and Putz, O. (2008). Molecular signaling pathways that regulate prostate gland development. *Differentiation* **76**, 641-659.

Prins, G. S., Tang, W. L., Belmonte, J., and Ho, S. M. (2008). Perinatal Exposure to oestradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis. *Basic Clin. Pharmacol. Toxicol.* **102**, 134-138.

Reis, L. O., Billis, A., Ferreira, F. T., Ikari, L. Y., Stellini, R. F., and Ferreira, U. (2011). Female urethral carcinoma: evidences to origin from Skene's glands. *Urol. Oncol.* **29(2)**, 218-223.

Santos, F. C. A., and Taboga, S. R. (2006). Female prostate: a review about the biological repercussions of this gland in humans and rodents. *Anim. Reprod.* **3**, 3-18.

Santos, F. C. A., Leite, R. P., Custódio, A. M. G., Carvalho, K. P., Monteiro-Leal, L. H., Santos, A. B., Góes, R. M., Carvalho, H. F., and Taboga, S. R. (2006). Testosterone stimulates growth and secretory activity of the adult female prostate of the gerbil (*Meriones unguiculatus*). *Biol. Reprod.* **75**, 370-379.

Shaeffer, E. M., Marchionni, L., Huang, Z., Simons, B., Blackman, A., Yu, W., Parmigiani, G., and Berman, D. M. (2008). Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer. *Oncogene* **27**, 7180-7191.

Soder, O. (2005). Perinatal imprinting by estrogen and adult prostate disease. Proc. Natl. Acad. Sci. USA **102**, 1269-1270.

Staack, A., Donjacour, A. A., Brody, J., Cunha, G. R., and Carroll, P. (2003). Mouse urogenital development: a pratical approach. *Differentiation* **71**, 402-413.

Takeda, H., Mizuno, T., and Lasnitzki, I. (1985). Autoradiographic studies of androgen binding sites in the rat urogenital sinus and postnatal prostate. *J. Endocrinol.* **104(1)**, 87-92.

Thomson, A. A. (2008). Mesenchymal mechanisms in prostate organogenesis. *Differentiation* **76**, 587-598.

Thomson, A. A., Timms, B. G., Barton, L., Cunha, G. R., and Grace, O. C. (2002). The role of smooth muscle in regulating prostatic induction. *Development* **129**, 1905-1912.

Timms, B. G. (2008). Prostate development: a historical perspective. *Differentiation* **76**, 565-577.

Timms, B. G., and Hofkamp, L. (2011). Prostate development and growth in benign prostatic hyperplasia. *Differentiation* **82**, 173-183.

Timms, B. G., Kembra, L. H., Barton, L., Bradley, S., Richter, C. A., and vom Saal, F. S. (2005). Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. Proc. Natl. Acad. Sci. USA **61**, 200-208.

Timms, B. G., Lee, C. W., Aumüler, G., and Seits, J. (1995). Instructive induction of prostate growth and differentiation by a defined urogenital sinus mesenchyme. *Microsc. Res. Tech*. **30**, 319-332.

Timms, B. G., Mohs, T. J., and Didio, L. J. A. (1994). Ductal budding and branching patterns in the developing prostate. *J. Urol.* **151(5)**, 1427-1432.

Weibel, E. R. (1963). Principles and methods for the morphometric study of the lung and other organs. *Lab. Invest.* **12**, 131-155.

Welsh, M., Saunders P. T. K., Fisken, M., Scott, H. M., Hutchison, G. R., Smith, L. B., and Sharpe R. M. (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J. Clin. Invest.* **118(4)**, 1479-1490.

Wilhelm, D., and Koopman, P. (2006). The makings of maleness towards an integrated view of male sexual development. Nat. Genet. **7**, 620-631.

Wilson, J. D. (2011). The critical role of androgens in prostate development. *Endocrinol Metab. Clin. N. Am.* **40**, 577-590.

Wolf, C. J., Hotchkiss, A. K., Ostby, J. S., LeBlanc, G. A., and Gray, LEJr. (2002). Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study. *Toxicol. Sci.* **65**, 71-86.

Zanatelli, M., Silva, D. A., Shinohara, F. Z., Góes, R. M., Santos, F. C., Vilamaior, P. S., and Taboga, S. R. (2013). Actions of oestradiol and progesterone on the prostate in female gerbils: reversal of the histological effects of castration. Reprod. Fertil. Dev. (Epub ahead of print).

Zaviačič, M. (1999). The Female Prostate: From vestigial Skene's paraurethral glands and ducts to woman's functional prostate. pp. 31-49. (Slovack Academic Press, Bratislava, Slovakia.)

8. Legends

Figure 1. Schematic representation of the experimental protocol employed in this study. The interval between day 0 (day of mating) and day P0 (day of birth) represents the gerbil gestational

period. Days 2 and 4 represent the prenatal days of testosterone treatment (groups TG and TG + T). 6^{th}, 7^{th}, and 8^{th} represent the pubertal weeks in which the animals were treated once per week with testosterone (groups C + T and TG + T). The end of the timeline (one year old) represents the animals' age at death.

Table 1. Biometrical data of female gerbils (N = 6 animals/group; mean ± SEM[§]). Superscript letters (a, b, c) represent statistically significant differences ($p \leq 0.05$) between the experimental groups. Values labeled with the same letters are not significantly different. [§]Standard error of mean. [†]Relative weight corresponds to the ratio between the weight of the prostate and that of the whole body.

Figure 2. Stereological analysis of prostate compartments (epithelium, lumen, muscle stroma and non-muscle stroma). Values represent the mean ± standard error of mean (SEM). Superscript letters (a, b, c) represent statistically significant differences ($p \leq 0.05$). Values labeled with the same letters are not significantly different. The values are expressed as percentages (%) and represent the relative volume of prostate compartments in each experimental group. N = 5 animals/group.

Figure 3. General histological view of the female urogenital tract in all experimental groups (Figs. 3A–D). Figs. 3A, B represent the female urogenital tract in the C and C + T groups, showing a normal paraurethral localization of the prostate in the female gerbil. In the groups TG and TG + T, in contrast, we observed the formation of a dorsolateral prostate around the vaginal wall, in addition to the formation of abnormal prostate acini above the urethra (Figs. 3C, D). V (ventral), D (dorsal), U (urethra), Va (vagina), arrows (paraurethral prostatic acini), large arrows (ectopic prostate around the vaginal wall), arrowhead (abnormal prostate acini at the ventral localization).

Figure 4. Morphological characterization of the prostate by the HE and PAS techniques. Figs. 4A–H show morphological aspects of C and C + T prostates. Although we observed some hyperplasic areas in the C and C + T groups (Figs. 4C, G), the TG and TG + T groups showed more severely injured glands affected by adenomatous hyperplasia (Figs. 4I–O). We observed several invasive prostatic acini in addition to the expressive number of areas with inflammation foci in prostates in the TG and TG + T groups (Figs. 4I–O). The pattern of secretion was similar between all experimental groups (Figs. 4D, H, L, O). Ac (acini); Ep (epithelium); L (lumen); S (stroma); arrows (invasive acini characteristic of adenomatous hyperplasia); large arrows (vesicular nuclei present in adenomatous hyperplasia); asterisk (glycoprotein secretion stained by PAS).

Figure 5. Gömöri's reticulin, picrosirius, and resorcin-fuchsin techniques for identification of reticular fibers (collagen III) (Figs. 6A–D), general collagen (Figs. 5E–H), and elastic fibers (Figs. 5I–L). Figs. 5A, B show regular localization of reticular fibers adjacent to prostatic epithelium in the C and C + T groups. The TG and TG + T groups, on the other hand, showed a intense reshuffling of these fibers, mainly in regions affected by lesions (Figs. 5C, D). Regarding general collagen, prostates in the TG and TG + T groups exhibited a drastic reshuffling of these fibers, especially in injured regions (Figs. 5G, H). Regarding the elastic system, we observed several regions with a changed pattern of these stromal components in both the TG and TG + T groups, showing a expressive increase in elastic fiber thickness (Figs. 5K, L). Ep (epithelium); L (lumen); S (stroma); arrows (reticular fibers); asterisk (regions lacking reticular layer); short arrows (general collagen); large arrows (elastic fibers).

Figure 6. Immunohistochemical analysis of AR, ERα, and p63 protein in the female prostate. Figs. 6A–D show AR immunolocalization in both epithelial and stromal compartments of the prostate in all experimental groups. Figs. 6E–H show the stromal expression of ERα, which was similar in all experimental groups. Figs. 6I–L demonstrate the immunolocalization of p63 protein in the basal cell population in the prostate epithelium. We observed that the regularity of this cellular layer was lost in some injured areas of the prostate in the TG and TG + T groups (Figs. 6K, L). Ep (epithelium); L (lumen); S (stroma); large arrows (AR-positive cells), small arrows (ERα-positive cells); arrows (p63-positive cells).

Figure 7. Immunofluorescence for smooth muscle α-actin. Figs. 7A–D show the normal pattern of smooth muscle surrounding the epithelium in prostates in the C and C + T groups. Note the increased thickness of the smooth muscle layer (SML) in prostates in the TG and TG + T groups (Figs. 7E–H) as well as the proliferative regions lacking adjacent SML. Ac (acini); Ep (epithelium); L (lumen); S (stroma); arrows (smooth muscle layer); large arrows (regions lacking smooth muscle); U (urethra); (Scale bar: 200μm - Figs. 7A, C, E, G; Scale bar: 20μm - Figs. 7B, D, F, H).

Figure 1

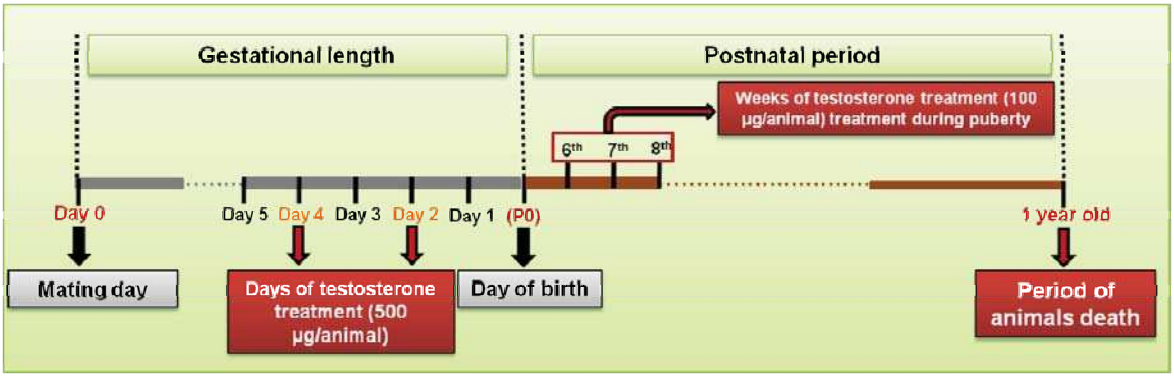

Gestational length
Postnatal period
Weeks of testosterone treatment (100 µg/animal) treatment during puberty
6th 7th 8th
Day 0
Day 5 Day 4 Day 3 Day 2 Day 1 (P0)
1 year old
Mating day
Days of testosterone treatment (500 µg/animal)
Day of birth
Period of animals death

Table 1

Variables	Groups			
	C	**C + T**	**TG**	**TG + T**
Body weight (g)	62 ± 2,37	65.67 ± 2.03	68.67 ± 1.61	60.80 ± 2.58
Prostatic complex (PrC) weight (g)	0.103 ± 0.01^{a}	$0.122 \pm 0.01^{a,c}$	0.243 ± 0.03^{b}	$0.223 \pm 0.06^{c,b}$
†***Relative weight (x10^{-3})***	1.6 ± 0.10^{a}	1.9 ± 0.14^{a}	3.6 ± 0.49^{b}	3.7 ± 1.10^{b}
Ovary weight (g)	0.04 ± 0.050	0.045 ± 0.004	0.046 ± 0.009	0.037 ± 0.011
Adrenal weight (g)	0.037 ± 0.003	0.038 ± 0.002	0.039 ± 0.003	0.041 ± 0.002
Anogenital distance (AGD) (mm)	1.96 ± 0.15^{a}	2.22 ± 0.07^{a}	4.05 ± 0.35^{b}	4.06 ± 0.27^{b}

Figure 2

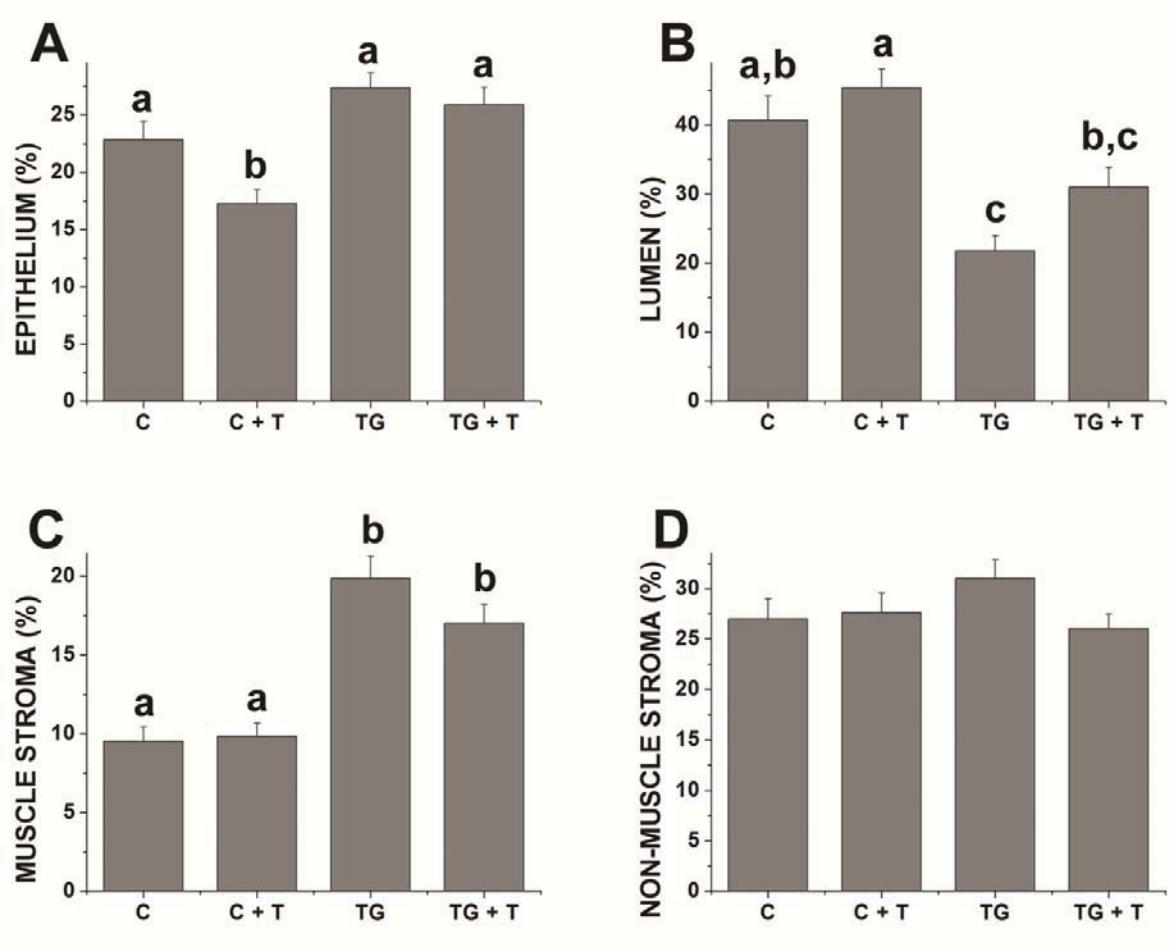

A
EPITHELIUM (%)
a
b
a
a
C
C + T
TG
TG + T
B
LUMEN (%)
a,b
a
c
b,c
C
MUSCLE STROMA (%)
a
a
b
b
D
NON-MUSCLE STROMA (%)

Figure 3

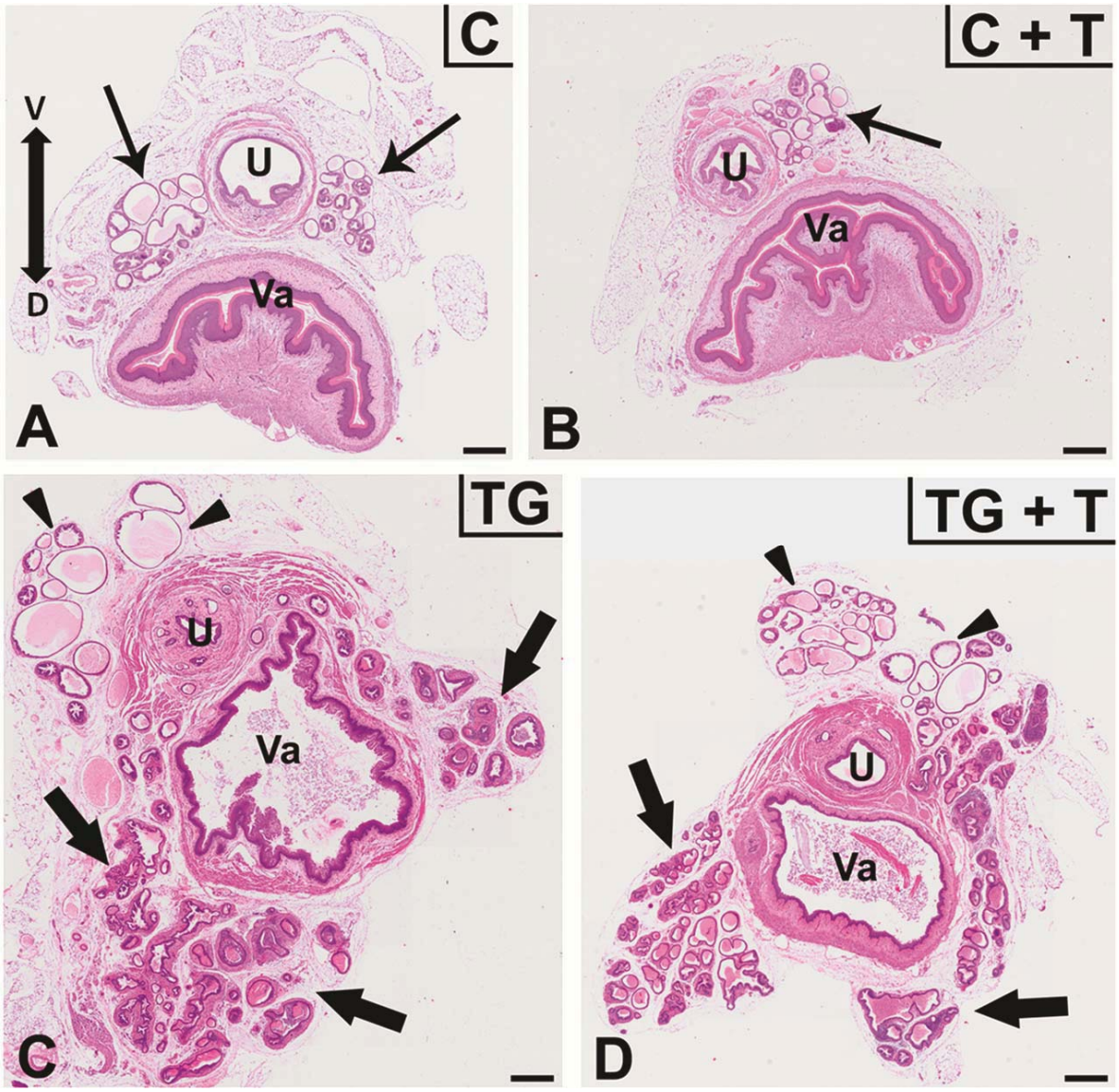


Figure 4

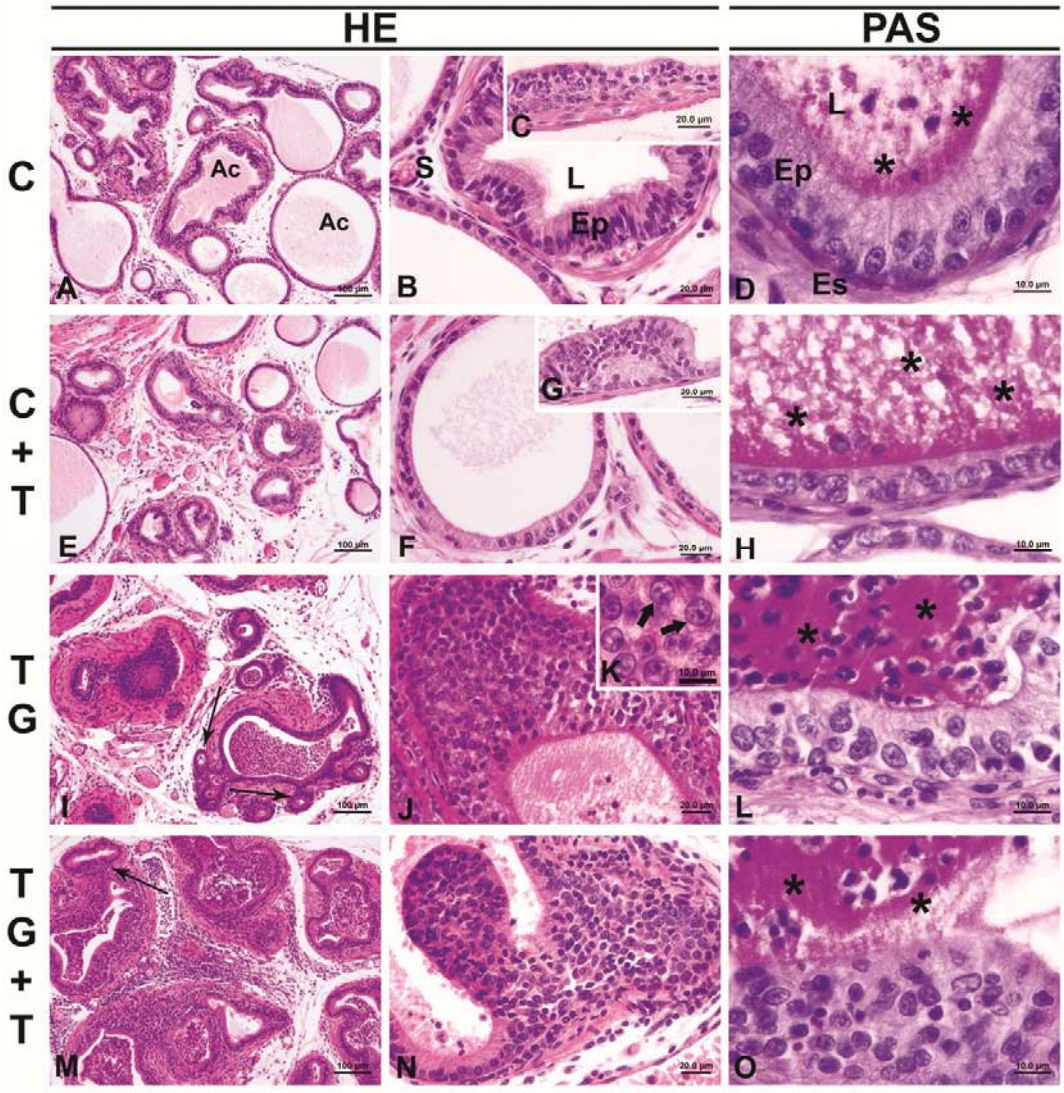

HE
PAS
C
C + T
T G
T G + T
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Ac
Ac
S
L
Ep
L
Ep
Es
100 µm
20.0 µm
10.0 µm

Figure 5

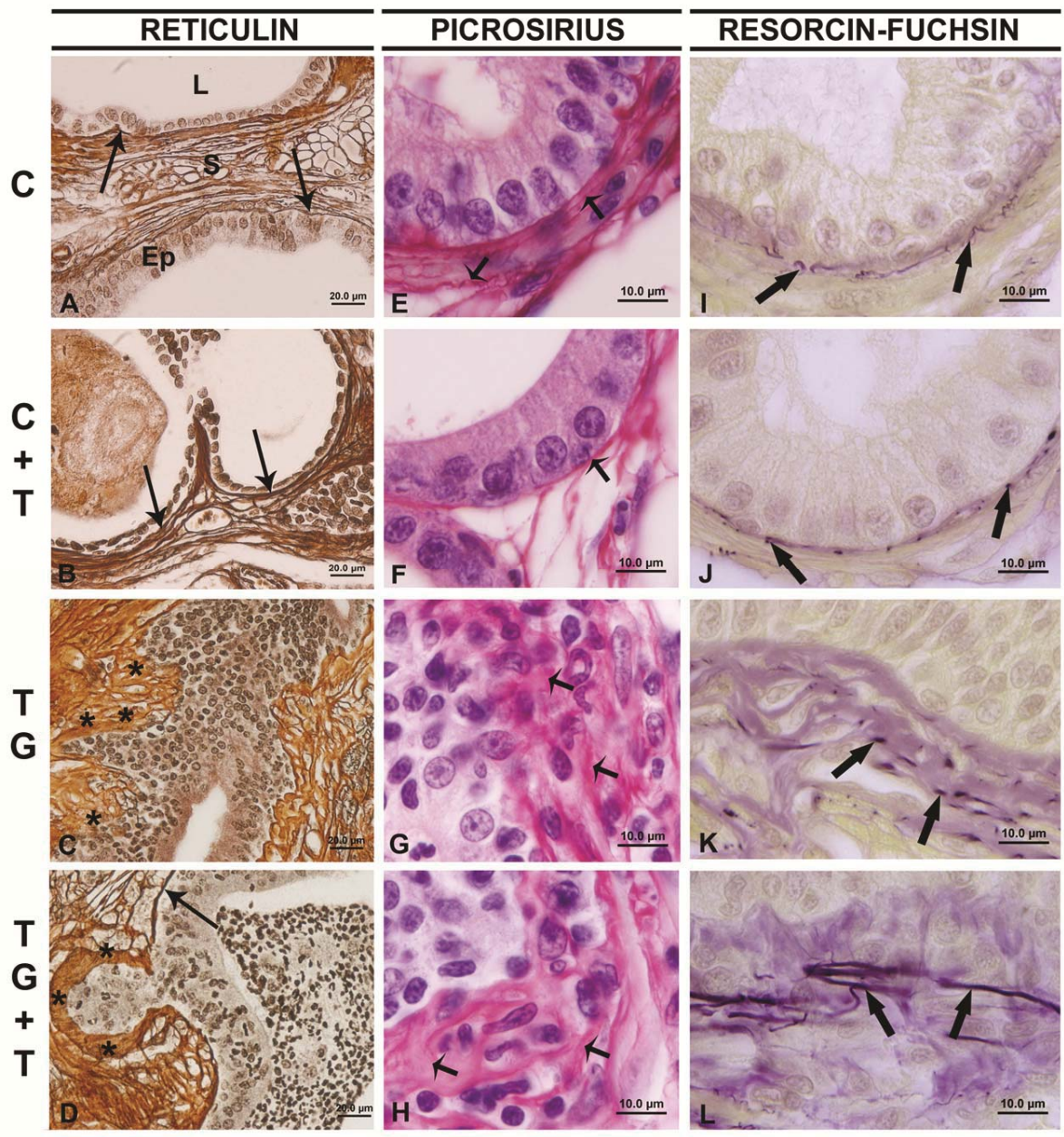

RETICULIN
PICROSIRIUS
RESORCIN-FUCHSIN
C
C + T
T G
T G + T
L
S
Ep
A
B
C
D
E
F
G
H
I
J
K
L
20.0 µm
10.0 µm

Figure 6

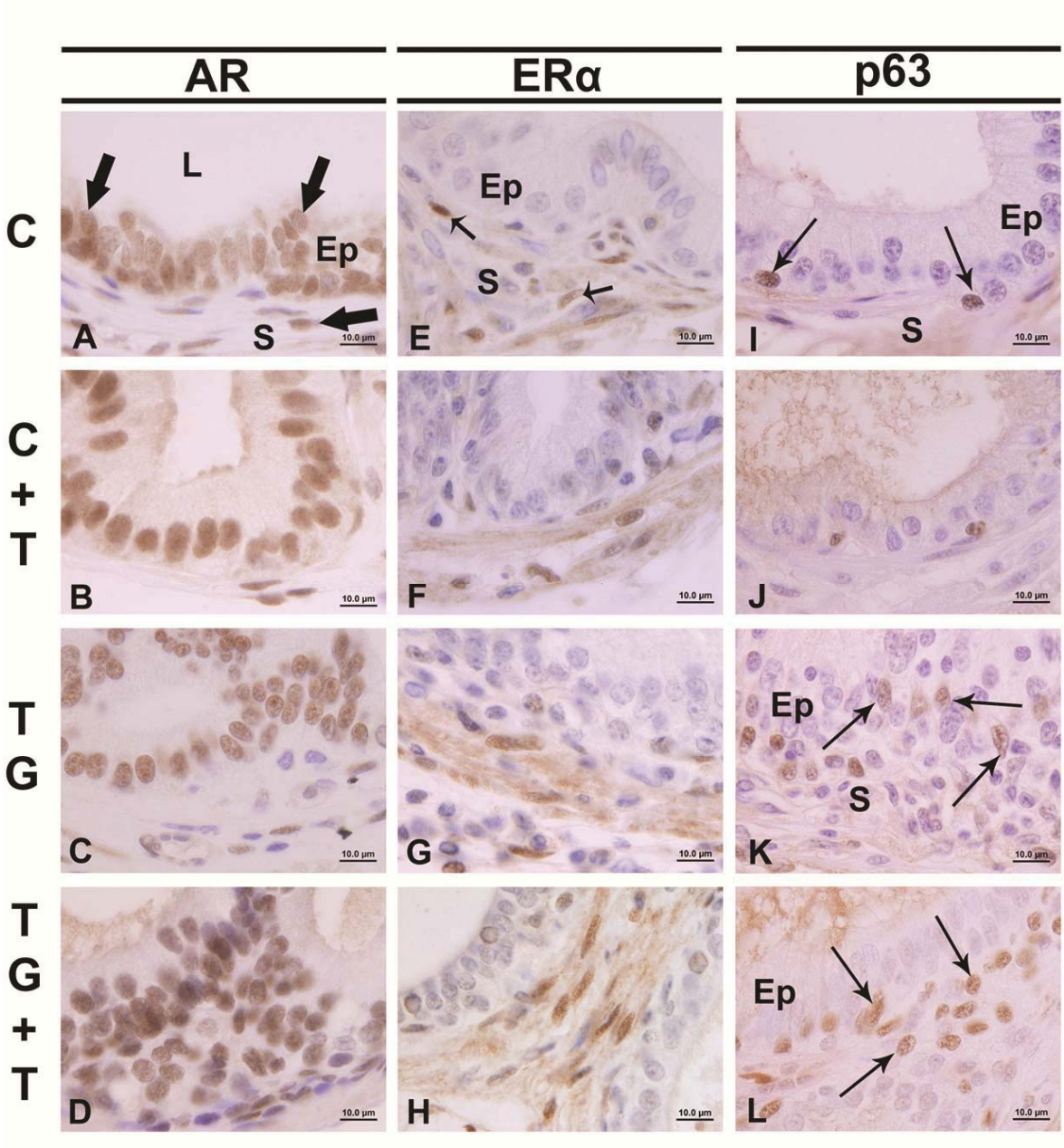
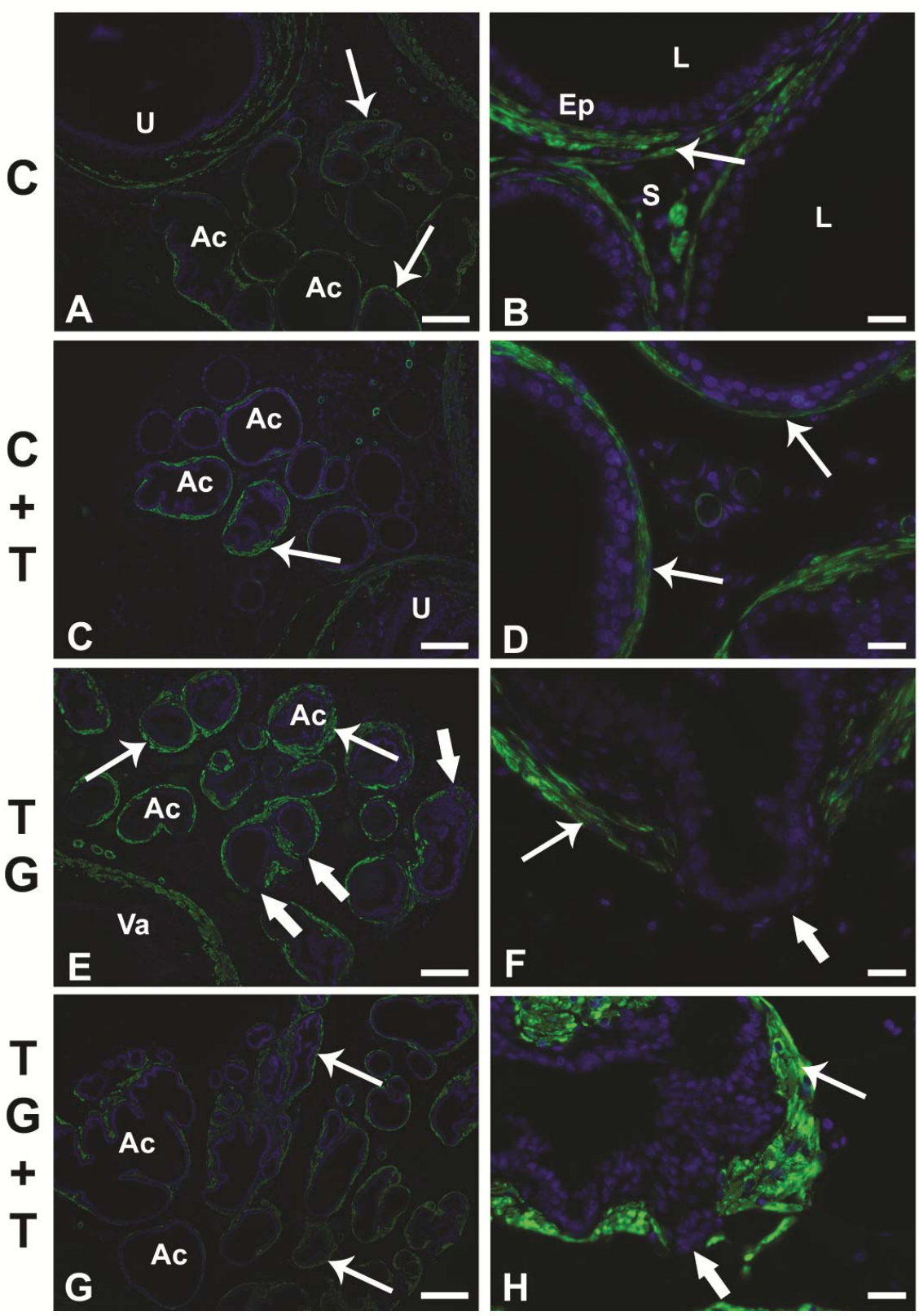


Figure 7



V.2 Effects of prenatal and pubertal testosterone exposure on the ventral prostatic lobe of old male gerbils

Manoel F. Biancardi[1], Ana P. S. Perez[1], Cássia Regina Suzuki Caires[2], Luiz Roberto Falleiros Júnior[2], Rejane M. Góes[2], Patrícia S. L.Vilamaior[2], Diógenes Rosa de Freitas Junior[3], Fernanda C. A. Santos[4], Sebastião R. Taboga[1,2]

[1]*Department of Cell Biology, State University of Campinas, Av. Bertrand Russel s/n, Campinas, São Paulo, 13084864, Brazil.*
[2]*Univ. Estadual Paulista – UNESP*, *Department of Biology, Laboratory of Microscopy and Microanalysis, Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo, 15054000, Brazil.*
[3]*Medical School, Federal University of Goiás,* Colemar Natal e Silva, *Goiânia, Goiás, 74001970, Brazil.*
[4]*Department of Morphology, Federal University of Goiás,* Samambaia II, *Goiânia, Goiás, 74001970, Brazil.*

Corresponding author.
Dr. Sebastião R. Taboga: *Department of Biology, Laboratory of Microscopy and Microanalysis, São Paulo State University, 2265 Cristóvão Colombo Street, Jardim Nazareth, São José do Rio Preto, São Paulo, 15054-000, Brazil. E-mail address: taboga@ibilce.unesp.br.* Tel: +55 17 32212386; Fax: +55 17 32212390.

Abstract

The prostate is an accessory sex gland that develops under precise androgenic control. It is known that hormonal imbalance may disrupt its development predisposing this gland to develop diseases during aging. Although the hypothesis regarding earlier origins of prostate diseases was proposed many years ago, the mechanisms underlying this complex phenomenon are poorly understood. Therefore, the aim of this study was to evaluate the prostates of old male gerbils exposed to testosterone during intrauterine and postnatal life using morphological, biometrical, stereological, cariometrical, immunohystochemical, and immunofluorescence analyses. Our findings demonstrate that prenatal and pubertal exposure to testosterone increases the susceptibility to the development of prostate diseases during aging. The presence of a more proliferative gland associated with foci of adenomatous hyperplasia in animals exposed to testosterone during prenatal and pubertal phase, show that the utero life and the pubertal period are important phases for prostatic morphophysiology establishment, which is a determinant for the health of the gland during aging. Therefore, these findings reinforce the idea that prostate disease may result from hormonal disruptions in early events during prostate development, which imprint permanently on the gland predisposing it to develop lesions in later stages of life.

Keywords: Prostate development, gerbil, testosterone, endocrine-disrupting chemicals, adenomatous hyperplasia.

1. Introduction

Studies have been emphasizing that prostatic diseases such as benign prostatic hyperplasia (BPH) and prostate cancer originate from direct influences during uterine life (Gardner, 1995), with androgenic imbalance as one of the most critical causes (Schaeffer et al., 2008). Although the mechanisms underlying the early origin of prostatic diseases are still poorly understood, hormonal influences are one of the most critical causes for these diseases (Singh and Handelsman, 1999).

There are several factors which may be responsible for disrupting critical moments of the prenatal period of prostate development such as hyperandrogenism, polycystic ovary syndrome (PCOS) (Yarak et al., 2005), dietary and steroid intake, besides exposure to endocrine-disrupting chemicals (EDCs) (Prins et al., 2008). Although most literature reports have emphasized the influence of EDCs with estrogenic potential (Timms et al., 2005; Prins et al., 2008; Perez et al., 2010), some studies have shown the impact of disruption, by androgenic compounds, on prostate development in rodents (Wolf et al., 2002; Hotchkiss et al., 2007a; b).

A recent study by our research group showed that abnormal testosterone exposure during prenatal life may be directly related to the development of premalignant prostate diseases during adult life in the Mongolian gerbil (Biancardi et al., 2012). However, there is a lack of studies on the impact of androgenic imbalance during early moments of life and the consequences on the prostate gland of old individuals.

Thus, considering that prostate development is an event that depends on fine hormonal control (Timms, 2008), sensible interferences may imbalance programs of gene expression leading to irreversible changes in prostate morphogenesis and maintenance during aging. Therefore, an understanding of events that may interfere with prostate organogenesis may help elucidate the mechanisms underlying the origin of prostate diseases.

Recent evidence has demonstrated that exposure to oestradiol and bisphenol-A (BPA) during prostate development is directly linked to an epigenetic alteration of this gland, increasing the susceptibility to prostate carcinogenesis with aging (Prins et al.,

2008). This evidence is very important to gain insight into the early events of prostate development and its relation with the origin of prostate diseases.

Considering all these aspects, our hypothesis is that abnormal prenatal exposure to testosterone may disrupt the normal process of prostate development increasing the susceptibility to the development of prostatic diseases during aging. Thus, the aim of this study was to evaluate the prostate of old gerbils that were exposed to testosterone during prenatal and pubertal life.

2. Material and Methods

2.1 Animals and experimental design

The animals were provided by the São Paulo State University (UNESP) (São José do Rio Preto) and maintained in polyethylene cages under controlled conditions of light and temperature, provided with filtered water and rodent food *ad libitum*. Animal handling and experiments were performed according to the ethical guidelines of the São Paulo State University (UNESP) (Ethical committee number 021/09 CEUA), following the Guide for Care and Use of Laboratory Animals. During all experiments, we provided filtered water in glass bottles to avoid the release of endocrine-disrupting chemicals such as bisphenol-A from plastic material.

We used 20 adult females and 20 adult males (between 3 and 4 months old) of gerbils (*Meriones unguiculatus*, Muridae: Gerbillinae) for mating. We matched, randomly, one male and one female to form independent families. Five couples were put into each group. Pregnant females from these couples underwent different manipulations, and their offspring formed all experimental groups, as follows: C (control) group: offspring from non-manipulated pregnant females; C + T (testosterone during puberty) group: offspring from non-manipulated pregnant females. The littermate was treated with subcutaneous injections of 100 µg of T (testosterone cypionate – deposteron; EMS) diluted in 100 µl of mineral oil during the 6^{th}, 7^{th} and 8^{th} weeks of life; TG (testosterone during gestation) group: offspring from mothers exposed to subcutaneous injections of 500 µg of T during gestation. TG + T (testosterone during gestation plus puberty) group: offspring from

mothers exposed to subcutaneous injections of 500 μg of T during gestation plus subcutaneous injections of 100 μg of T during the 6th, 7th and 8th weeks of life. Only the pups exposed to testosterone that were born four days after treatment were employed in this study. All animals utilized in this study were killed after 1 year of birth. The protocol of T treatment was adapted from Wolf et al. (2002). The overall experimental design, treatment details with T and the age at which the animals were killed are shown in Figure 1.

All animals were killed by CO_2 inhalation followed by decapitation. Body, prostatic complex (PrC - correspondent urethral segment, ventral, dorsolateral and dorsal prostate lobes), testis, and adrenals were weighed. These fragments were dissected out using a Leica stereoscopic microscope (Leica, Germany) to remove adipose tissues and isolate the urethral segment plus the associated prostatic tissue. The anogenital distance measurements were obtained with a digital caliper rule.

2.2 Light microscopy

The PrCs from gerbils were fixed by immersion in 4% paraformaldehyde (buffered in 0.1 M phosphate, pH 7.2) or in metacarn (proportions: methanol 60%, chloroform 30% and acetic acid 10%) for 3 hours. After fixation, the tissues were washed in water, dehydrated in ethanol, clarified in xylene and embedded in paraffin (Histosec, Merck, Dermstadt, Germany). Serial tissue sections of 5 μm were obtained for all tissue fragments using an automatic rotator microtome (Leica RM2155, Nussloch, Germany). The sections were stained with hematoxylin-eosin (HE) and picrossirius for general morphological analysis. Prostatic reticular fibers and elastic fibers were identified, respectively, by Gömöri's reticulin and resorcin-fucsin techniques. The specimens were analyzed with an Olympus BX60 light microscope (Olympus, Hamburg, Germany), and the images were digitalized using the software DP-BSW V3.1 (Olympus).

2.3 Stereological and kariometrical analysis

The stereological analyses were carried out using Weibel's multipurpose graticulate with 130 points and 10 test lines (Weibel, 1963) to compare the relative proportion (relative

volume) of each component of the prostatic tissue (epithelium, lumen and muscle and non-muscle stroma), as described by Huttunen et al. (1981). We chose thirty microscopic fields at random from each experimental group (6 fields per animal; N = 5). In summary, we determined the relative values by counting the coincident points in the test grid and dividing them by the total number of points.

Nuclear area, perimeter and form factor data were obtained from fifty microscopic fields (subjected to Feulgen reaction) randomly taken from each experimental group (10 fields/animal; N = 5). From these fifty microscopic fields, we randomly obtained the area and perimeter data from 400 nuclei for each group. Indeed, we obtained form factor data employing [4π x nuclear area/(nuclear perimeter)^2]. Stereological and kariometric analyses were taken using the software Image-Pro Plus version 6.1 for Windows.

2.4 Immunohistochemistry

Tissue sections were subjected to immunohistochemistry for the detection of androgen receptor (AR), estrogen receptor-alpha (ER-α) and proliferating cell nuclear antigen (PCNA). Primary antibodies reactive to AR (rabbit polyclonal IgG, N-20, Santa Cruz Biotechnology, CA, USA), ER-α (rabbit polyclonal IgG, MC-20, Santa Cruz Biotechnology), p63 (mouse monoclonal IgG_{2a}, sc-843, 4A4, Santa Cruz Biotechnology, CA, USA) and PCNA (mouse monoclonal IgG_{2a}, SC 56, Santa Cruz Biotechnology, CA, USA) were employed at a dilution of 1:100. Peroxidase-conjugated specific antibodies (Sigma Chemical Co., Saint Louis, MO, USA) or polymers (Post Primary Block and polymer, Novocastra, Newcastle Upon Tyne, UK; DAKO Envision^{tm} + Dual link system-HRP, K4061) were used as secondary antibodies and incubated with samples for 45 minutes at 37 °C. The sections were reacted with diaminobenzidine and counterstained with Harris's hematoxylin. The histological sections were analyzed with a Olympus BX60 light microscope (Olympus, Hamburg, Germany).

2.5 AR and PCNA quantification

For AR quantification, thirty microscopy fields (magnification of 400x) were used for each experimental group. In each field, the total number of positive epithelial and stromal cells was obtained as a relative frequency (%) in relation to the total number of negative epithelial or stromal cells. Between positive and negative cells, we counted a mean of 5,300 epithelial cells and 1,300 stromal cells for each experimental group.

Regarding PCNA quantification, we employed fifty microscopy fields (magnification of 400x) for each experimental group. In each field, the total number of positive epithelial cells was obtained as a relative frequency (%) in relation to the total number of epithelial cells of the acini. Between positive and negative cells, we counted a mean of 6,000 epithelial cells for each experimental group. All these analyses were performed using the image analysis system previously described.

2.6 Immunofluorescence

Tissue sections were subjected to immunofluorescence for the detection of smooth muscle α-actin (mouse monoclonal IgG_{2a}, sc-32251, IA4, Santa Cruz Biotechnology, CA, USA), which was incubated at a dilution of 1:100 overnight. The next morning, the sections were incubated with fluorochrome-conjugated specific secondary antibodies (anti-mouse, sc-2010, IgG-FITC, Santa Cruz Biotechnology, CA, USA) for 2 hours at room temperature. DAPI was employed to identify the cell nuclei. The histological sections were analyzed with a Zeiss Imager M2 fluorescence microscope (Zeiss, Alemanha) coupled to the AxioVision (Zeiss, Alemanha) software.

2.7 Statistical analyses

The hypothesis tests employed to determine statistical significance were the Kruskal-Wallis test for non-parametric distributions and ANOVA for parametric distributions. Further determination of the significant statistical differences between experimental groups was done using Dunn's test for non-parametric distributions and

Tukey's test for parametric distributions. The data were analyzed using Statistica 6.0 (StarSoft, Inc., Tulsa, OK) and BioEstat 5.0 (*free statistical program*) software. The level of significance was set at 5% ($P \leq 0.05$). Values are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 Biometrical and stereological analysis

Regarding the biometrical analyses, we did not observe statistically significant differences between the variables of experimental groups (Table 1). Stereological analyses of prostate compartments demonstrated statistically significant differences only for muscle relative volume between the C + T and TG groups (Table 1).

3.2 Kariometrical analysis

Regarding kariometrical analysis, we did not observe any significant differences between the nuclear perimeter of the treated groups (C + T, TG and TG + T) in comparison with the C group (Fig. 2A). The only differences were between the TG and TG + T groups in comparison with the C + T group (Fig. 2A). Regarding nuclear area, the group C + T presented the highest statistically significant value in comparison with the C, TG and TG + T groups (Fig. 2B). Indeed, nuclear form factor data were significantly increased in all treated groups (Fig. 2C).

3.3 Morphological aspects of the ventral prostate

Through morphological characterization (Figs. 3A–P) of H&E-stained sections, we observed the presence of hyperplasic lesions in all experimental groups. However, lesions characterized by adenomatous hyperplasia were found only in the prostate of the TG + T group (Figs. 3M, O).

Stromal analyses employing Gömöri's reticulin, picrossirius and resorcin-fuchsin techniques (Figs. 4A–N) showed an intense stromal reshuffling that affected regions with lesions, especially in the prostates of the TG and TG + T groups. Regarding the collagen fibers, we identified foci with intense collagen remodeling in injured regions (Figs. 4C, E, I, J) of these groups. In addition, in the TG + T group we observed a severe collagen alteration surrounding regions affected by adenomatous hyperplasia, where the fibers presented a fragmented disposing (Fig. 4J).

Regarding the elastic system, the C and C + T groups presented prostates with elastic fibers normally disposed around the acini (Figs. 4K, L), except in some cases of prostatic lesions, in which we observed a significantly altered pattern of elastic components. For the groups TG and TG + T, these fibers were dispersed and almost absent in regions with lesions (Figs. 4M, N).

3.4 Immunohistochemical aspects of the male ventral prostate

Androgen receptor (AR) immunolocalization showed a similar pattern in all experimental groups in both epithelial and stromal compartments (Figs. 5A, B, C, D). However, the AR quantification showed a significant increase in the number of AR-positive cells in both epithelial and stromal compartments of the C + T group in comparison with the C and TG + T groups (Figs. 6A, B). Quantitative analysis has also shown an increase of AR-positive cells in the stromal compartment in the TG group in comparison with the C and TG + T groups (Fig. 6B).

Using immunohistochemistry for PCNA, we observed a statistically significant increase of the proliferative cells in the prostates of the TG + T (Fig. 5H) group in comparison with the C, C + T and TG groups (Figs. 5E, F, G). This was confirmed by the quantification of the PCNA-positive cells distributed in the epithelial compartment of the gland (Fig. 6C).

Regarding ERα, we observed a similar pattern of immunolocalization in the C and C + T prostates (Figs. 7A, B), in which we noted a rare presence of this receptor only in the stromal compartment. On the other hand, in the prostate of TG and TG + T, in addition to

stromal immunolocalization, we also detected a marking of ERα in the epithelial compartment of the acini (Figs. 7C, D).

P63 protein showed a similar pattern of immunolocalization in the prostate of the C, C + T, and TG groups (Figs. 7E–G), being characterized by the presence of basal cells in the basal acinar layer. However, we observed an abnormal immunolocalization of p63-positive cells in some foci of TG and TG + T prostates (Figs. 7G, H). Indeed, in some regions of TG + T prostates we did not detect the presence of p63-positive basal cells (Fig. 7H).

3.5 Immunofluorescence analysis of smooth muscle α-actin

Immunofluorescence analysis of smooth muscle α-actin has shown a high heterogeneity of this stromal element in the prostates of the experimental groups. In the C and C + T prostates, we detected a normally smooth muscle distributed around the gland acini (Figs. 8A, B, C, D). However, in the TG and TG + T prostates this stromal element was absent in some areas, especially around regions with lesions (Figs. 8E, F, G, H). Furthermore, in some cases relating TG + T prostates, we did not detect the presence of a smooth muscle layer in adjacent regions affected by adenomatous hyperplasia (Figs. 8G, H).

4. Discussion

This study showed that old male gerbils exposed to testosterone during prenatal and pubertal phases (TG + T group) had a higher propensity to develop prostatic lesions during aging. Although we detected hyperplasic foci in the prostates of all experimental groups, lesions such as adenomatous hyperplasia associated with inflammatory foci were observed only in prostates of the TG + T group. Indeed, we observed an increase in the proliferative rate in the glands of the TG + T group, as determined by PCNA counting. These findings suggest that the association of both periods of treatment (prenatal and pubertal) are necessary to increase the susceptibility of the gland to developing lesions with aging.

Regarding morphological analyses, the prostates of all experimental groups have presented, somehow, lesions with hyperplastic foci. We found both epithelial and stromal compartments changed in regions containing lesions either in control or treated groups. The presence of spontaneous prostatic lesions in old gerbils has been well characterized by researchers of our group in previous studies (Campos et al., 2008). These results showed that gerbils, in contrast to other rodents such as rats and mice, have a biological condition to develop prostatic lesions during aging. This congenital condition of male gerbils to develop prostatic lesions is still poorly understood, lacking of a definitive reference available in the literature.

The most differential morphological aspect observed was the presence of adenomatous hyperplasia in the prostates of TG + T animals. This pathological condition is characterized by the proliferation of small acini from the normal acini, which is easily mistaken with prostatic adenocarcinoma (Bostwick et al., 1994; 1996; Zhang et al., 2011). Although the precursor nature of adenomatous hyperplasia remains uncertain, some recent evidence supports that adenomatous hyperplasia may be a precursor lesion of prostate cancer (Zhang et al., 2011).

Although the data of biometrical and stereological analyses have not presented any significant aspect between the experimental groups, the kariometrical data showed some differences throughout the groups. We did not find any statistically significant difference regarding nuclear perimeter between treated groups (C + T, TG, TG + T) in comparison with the C group. Indeed, although we observed an increase of the nuclear area of all treated groups, only the C + T group showed a statistically significant difference of this parameter in comparison with the C group. On the other hand, the most significant change was the increase of the nuclear form factor index in all treated groups in comparison with the C group.

The form factor has been used as a measure to check the roundness status of the cell nucleus. Nuclear form factor values close to 1 index indicate that the cell nucleus is more round and less elliptic (Santos et al., 2006). Some studies have employed this parameter in cases of BPH and prostate adenocarcinoma (Taboga et al., 2003), or in studies showing the synthesizing status of the prostatic cells when subjected to testosterone supplementation (Santos et al., 2006). Here, we observed an increase of the circular shape in the nuclei of

epithelial prostatic cells of all treated groups, showing that this parameter was, somehow, affected by the treatments.

Regarding immunohistochemical analyses for AR, we observed a similar pattern of immunolocalization in both epithelial and stromal cells between the experimental groups. However, employing AR quantification we detected an increase of AR-positive cells in both epithelial and stromal compartments of the prostate in C + T animals, whereas only AR-positive stromal cells showed an increase in the prostates of the TG group in comparison with the C group. Although the glands of C + T animals contained a higher number of AR-positive cells, we did not observe any association of this fact with a possible increase in the proliferative rate in this experimental group or with the development of lesions such as adenomatous hyperplasia. Although we are unable to explain the molecular basis of these differences regarding the increase of AR-positive cells in C + T and TG groups, some events of DNA imprinting may be involved.

Recent studies have been investigating the influence of endocrine disruptors on the epigenome during early stages of prostate development (Prins et al., 2008). According to Prins et al. (2008), exposure to estrogenic compounds (oestradiol and BPA) imprint the prostate to an increased susceptibility to the development of carcinogenesis during aging. Prins et al. (2008) have shown a changed pattern of DNA methylation in multiple cell signaling genes, which suggested an epigenetic mechanism of action. Because the mechanisms underlying the origin of prostate diseases are unknown, an understanding of the molecular basis of these events responsible to imprint prostates may open new strategies to prostate disease therapy.

Another relevant aspect was the increase of proliferation in the epithelial compartment of TG + T prostates, as determined by PCNA-positive cell counting. The data showed a statistically significant increase in the proliferative rate only in the prostates of the TG + T groups in comparison with other experimental groups. These data are in consistent with the presence of several foci of adenomatous hyperplasia in the glands of these animals, as shown in H&E sections.

Regarding ERα immunolocalization, we observed a different pattern of localization between the groups. Whereas we observed only stromal cells positive to ERα in C and C + T prostates, in TG and TG + T we detected ERα-positive cells in both stromal and

epithelial compartments. Because ERα has been associated with an aberrant proliferative role, which may lead to the development of lesions in the prostate (Ellem and Risbridger, 2009), these findings suggest that the prostates of TG and TG + T were influenced by ERα pathways related to an increase in the proliferative status.

Regarding p63 immunomarking, we observed an altered pattern of p63 immunolocalization in the prostates of TG and TG + T. In these prostates, we detected an alteration of p63 immunolocalization in the basal layer, being possible to localize p63-positive cells out of the basal layer, especially in foci of lesions. This evidence is consistent with literature studies that showed the loss of p63 expression in the basal layer, causing a changed pattern of p63 expression in these cases (Grisanzio and Signoretti, 2008). Moreover, in some regions affected by adenomatous hyperplasia in the TG + T prostates, we observed a scattered pattern of p63-positive basal cells in some acini. These findings are in consistent with recent findings by Zhang et al. (2011), who showed a fragmented pattern of the basal layer in regions of the human prostate affected by atypical adenomatous hyperplasia (AAH).

The immunofluorescence analyses for smooth muscle α-actin showed several regions with an interruption or absence of the smooth muscle layer surrounding lesioned regions in the prostates of TG and TG + T. Furthermore, in TG + T prostates we observed large regions of adenomatous hyperplasia with a complete absence of smooth muscle α-actin, which suggests the presence of new invasive buds in the adjacent stroma.

In addition to the aforementioned results, we also detected the presence of some inflammation foci in the prostate of all experimental groups, in particular associated with the lesions of the TG + T group. However, it is unclear to us if this inflammatory state may have occurred due to a primary effect of the treatment or if it occurred secondarily due to the presence of lesions in the prostate. These questions need to be further explored in future studies, since some reports have proposed a new hypothesis for prostate carcinogenesis, which changes the view regarding the origin of prostate cancer. A review by De Marzo et al. (2007) showed some important aspects of the relation between inflammation and prostate carcinogenesis. According to De Marzo et al. (2008), there are several potential sources for the inflammation, such as direct infection, reflux urine, inducing chemical and physical trauma, dietary factors, estrogens, or a combination of these. Any of these factors

have the potential to break the immune tolerance leading to an autoimmune reaction in the prostate (De Marzo et al., 2008).

Recent researches have given special attention to questions regarding the prenatal origins of prostate diseases. The evidence shows that early prostate developmental processes are similar to the program controlling prostate cancer installation (Schaeffer et al., 2008). These events share similar gene expression programs, such as the acid phosphatase pathway, Wnt, besides others associated with angiogenesis, apoptosis, migration and cell proliferation (Schaeffer et al., 2008).

These studies have demonstrated that embryonic gene expression, sensitive to androgens, is activated during the processes involved in the initiation and progression of prostate cancer. Because prostate development is an event that depends on a finely regulated hormonal environment (Thomson, 2008; Timms, 2008; Timms and Hofkamp; 2011), sensitive interferences may disrupt gene expression programs leading to irreversible changes in the pattern of prostate development and increasing the susceptibility to diseases with aging. Therefore, an understanding of the events that may interfere with prostate organogenesis may highlight the mechanisms involved during prostate disease.

The present study identifies the early phases of prostate development as a determinant aspect for prostate gland morphogenesis. However, as shown in previous studies by our group (Biancardi et al., 2012; Perez et al., 2012), the female fetus is affected differently by testosterone exposure during the prenatal period, being masculinized and developing ectopic prostatic tissue around the vaginal wall. However, this study showed that although we did not observe any malformation of the male reproductive system, testosterone exposure during the prenatal phase associated with androgenic exposure during puberty, seems to be associated with increased susceptibility to the development of benign diseases in these animals.

Moreover, evidence of a more proliferative prostate gland in animals exposed both prenatally and postnatally to testosterone, indicate that the period of development of the prostate is determinant for the health of the gland during aging. We believe that the present study, although fundamentally based on morphological approaches, may contribute some additional information to a lacking area of research regarding the effects of prenatal androgenic imbalance and the consequences for the health of the prostate during aging

periods. However, new studies are necessary to clarify fundamental questions regarding the molecular biology of the effects of these androgen-induced changes during determinant periods of prostate development and its relation with the development of prostatic diseases in later stages.

Altogether, the evidence reinforces the potential that abnormal androgen exposure during prenatal phases associated with pubertal exposure may have on the prostate in terms of lesion development during aging. Although the hypothesis of the prenatal origins of prostate cancer has been debated lately as a strong candidate for the origin of prostate diseases (Schaeffer et al., 2008), conclusive studies are necessary in order to highlight the mechanisms underlying this complex field.

Furthermore, studies focusing on the aromatization of testosterone in estradiol by aromatase (p450), or even testosterone conversion in dihydrotestosterone by 5α-reductase are approaches that need to be evaluated in future studies focusing on exogenous exposure to androgenic compounds, so that they may provide more insight into how these substances are metabolized and act inside the organism in critical moments of the developmental period.

5. References

Biancardi MF, Perez APS, Góes RM, Santos FCA, Vilamaior PSL, Taboga SR. 2012. Prenatal testosterone exposure as a model for the study of endocrine-disrupting chemicals on the gerbil prostate. Exp Biol Med, 237:1298-1309.

Bostwick DG, Algaba F, Amin MB, Ayala A, Eble J, Goldstein N, Helpap B, Humphrey P, Grignon D, Jones EC, McNeal J, Montironi R, Qian J, Ro J, Srigley J, Tetu B, Troncoco P, True L, Wheeler T, Young RH. 1994. Consensus statement on terminology: recommendation to use atypical adenomatous hyperplasia in place of adenosis of the prostate. Am J Surg Pathol, 18:1069-1070.

Bostwick DG. 1996. Prostatic intraepithelial neoplasia and atypical adenomatous hyperplasia. Cancer, 78:330-336.

Campos SGP, Zanetoni C, Scarano WR, Vilamaior PS, Taboga SR. 2008. Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. Int J Exp Pathol, 89(1):13-24.

De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. 2007. Inflammation in prostate carcinogenesis. Nature, 7:256-269.

Ellem SJ, Risbridger GP. 2009. The dual, opposing roles of estrogen in the prostate. Ann N Y Acad Sci, 1155:174–86.

Gardner WA. 1995. Hypothesis: the prenatal origins of prostate cancer. Hum Pathol, 26:1291-1292.

Grisanzio C, Signoretti S. 2008. p63 in prostate biology and pathology. J Cell Biochem, 103:1354–1368.

Hotchkiss AK, Furr J, Makynen EA, Ankley GT, Gray LE Jr. 2007a. In utero exposure to the environmental androgen trenbolone masculinizes female Sprague-Dawley rats. *Toxicol. Lett.* 174, 31-41.

Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenberg JG, Gray LE. Jr. 2007b. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicol. Sci.* 96(2), 335-345.

Huttunen E, Romppanen T, Helminen HJ. 1981. A histoquantitative study on the effects of castration on the rat ventral prostate lobe. J Anat, 3:357-370.

Perez APS, Biancardi MF, Vilamaior PSL, Góes RM, Santos FCA, Taboga SR. 2012. Microscopic comparative study of the exposure effects of testosterone cypionate and ethinylestradiol during prenatal life on the prostatic tissue of adult gerbils. Microsc Res Tech, 75(8):1084-1092.

Prins GS, Tang WL, Belmonte J, Ho SM. 2008. Perinatal Exposure to oestradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis. Basic Clin Pharmacol Toxicol, 102:134-138.

Shaeffer EM, Marchionni L, Huang Z, Simons B, Blackman A, Yu W, Parmigiani G, Berman DM. 2008. Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer. Oncogene, 27:7180-7191.

Shappell SB, Thomas GV, Roberts RL, Herbert R, Ittmann MM, Rubin MA, Humphrey PA, Sundberg JP, Rozengurt N, Barrios R, Ward JM, Cardiff RD. 2004. Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. Cancer Res, 64:2270–305.

Singh J, Handelsman DJ. 1999. Imprinting by neonatal sex steroids on the structure and function of the mature mouse prostate. Biol Reprod, 61:200-208.

Thomson AA. 2008. Mesenchymal mechanisms in prostate organogenesis. Differentiation, 76:587-598.

Timms BG, Hofkamp L. 2011. Prostate development and growth in benign prostatic hyperplasia. Differentiation, 82:173-183.

Timms BG, Howdeshell KL, Barton L, Bradley S, Richter C, vom Saal FS. 2005. Estrogenic chemical in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. Proc Natl Acad Sci USA, 102(19):7014-7019.

Timms BG. Prostate development: a historical perspective. Differentiation, 2008, 76:565-577.

Weibel ER. 1963**.** Principles and methods for the morphometric study of the lung and other organs. Lab Invest, 12:131-155.

Wolf CJ, Hotchkiss AK, Ostby JS, LeBlanc GA, Gray LE Jr. 2002. Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study. Toxicol Sci, 65:71-86.

Yarak S, Bagatin E, Hassun KM, Parada MOAB, Filho ST. 2005. Hyperandrogenism and skin: polycysic ovary syndrome and peripheral insulin resistance. An Bras Dermatol, 80(4):395-410.

Zhang C, Montironi R, MacLennan, Lopez-Beltran A, Li Y, Tan Puay-Hoon, Wang M, Zhang S, Iczkowski KA, Cheng L. 2011. Is atypical adenomatous hyperplasia of the prostate a precursor lesion? Prostate, 71:1746-1751.

6. Legends

Figure 1. Schematic representation of the experimental protocol employed in this study. The interval between day 0 (day of mating) and day P0 (day of birth) represents the gerbil gestational period. The days 4 and 2 represent the prenatal days of testosterone treatment (groups TG and TG + T). The 6^{th}, 7^{th}, and 8^{th} represent the pubertal weeks in which the animals were weekly treated with testosterone (groups C + T and TG + T). The end of the timeline (1 year old) represents the age of the animals' death.

Table 1. Biometrical and stereological data of the experimental groups. Values are expressed as mean ± standard error of the mean (SEM) (N = 6 animals/group). Superscript letters (a and b) represent statistically significant ($P \leq 0.05$) differences between the experimental groups. Experimental groups with values that have the same letters did not show statistically significant differences.

Figure 2. Kariometrical analysis (nuclear perimeter, nuclear area and nuclear form factor) of secretory prostate cells. Asterisks indicate statistically significant differences between experimental groups (*$p \leq 0.05$; **$p \leq 0.01$). The values are expressed as the mean ± standard error of the mean (SEM). N = 400 nuclei/group.

Figure 3. Morphological characterization of prostates by HE and PAS techniques. (Figs. 3A–H) show morphological aspects of the C and C + T prostates. Although we observed some prostatic lesions in the C, C + T and TG groups (Figs. 3B, F, K), the TG + T group showed a more severely injured gland, affected mainly by adenomatous hyperplasia associated with inflammatory foci (Figs. 3M, O). Note the presence of inflammatory foci in prostates of all groups (Figs. 3B, F, I, M). The pattern of secretion showed a similar pattern in all experimental groups (Figs. 3D, H, L, P). Ac (acini); Ep (epithelium); L (lumen); S (stroma); IF (inflammatory foci); arrows (prostatic lesions); large arrows (adenomatous hyperplasia); asterisk (glycoprotein secretion stained by PAS); PA (proliferative area).

Figure 4. Gömöri's reticulin, picrossirius, and resorcin-fucsin techniques for identification of reticular fibers (collagen III) (Figs. 4A–E), general collagen (Figs. 4G–J), and elastic fibers (Figs. 4K–N). Figures 4A, B show the regular localization of reticular fibers adjacent to the prostatic epithelium in the C and C + T groups. On the other hand, the groups TG and TG + T showed some areas with reshuffling of these fibers, mainly in regions affected by lesions (Figs. 4C, E). Inserts show the disposing of collagen III in unaffected TG and TG + T prostates (Figs. 4D, F). Regarding general collagen, the prostates of TG and TG + T presented a high reshuffling of these fibers, specifically in injured regions (Figs. 4I, J). Regarding the elastic system, we observed several regions with a changed pattern of these stromal components in both TG and TG + T (Figs. 4M, N). Regions affected by adenomatous hyperplasia showed a reduced quantity of elastic fibers (Fig. 4N). Ep (epithelium); L (lumen); Es (stroma); arrows (reticular fibers); short arrows (general collagen); large arrows (elastic fibers).

Figure 5. Immunohistochemistry for AR and PCNA. Figures 5A–D show AR immunolocalization in both epithelial and stromal compartments of the prostate in all experimental groups. Figures 5E–H demonstrate the immunolocalization of PCNA in the prostates. Observe the high number of PCNA-positive cells in the prostates of the TG + T group (Fig. 5H). Ep (epithelium); L (lumen); S (stroma); arrows (positive cells to AR and PCNA).

Figure 6. Graphical representation of AR and PCNA quantification. Observe the significant increase of AR-positive cells in both epithelial (Fig. 6A) and stromal (Fig. 6B) compartments of C + T prostates in comparison with the C group. AR-positive stromal cells have also increased in the prostates of the TG group in comparison with the C group (Fig. 6B). Regarding PCNA quantification, the TG + T group showed a significant increase of PCNA-positive cells in comparison with the C, C + T and TG groups (Fig. 6C). Asterisks indicate statistically significant differences between experimental groups (*$p \leq 0.05$; **$p \leq 0.01$). The values are expressed as the mean ± standard error of the mean (SEM).

Figure 7. Immunohistochemistry for ERα and p63 proteins. Figures 7A–D show the stromal expression of ERα for all experimental groups. Observe the expression of ERα in the epithelium of TG and TG + T prostates (Figs. 7C, D). Figures 7E–H demonstrate the immunolocalization of p63 protein in the basal cell population in the prostate epithelium. We observed that the regularity of this cellular layer is lost in some injured areas of the prostate in the TG and TG + T groups (Figs. 7G, H). Observe the absence of basal cells in some regions of the adenomatous hyperplasia (Fig. 7H). Ep (epithelium); L (lumen); S (stroma); small arrows (ERα-positive cells); arrows (p63-positive cells).

Figure 8. Immunofluorescence for smooth muscle α-actin. Figures 8A–D show the normal pattern of smooth muscle surrounding the epithelium in the prostates of the C and C + T groups. Observe the absence of the smooth muscle layer (SML) in the prostates of TG and TG + T injured by lesions (Figs. 8E–H). Ac (acini); Ep (epithelium); L (lumen); S (stroma); arrows (smooth muscle layer); large arrows (regions lacking smooth muscle); (Scale bar: 200μm - Figs. 8A, C, E, G; Scale bar: 20μm - Figs. 8B, D, F, H).

7. Conflict of interest

The authors declare no conflict of interest to this work.

Figure 1

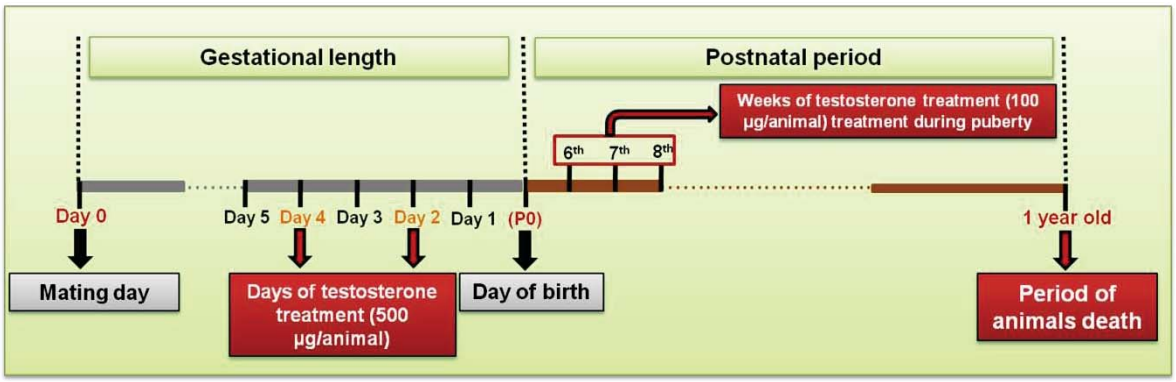

Gestational length
Postnatal period
Weeks of testosterone treatment (100 µg/animal) treatment during puberty
6th 7th 8th
Day 0
Day 5 Day 4 Day 3 Day 2 Day 1 (P0)
1 year old
Mating day
Days of testosterone treatment (500 µg/animal)
Day of birth
Period of animals death

Table 1

	GROUPS			
Variables	**C**	**C + T**	**TG**	**TG + T**
Biometrical analysis				
Body weight (g)	82.7 ± 3.4	84.4 ± 5.7	80.7 ± 4.4	77 ± 2.0
Prostatic complex (PrC) weight (g)	0.96 ± 0.09	1.03 ± 0.04	0.99 ± 0.79	1.01 ± 0.03
Testis weight (g)	1.19 ± 0.04	1.17 ± 0.05	1.17 ± 0.03	1.19 ± 0.03
Adrenal weight (g)	0.044 ± 0.003	0.046 ± 0.002	0.048 ± 0.002	0.045 ± 0.003
AGD (mm)	16.03 ± 0.39	15.37 ± 0.41	16.24 ± 0.29	15.30 ± 0.20
Stereological analysis				
Epithelium (%)	23.26 ± 1.84	21.77 ± 2.38	27.05 ± 2.58	25.95 ± 1.88
Lumen (%)	45.54 ± 3.01	47.72 ± 3.84	42.31 ± 3.63	42.02 ± 3.37
Muscle stroma (%)	9.13 ± 0.69[a,b]	7.26 ± 0.81[b]	10.31 ± 0.85[a]	8.48 ± 0.77[a,b]
Non-muscle stroma (%)	22.08 ± 2.14	23.26 ± 2.31	20.33 ± 1.79	23.54 ± 2.05

Figure 2

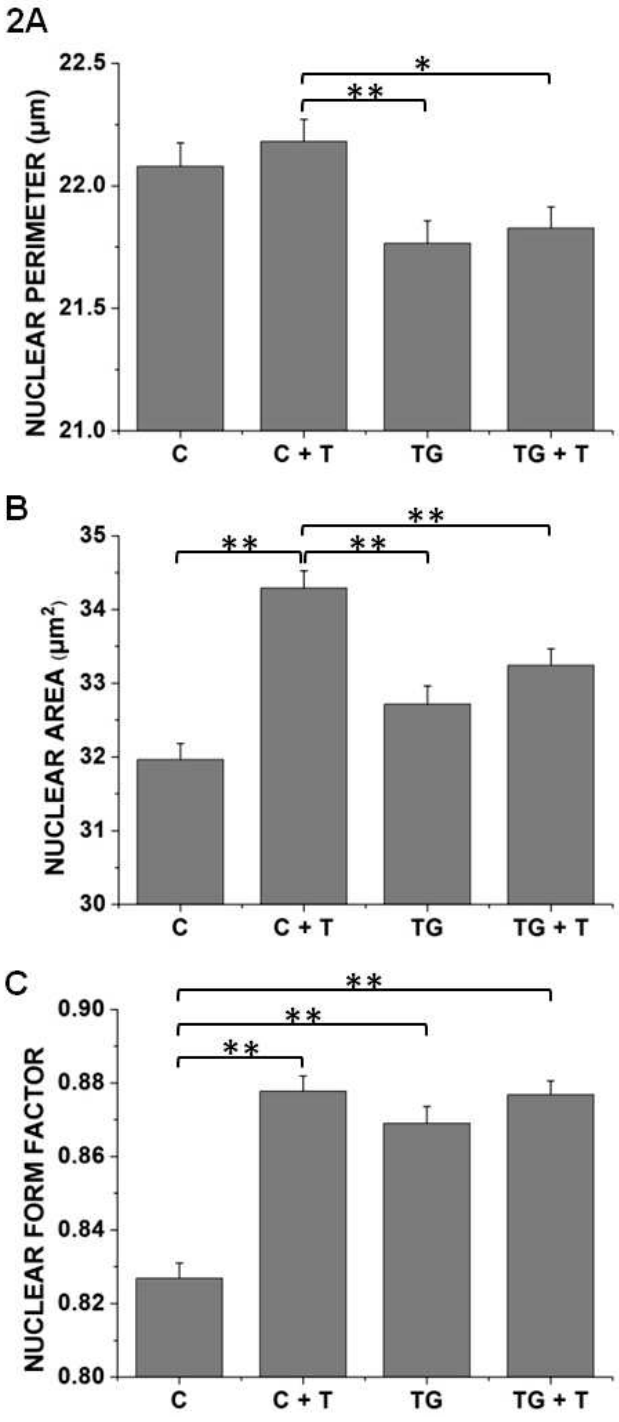

2A
NUCLEAR PERIMETER (µm)
22.5
22.0
21.5
21.0
*
**
C
C + T
TG
TG + T
B
NUCLEAR AREA (µm²)
35
34
33
32
31
30
**
**
**
C
C + T
TG
TG + T
C
NUCLEAR FORM FACTOR
0.90
0.88
0.86
0.84
0.82
0.80
**
**
**
C
C + T
TG
TG + T

Figure 3

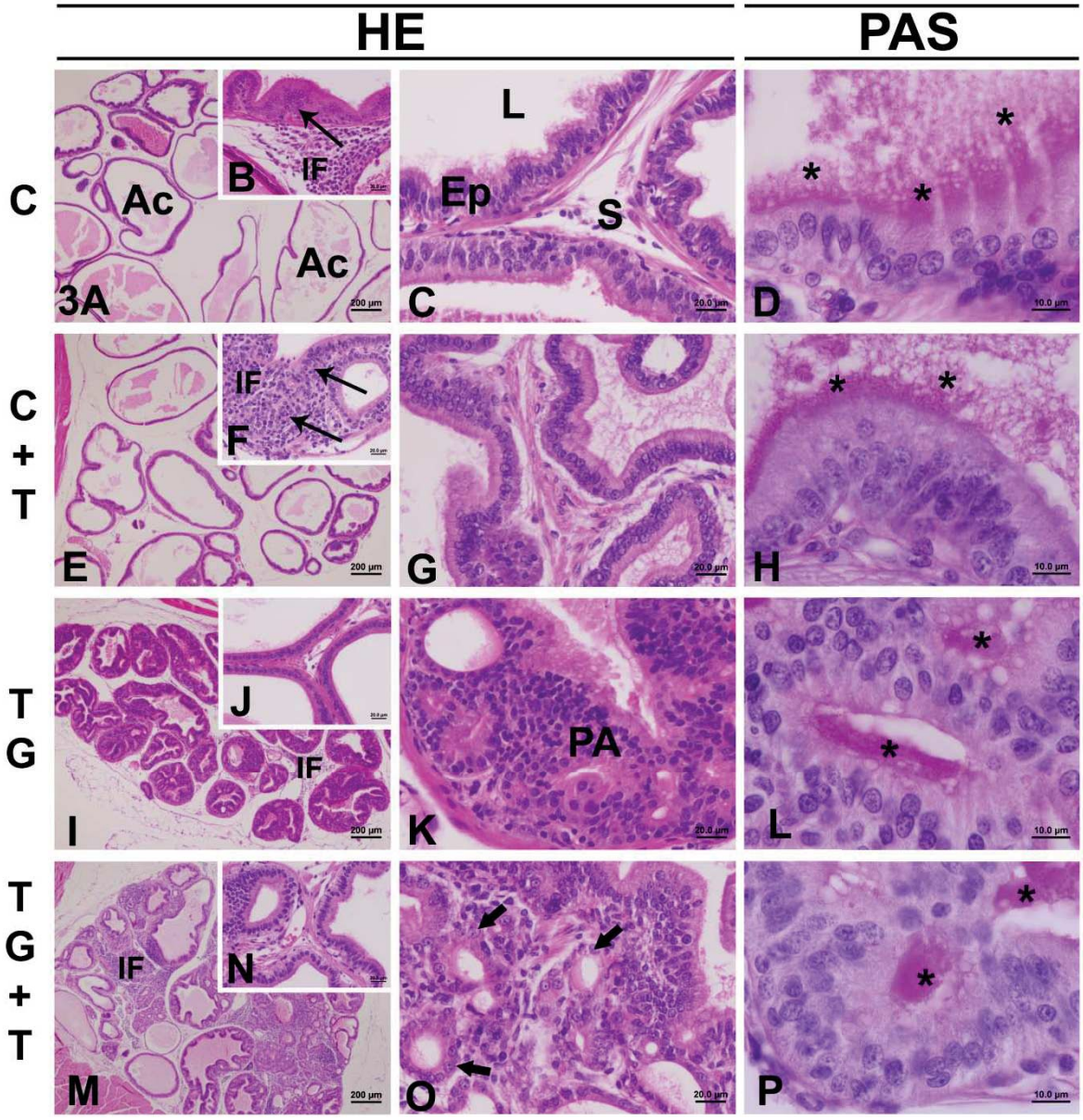

HE
PAS
C
C + T
T G
T G + T
Ac
Ac
3A
B
IF
L
Ep
S
C
D
IF
F
E
G
H
IF
J
PA
I
K
L
IF
N
M
O
P
200 µm
20.0 µm
10.0 µm

Figure 4

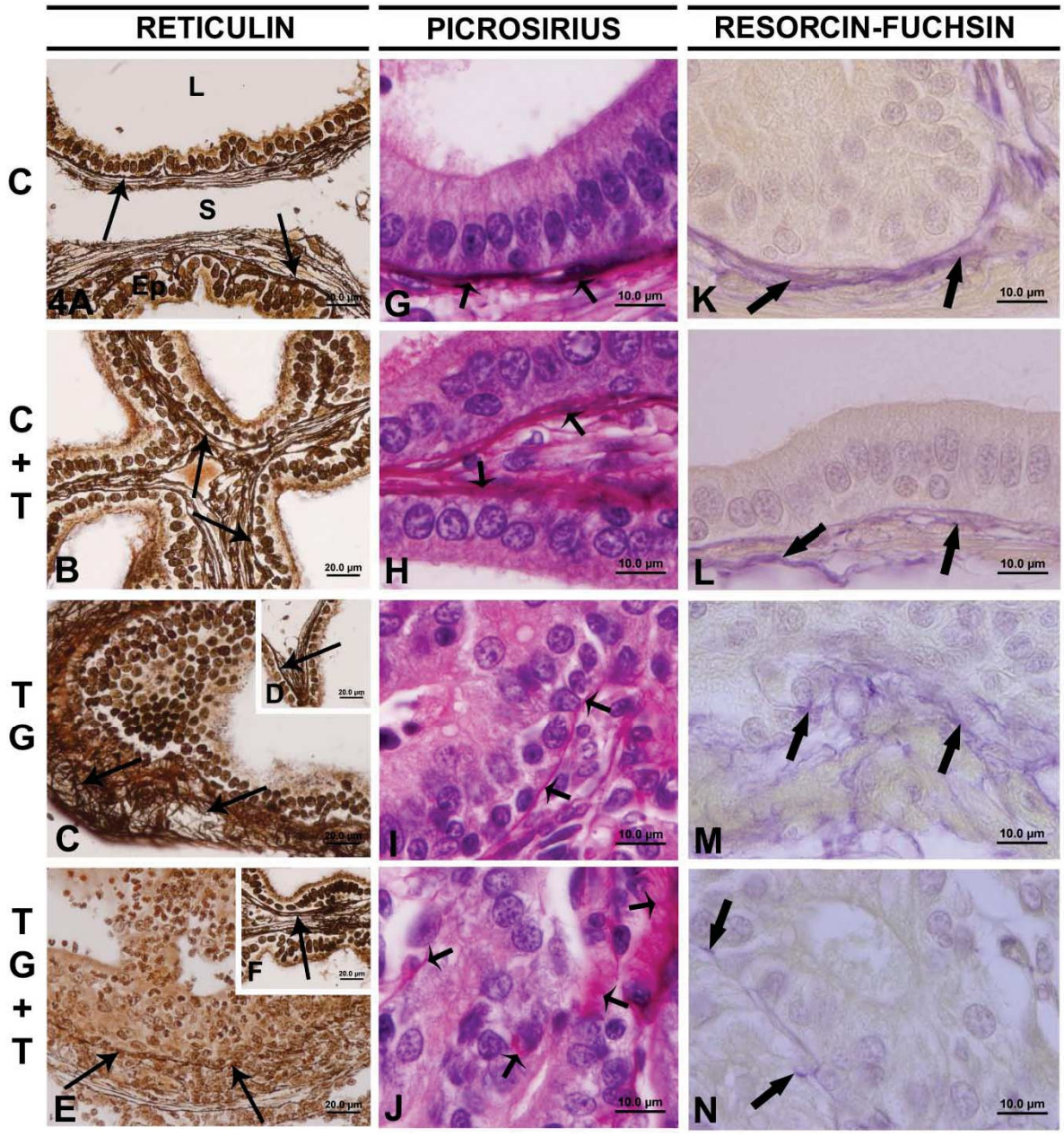

RETICULIN
PICROSIRIUS
RESORCIN-FUCHSIN
C
C + T
T G
T G + T
L
S
Ep
4A
G
K
B
H
L
D
C
I
M
F
E
J
N
20.0 µm
10.0 µm

Figure 5

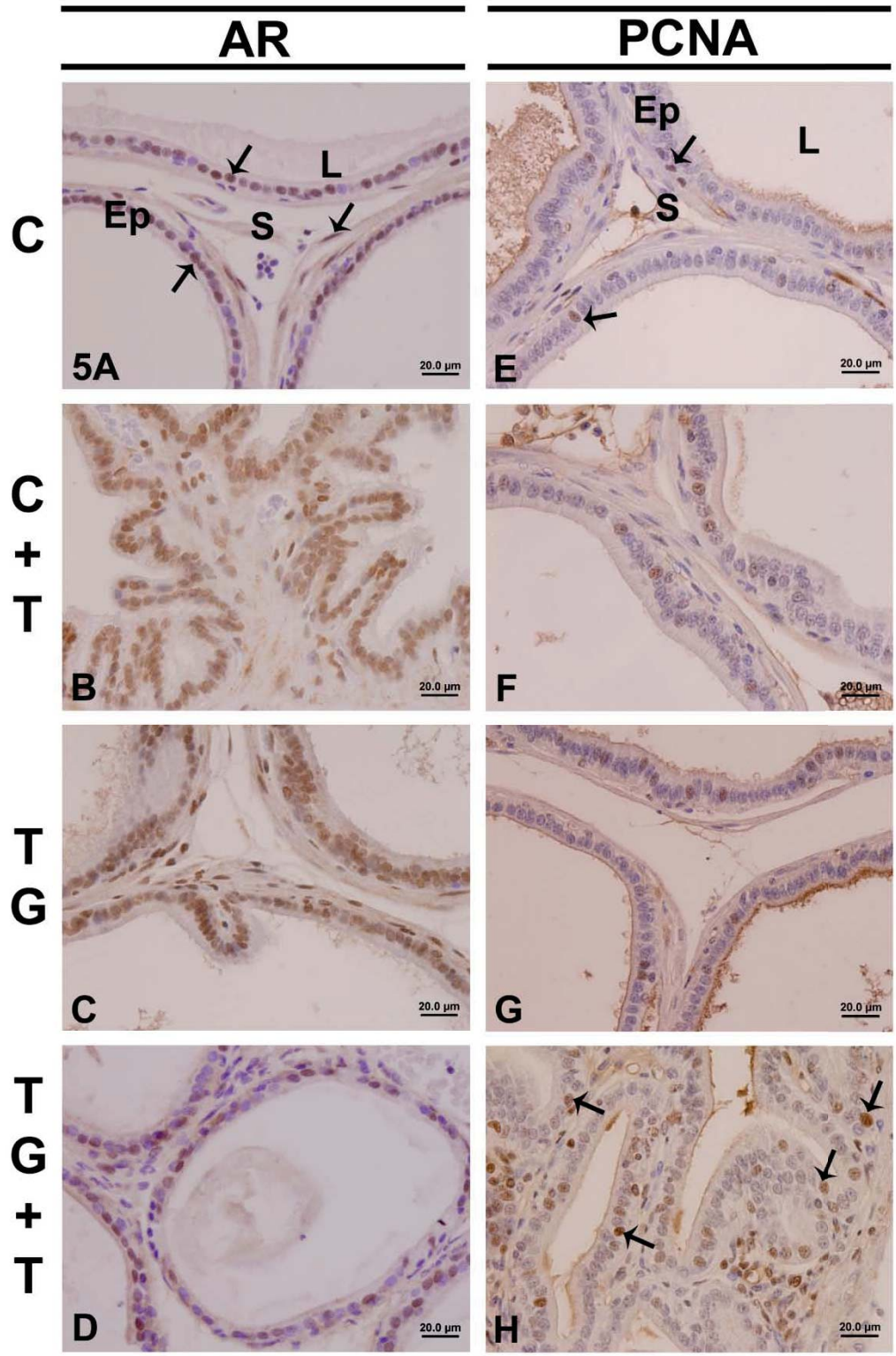


Figure 6

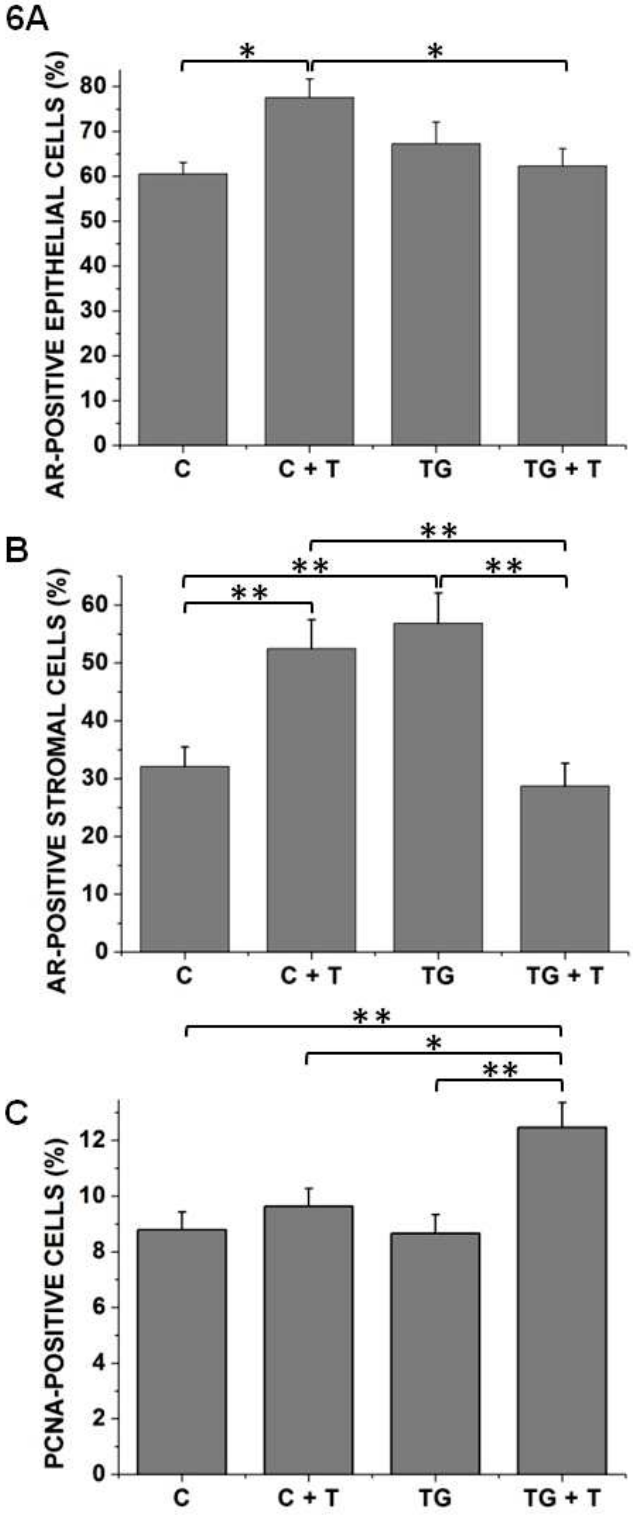

6A
AR-POSITIVE EPITHELIAL CELLS (%)
0
10
20
30
40
50
60
70
80
*
*
C
C + T
TG
TG + T
B
AR-POSITIVE STROMAL CELLS (%)
0
10
20
30
40
50
60
**
**
**
C
C + T
TG
TG + T
C
PCNA-POSITIVE CELLS (%)
0
2
4
6
8
10
12
**
*
**
C
C + T
TG
TG + T

Figure 7

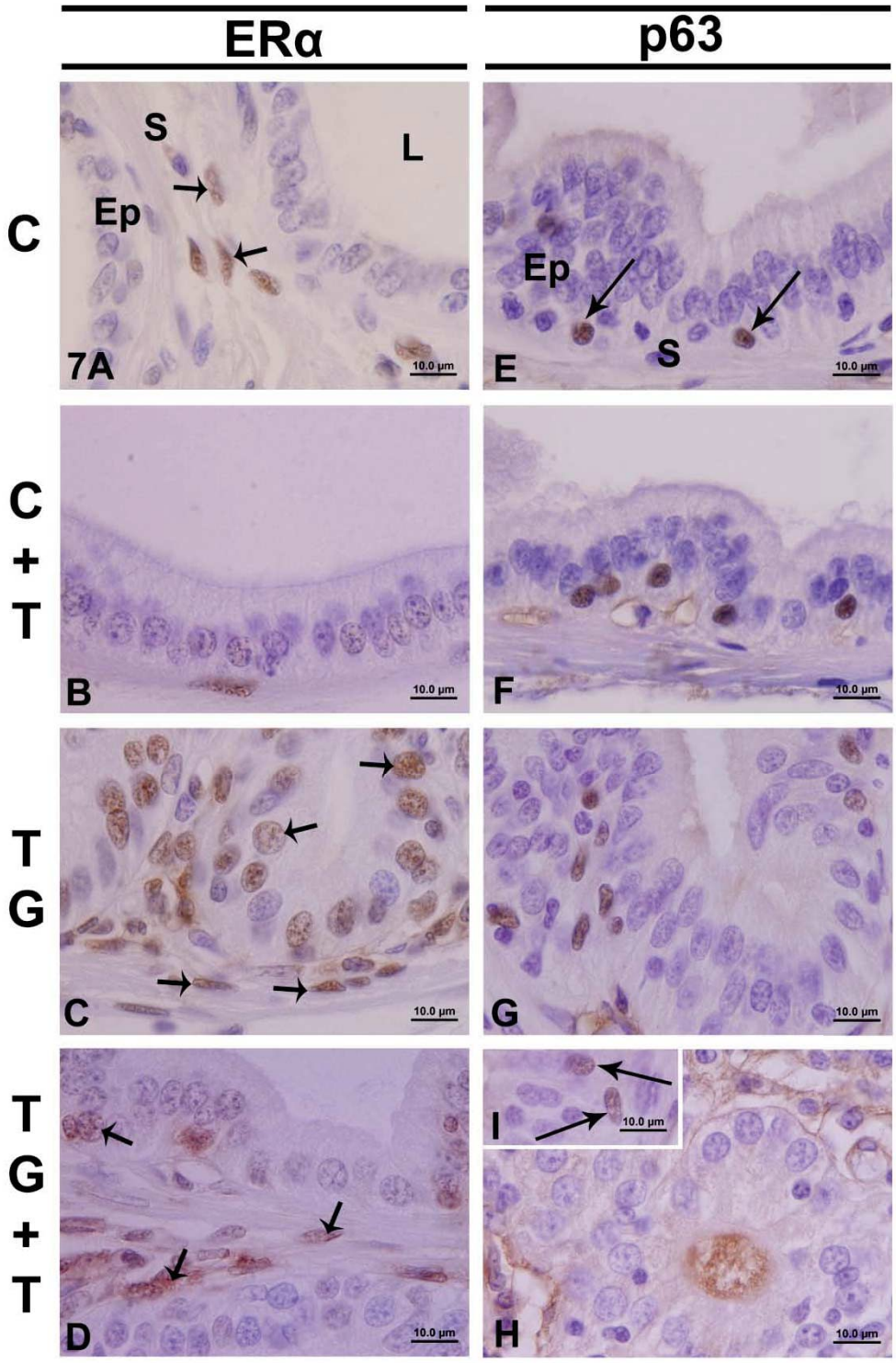
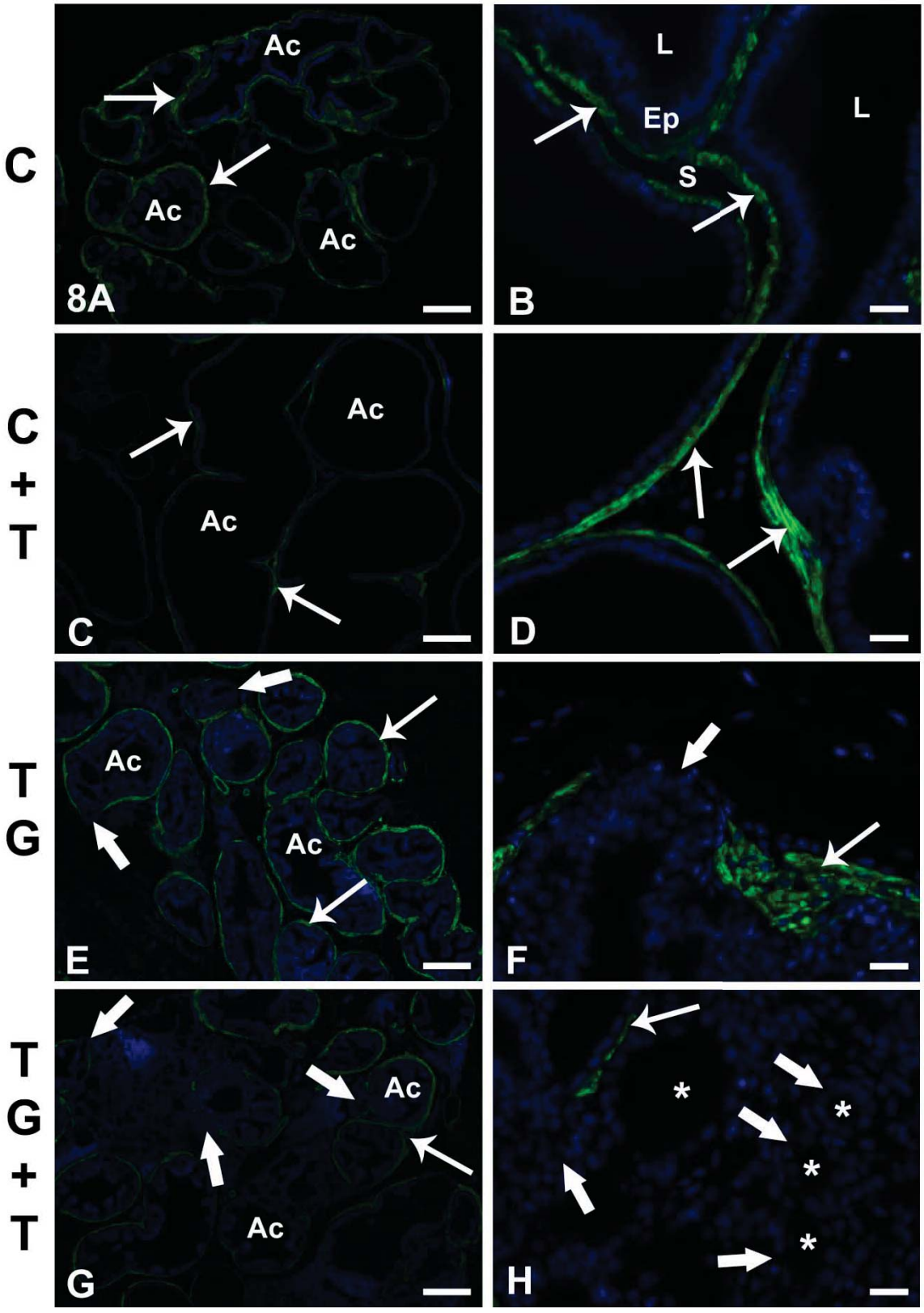


Figure 8



V.3 Budding Process During the Organogenesis of the Ventral Prostatic Lobe in Mongolian Gerbil

Bruno Domingos Azevedo Sanches[1#], Manoel Francisco Biancardi[2#], Fernanda Cristina Alcantara dos Santos[3], Rejane Maira Góes[1], Patrícia Simone Leite Vilamaior[1], Sebastião Roberto Taboga[1,2]

[1]*Univ. Estadual Paulista – UNESP*, *Department of Biology, Laboratory of Microscopy and Microanalysis, Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo, 15054000, Brazil.*
[2]*Department of Structural and Functional Biology, State University of Campinas, Av. Bertrand Russel s/n, Campinas, São Paulo, 13084864, Brazil.*
[3]*Department of Morphology, Federal University of Goiás,* Samambaia II, *Goiânia, Goiás, 74001970, Brazil.*

Running Title: Ventral prostate organogenesis.

Corresponding author.

Dr. Sebastião R. Taboga: *Department of Biology, Laboratory of Microscopy and Microanalysis, São Paulo State University, 2265 Cristóvão Colombo Street, Jardim Nazareth, São José do Rio Preto, São Paulo, 15054-000, Brazil. E-mail address: taboga@ibilce.unesp.br.* Tel: +55 17 32212386; Fax: +55 17 32212390.

[#]**BDAS** and **MFB** contributed equally to this work.

Keywords: Gerbil prostate, ventral mesenchymal pad, smooth muscle layer, androgen receptor, estrogen receptor alpha

Abstract

The prostate is a mammalian gland that shows a complex process of organogenesis. Here, we conducted a morphological study to characterize the organogenesis of the ventral prostate lobe in male gerbils. The urogenital sinus (UGS) was dissected out and processed for paraffin embedding. Histological sections were subjected to cytochemical, immunofluorescence, immunohistochemical, and three-dimensional reconstruction techniques. We found that the first ventral buds emerged from the ventral urethral epithelium between the 20th and 21st days of prenatal life, reaching the ventral mesenchymal pad and initiating the branching process on the first day of postnatal life. The buds presented a V-shaped elongation, suggesting that the smooth muscle layer (SML) plays an important role during budding events. Indeed, whereas the androgen receptor (AR) was preferentially found in the urogenital sinus mesenchyme (UGM), estrogen receptor alpha (ERα) was localized in both the UGM and in the emerging buds. The present study characterized the morphological aspects of the budding process in a different rodent from rat and mice, serving as a new model for future studies on developmental biology of the prostate.

Introduction

Prostate organogenesis, which is responsible for prostate formation, is a complex process in which an intricate way of paracrine signaling regulates precise gene expression either spatially or temporally (Meeks and Schaeffer, 2001; Prins and Putz, 2008). In rats, the complete prostate development encompasses five sequential events, which involve determination, initiation and budding, branching morphogenesis, differentiation, and maturation (Prins and Putz, 2008). The formation of a normal adult prostate depends on the exact occurrence of these events, so that sensible interferences may disrupt normal prostate development, affecting the formation of an adult normal prostate either in terms of architecture establishment or prostatic lesion development (Schaeffer et al., 2008; Timms, 2008; Bruni-Cardoso et al., 2010; Biancardi et al., 2012). Taking this into account, efforts have been made to improve knowledge relating to prostate development, which may be a determinant in defining the mechanisms underlying prostate disease physiology (Wu et al., 2011; Huang et al., 2012; Ousset et al., 2012).

There are essential pathways controlled by steroid receptors such as the androgen receptor (AR) (Cunha, 2008) and estrogen receptors (ER) (McPherson et al., 2008). Although complete understanding of the action of these steroid receptors has not been reached, it has been shown that the AR is expressed prior in mesenchymal tissue than in the urethral epithelium and the buds (Cunha et al., 2003). This suggests that the androgens initially act in the mesenchymal cells which, in response to the stimulus, produce paracrine factors that act in the emerging buds (Cunha and Chung, 1981; Takeda et al., 1985; Timms, 2008). Estrogen receptor alpha (ERα) has been shown to be expressed in both the UGM and buds initially during prostate development, and is suppressed by the action of androgens when the production of this hormone increases (McPherson, 2008). On the other hand, estrogen receptor beta (ERβ) is not expressed during the early stages of prostate development, being important later during the maturation and maintenance of the prostate throughout its lifetime (McPherson, 2008).

Several works have been carried out by our research group employing the Mongolian gerbil (*Meriones unguiculatus*) as an experimental model in prostate studies involving drugs and hormonal manipulation (Corradi et al., 2009; Perez et al., 2012; Biancardi et al., 2012). However, the biology of prostate organogenesis of this species remains poorly understood. Thus, there is the need to elucidate the mechanisms underlying prostate development in gerbils, since a growing number of studies in the prostate field have employed this animal model. Taking into account all these evidences, our study aimed to evaluate early development of the ventral lobe in male gerbils. In this sense, we believe that

new models for studying prostate organogenesis may provide novel sources of data for understanding the biology of this gland.

Material and Methods

Animals and experimental design

The animals were provided by the São Paulo State University (UNESP) (São José do Rio Preto), maintained in polyethylene cages under controlled conditions of light and temperature, and provided with filtered water and rodent food *ad libitum*. Animal handling and experiments were performed according to the ethical guidelines of the São Paulo State University (UNESP) (Ethical committee number 052/2011 CEUA), following the Guide for the Care and Use of Laboratory Animals (The National Academies Press, 2011, Washington, D.C., USA).

We used 18 adult female and 18 adult male (between 3 and 6 months old) gerbils (*M. unguiculatus*, Muridae: Gerbillinae) for mating. We matched, randomly, one male and one female to form independent families. We divided the animals into three couples for each group, as follows: E20, E21, E22, E23, E24, and P1. The letter "E" denotes embryonic day and the letter "P" denotes postnatal day. The groups between E20–24 represent the groups composed of the fetuses that were obtained from the pregnant female during the gestational period. The group P1 represents the newborns at 1 day of life. The mating day was determined by the presence of spermatozoids in the vaginal smears. This day was considered day 0, being the initial day of the pregnancy period and for establishment of the days for obtaining the experimental groups.

All adult animals (pregnant females) were killed by CO_2 inhalation followed by decapitation. All pups were killed by a lethal dosage of anesthesia (0,1 mL/pup), which was prepared with a mixture (proportion of 1/1) of anesthetic (Dopalen, Vetbrands, Brazil) and muscle relaxant (Rompun, Bayer S.A., Brazil). The gender of the fetuses was assessed through visualization of the testes during the dissection. After this, the animals were dissected and the urogenital sinus (UGS) plus bladder and a segment of the pelvic part of the urethra of males was collected and fixed. These fragments were dissected out using a Leica stereoscopic microscope (Leica, Germany) to isolate the UGS. We designated, randomly, six pups from each of three different litters for each experimental group.

Abbreviations: PM (periurethral mesenchyme); VMP (ventral mesenchymal pad); UGM (urogenital sinus mesenchyme - PM + VMP); UR (urethral epithelium); SML (smooth muscle layer); AR (androgen receptor); Estrogen receptor alpha (ERα); estrogen receptor beta (ERβ); PCNA (proliferating cell nuclear antigen).

Light microscopy

Tissue fragments from males were fixed in 4% paraformaldehyde for 24 hours or in methacarn (proportions: 60% methanol, 30% chloroform, and 10% acetic acid) for 3 hours. After fixation, the tissue were washed in water, dehydrated in ethanol, clarified in xylene, and embedded in paraffin (Histosec, Merck, Dermstadt, Germany). Fragments fixed in 4% paraformaldehyde were employed for general morphological analysis and for immunohistochemical studies of the AR. Fragments fixed in methacarn were employed for general morphological studies, three-dimensional reconstruction, immunofluorescence, and for immunohistochemical procedures for estrogen receptor alpha (ERα) and proliferating cell nuclear antigen (PCNA).

The tissue fragments were serially sectioned (5 μm thickness) in a rotary microtome to obtain sections of the UGS and its structures. Tissue sections were subjected to cytochemical techniques such as hematoxylin-eosin (H&E) staining for general morphological analysis, and to immunohistochemical studies. The specimens were analyzed with an Olympus BX60 light microscope (Olympus, Japan), and the images were digitalized using the software DP-BSW V3.1 (Olympus) and Image-Pro Plus version 6.1 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA).

Immunohistochemistry

Tissue sections were subjected to immunohistochemistry for detection of the AR, as described in protocols applied to the prostate (adapted from Cordeiro et al., 2008), ERα, and PCNA. Primary antibodies reactive to the AR (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology, CA, USA), ERα (rabbit polyclonal IgG, MC-20, sc-542, Santa Cruz Biotechnology), and PCNA (mouse monoclonal IgG_{2a}, PC10, sc-56, Santa Cruz Biotechnology, CA, USA) were employed at a dilution of 1:100. Polymer (DAKO Envision^{tm} + Dual link system-HRP, K4061; DAKO, North America, Inc., Carpinteria, California, USA) was used as secondary antibodies; incubation was for 45 minutes at 37°C. The sections were revealed with diaminobenzidine and counterstained with Harris's hematoxylin. The histological sections were analyzed with the Olympus BX60 light microscope (Olympus, Japan).

Immunofluorescence

Tissue sections were subjected to immunofluorescence for the detection of smooth muscle α-actin (mouse monoclonal IgG_{2a}, sc-32251, IA4, Santa Cruz Biotechnology, CA, USA), which were incubated with α-actin antibody at a dilution of 1:100, overnight at 4°C. The next morning, the sections were incubated with fluorochrome-conjugated specific secondary antibodies (anti-mouse, sc-2010, IgG-FITC, Santa Cruz Biotechnology, CA, USA) for 2 hours at room temperature. DAPI (4',6-diamidino-2-phenylindol) was employed to allow the visualization of cell nuclei. The histological sections were analyzed with the Zeiss Imager M2 fluorescence microscope (Zeiss, Göttingen, Germany), and Laser Scanning microscope LSM 710 (Zeiss, Jena, Germany).

Three-dimensional reconstruction

Three-dimensional reconstruction was performed to determine the pattern of prostatic budding and branching in male gerbils of all experimental groups. To this end, serial histological sections (5 μm thickness) were obtained from the UGS and further stained with H&E. The serial sections were analyzed using the Olympus BX60 light microscope (Olympus, Japan) and digitalized with the software DP-BSW V3.1 (Olympus, Japan). The alignment and the reconstruction of the UGS structures were performed with the Reconstruct software (Fiala, 2005).

Results

The region of the UGS corresponding to the region of the ventral prostate formation is composed of the urethral epithelium, PM, SML, and VMP

The prostatic buds arose from the urethral epithelium (UR), a tissue characterized by its stratified epithelium, which form a tube inside the UGS (Figs. 1A, B, C). The periurethral mesenchyme (PM), a connective tissue adjacent to the UR, was formed mostly by elongated cells, capillaries, and extracellular matrix (Fig. 1). Just below the VMP, we observed a group of cells that would differentiate into a layer of smooth muscle (SML), which varied in thickness through the UGS and could be distinguished from the other regions of the PM with cytochemical techniques such as H&E, although not so precisely (Fig. 1G). Right above the SML was a structure named the VMP, a kind of mesenchymal tissue, highly condensed and similar to the PM in morphological aspects (Figs.

1A, G; 2A–F). The VMP, a mesenchymal tissue, would differentiate into the connective tissue (stroma) throughout the prostate organogenesis process.

Budding process can be first visualized in fetuses between 20 and 21 days old. From this beginning, the buds undergo an intense process of elongation during the subsequent days of prenatal life

The ventral prostate budding process took place between the 20th and 21st days of fetal life (Figs. 1A–F; 2A, B). While fetuses of the E20 group did not show any visible buds (Figs. 1A–C; 2A), in the E21 group, we could observe the first prostatic buds emerging from the ventral region of the UGS (Figs. 1D–F; 2B). From the 21st day of fetal life, the ventral prostatic buds had increased either in number or in extension throughout the days until the first day of postnatal life (Figs. 1E–R; 2B–F). Between the groups E21–E23, we observed that the budding process was limited to the PM (Figs. 1D–L). However, in the E23 group, it was already possible to observe some buds reaching the lateral portions of the VMP (Fig. 1J).

The onset of the budding process was characterized by the intense cell division process of specific cells, leading to an invagination of the basal layer of UR in order to form solid buds that penetrated inside the adjacent PM (Figs. 1D–F). Following this incoming of the buds in the PM, they started elongating and undergoing an increase in number, which was observed in the E21 to P1 groups (Figs. 1D–R; 2B–F).

The branching process begins when the buds reach the VMP

Whereas the budding process was found to be limited to the periurethral mesenchyme from the 20th to the 23rd days of prenatal life (Figs. 1D–L), the branching process started only when the buds reached the VMP, an event that was observed exclusively for the P1 group (Figs. 1P–R, 2F). This event took place only after the buds had crossed either the periurethral mesenchyme or the space where SML had not yet been completely formed. Finally, the elongating buds reached the VMP, where they would undergo the branching process (Figs. 1P–R).

The SML seems to act as a barrier to the elongation of the prostatic buds, which need to move into a region lacking this layer

The budding process requires complex paracrine signaling, which is mediated either by the PM or the VMP. However, besides this requirement, the buds will reach the VMP only if they can cross the border between the SML and the upper VMP. Therefore, they need to turn away from the SML to reach the VMP. Applying immunofluorescence techniques (Figs. 3A–L), we observed that the SML acted as a natural barrier to the elongating process of the buds so that the buds needed to turn and cross a gap that formed between the SML and the PM (Figs. 4E–L). When the SML was crossed (Fig. 4K), the buds could reach the VMP and follow for the branching process.

The elongation of the buds follows a particular pattern of directionality, which suggests a specific signal mechanism, leading to a "V-shaped elongation"

We observed a particular characteristic of the elongation, in terms of the directionality of bud formation and elongation, which was characterized by a pattern of "V-shaped elongation". In fact, the buds originated from distal portions of the ventral urethral epithelium, showing a directional elongation in a "V" shape (Figs. 4A–D). This suggested directional growth of the buds in response to paracrine signaling (Fig. 4D).

AR and ERα have differential roles during prostate organogenesis

Both nuclear receptors of steroids, the AR and ERα, were found to be expressed in the UGS during the phases analyzed. While the AR was found to be preferentially expressed in the PM of the ventral region of the UGS (Figs. 5A–B), ERα was observed to be intensively expressed either in the UR and buds or in the PM (Figs. 5C–D). We observed weak expression of the AR in the cells lining the prostatic buds (Fig. 5B), which was different to the pattern observed for ERα, which showed intense expression in the buds (Fig. 5D).

PCNA is highly expressed predominantly in the basal layer of the urethral epithelium, in the buds, and in the periurethral mesenchyme

PCNA expression was characterized by an intense number of positive cells either in the epithelial compartments (UR and buds) or inside the PM (Figs. 5E–F). A particular finding regarding

PCNA was its preferential localization in the basal portion of the UR (Fig. 5E), showing that the most basal cells proliferated in order to form the emerging buds.

The VMP also expresses AR and ERα and the pattern of expression follows that seen in the periurethral mesenchyme and in buds

The VMP showed a similar pattern of AR and ERα expression as that seen before in the periurethral mesenchyme and in buds emerging from the UR. While the AR was found to be preferentially expressed in the VMP (Fig. 6A) and not in the buds (Fig. 6B), ERα showed marked expression either in the VMP (Fig. 6C) or in the buds (Fig. 6D).

Discussion

Overall, these data showed that the process of ventral prostate budding took place in the UGS of male gerbils between the 20th and 21st prenatal days, following by the elongation of the buds heading to the VMP. In gerbils at 20 days of prenatal life (E20), we did not find evidence of buds emerging from the UR. However, the UR of the E21 group showed the first emerging buds, indicating that the process of budding started between the 20th and 21st prenatal days in *M. unguiculatus*. Similar studies have been performed in rats of Wistar and Sprague Dawley strains (Thomson et al., 2002), which showed that the buds became visible between the 18th and 19th days of prenatal life. We propose that these differences between rats and gerbils may be explained by the fact that rats generally have a gestational period of 22 days, whereas gerbils have a gestational period of 24–26 days. This difference relating to gestational period may, somehow, explain these differences in terms of the precise moment when the buds begin to emerge from the UR, which is later in gerbils than in rats. According to Timms et al. (1994), studies with *Praomys natalensis* have shown that the UGS of this rodent presents the first ventral and dorsal buds after 20 days of prenatal life. This is similar to the gerbil, considering that *P. natalensis* has a gestational period of approximately 23 days. Altogether, these evidences show an approximate pattern for these rodents in terms of the duration of the initial prostate developmental program, with a mean duration of 3 or 4 days before birth.

Once the budding process took place, the buds underwent a fast elongation through the PM, reaching the VMP at approximately between the 23rd and 24th prenatal days. The elongation of the buds was a fast process that occurred until the moment when they reached the VMP, when the branching morphogenesis process begins. An interesting observation was the directionality of bud formation and elongation, which was characterized by a pattern of “V-shaped elongation”. The

ventral buds originated from the ventral urethral epithelium, showing directional elongation in a "V" shape. All our analyses showed a lack of bud elongation towards the SML region, which was clearly seen in cytochemical analysis, three-dimensional reconstruction, and immunofluorescence analysis. According to Thomson et al. (2002), the SML regulates paracrine signaling between the VMP and the buds. The buds receive a directional signal of the VMP, which crosses the gaps between the VMP and PM, allowing the elongation of the buds (Thomson et al., 2002). Indeed, according to Cunha et al. (2003), whereas the urogenital sinus mesenchyme (UGM) induces epithelial differentiation, the developing prostatic epithelium induces SML differentiation in the UGM.

After the buds cross the PM, they reach the VMP and begin another stage of the prostate developmental process known as branching morphogenesis (Prins and Putz, 2008). We could observe the beginning of the branching morphogenesis process only when the buds reached the VMP, which occurred in animals with 1 day of postnatal life (P1 group). These findings suggest that there is a drift in the pattern of signaling when the buds reach the VMP, which allows the beginning of the branching morphogenesis process. Some studies have shown the intricate network of paracrine signaling during branching morphogenesis, which involves several morphogens such as BMP4, FGF10, Shh, Nkx3.1, the Hox and Fox families, Notch, etc. (Thomson and Marker, 2006; Prins and Putz, 2008).

Differently of rodents, in humans the development of the prostate begins about the 10th week of gestation. During the sequential periods of fetal life, the prostatic buds elongate and initiates the branching morphogenesis process. Latter, during the postnatal period of life and under the influence of androgens, the buds undergo the process of luminal enlargement and the epithelial lining the acini initiates the synthesis of secretory products (Timms, 2008).

Several studies have shown the importance of the SML in regulating the initial part of prostate organogenesis (Timms et al., 1994; Thomson et al., 2002; Thomson, 2008), a determinant process for exact establishment of the glandular architecture. Our analysis employing three-dimensional reconstruction techniques showed that the smooth muscle presented an interruption between the SML of the bladder and the SML of the urethral segment in E20–E24 groups, which comprised exactly the region of the presence of the VMP. However, in the P1 group, part of this interval was absent, so that the SML of the urethral segment showed a continuity with the bladder SML. Only the region where the buds showed communication with the VMP remained lacking in SML. These findings suggest that the absence of smooth muscle is a determinant for prostatic budding, because it allows the elongation of the buds toward the VMP. These findings are in accordance with Thomson et al. (2002), who have shown that the UGS of male rats has a interrupted area lacking in smooth muscle, which comprises the exact area of the presence of the VMP.

According to Thomson et al. (2002), in male rats the SML in the UGS region remains interrupted from the 17th to 20th prenatal days, being maintained by the high concentration of testosterone secreted by the testis. Thomson et al. (2002) has proposed that the SML acts as a barrier between the VMP and the urethral epithelium, which prevents the development of the prostate in female rats. In this way, androgens play a central role in regulating prostatic induction by regulating differentiation of the SML and consequently signaling between the VMP and buds (Thomson et al., 2002). Taken together, these evidences show the importance of the smooth muscle and steroid receptors during the initial events of prostate development. However, the exact mechanism by which the SML regulates prostatic budding remains incomplete.

Besides smooth muscle regulating prostate signaling and the morphogens such as BMP4, FGF10, Shh, Nkx3.1, Hox, Fox, and Notch (Thomson and Marker, 2006; Prins and Putz, 2008), hormonal steroids such as androgens and estrogens are extremely important for the prostate developmental process (Thomson et al., 2002; McPherson et al., 2006; Wilson, 2011). Within this scenario, the androgen receptors (ARs) and estrogen receptors (ERs) play a fundamental role in prostate organogenesis, acting as transcriptional factors and being responsible for regulating prostate organogenesis and also during aging of this gland (Cunha, 2008; Ellem and Risbridger, 2009; Wilson, 2011). Thus, it is evident that androgens play a fundamental role in normal prostate morphogenesis, so that interferences may disrupt this process, leading the gland to develop an abnormal architecture and increasing the risk of development of prostatic diseases with aging (Ellem and Risbridger, 2009; Schaeffer et al., 2008; Timms and Hofkamp, 2011; Perez et al., 2011; Biancardi et al., 2012).

Our findings for the AR showed high expression of this receptor in both the PM and the VMP. On the other hand, the urethral epithelium and the buds showed weak or even no AR expression. These findings suggest that the AR is first required in the UGM, which acts to regulate the expression of paracrine factors that regulate the signaling towards the epithelial compartment. The requirement for the AR in the mesenchyme of the UGS and the absence of AR expression in the epithelial compartment have already been shown (Takeda et al., 1985; Cunha and Chung, 1981; Cunha, 2008).

Besides AR, we observed that the expression of ERα showed some peculiar differences. ERα was found to be expressed either in mesenchyme (PM or VMP) or in the epithelial (buds and urethral epithelium) compartments. These findings suggest that ERα acts in both compartments in order to regulate epithelium-mesenchyme paracrine signaling in both directions. According to McPherson et al. (2008), ERα is first expressed during early events of prostate development. These evidences are in accordance with studies reporting on the role of this receptor, which mediates cell proliferation (Morani et al., 2008; Ellem and Risbridger, 2009). Besides ERα, another variant of estrogen receptors

named ERβ is proposed to be expressed later, being important for later periods of development such as puberty and adulthood (McPherson et al.,2008).

Thus, we believe that the understanding of the detailed events that take place during prostate organogenesis may contribute valuable data relating to this complex biological process. Therefore, considering that prostate developmental processes may somehow mimic the tumorigenesis process, we believe that future investigations on other events related to prostate development and on the drugs used in cancer therapy may open new directions in understanding the mechanisms involved in prostatic diseases, such as benign prostate hyperplasia (BPH) and prostate cancer (PCa).

Besides all these aspects, the employment of gerbils (*M. unguiculatus*) as an experimental model has been increasing lately, mainly because these rodents show a propensity to develop prostatic lesions during aging (Campos et al., 2008). Moreover, these animals have contributed valuable data in experiments involving responses to drug administration (Corradi et al., 2009; Biancardi et al., 2012; Perez et al., 2011). Altogether, these aspects have drawn our attention to understanding prostate development in this rodent model, since new evidences regarding this process may contribute to the understanding of the complex events that regulate both normal and pathologic prostate growth. Therefore, the knowledge of prostate development in gerbils may open new perspectives in the prostatic biology field, since this animal model has shown relevant biological particularities in studies focusing on prostate diseases.

Conclusion

Overall, the present study shows that the budding process of the ventral prostate in gerbil (*M. unguiculatus*) is a key period for the normal establishment of prostate architecture. We conclude that prostatic budding in gerbil is a complex process, as previously described in other studies (Thomson et al., 2002), which comprises well-defined pathways that are activated spatially and temporally during the developmental process. Besides, prostate budding in gerbil is relatively fast developmental event that take place between the 20^{th} and 21^{st} prenatal days and proceed until the first day of postnatal life (P1), the moment at which the branching process starts in order to continue prostate formation. During this event, the cells lining the buds undergo an intense rate of cell division in order to allow the elongation of the buds towards the VMP, when the incoming buds start to undergo the process of branching morphogenesis.

Acknowledgements

We are grateful to Luiz Roberto Falleiros Júnior and Cássia Regina Suzuki Caires, as well as other researchers of the Laboratory of Microscopy and Microanalysis for their technical assistance.

This work was supported by grants from the Brazilian agency FAPESP (São Paulo Research Foundation, Procs. Nr. 2009/53990-2, 2009/16789-7, 2011/06335-9, 2011/20358-1) and CNPq (Brazilian National Research and Development Council, Proc. Nr. 301596/2011-5).

Conflict of interest

The authors declare no conflict of interest to this work.

Author Contributions

The work presented here was carried out as a collaboration. All of the authors (BDAS, MFB, FCAS RMG, PSLV, and SRT) participated in the design, interpretation of the results, and review of the manuscript. BDAS and MFB performed the experiments. MFB wrote the manuscript. FCAS, RMG, PSLV, and SRT equally contributed to the supervision of this work.

References

Biancardi MF, Perez APS, Góes RM, Santos FC, Vilamaior PSL, Taboga SR. 2012. Prenatal testosterone exposure as a model for the study of endocrine-disrupting chemicals on the gerbil prostate. Exp Biol Med 237:1298-1309.

Bruni-Cardoso A, Lynch CC, Rosa-Ribeiro R, Matrisian LM, Carvalho HF. 2010. MMP-2 contributes to the development of the mouse ventral prostate by impacting epithelial growth and morphogenesis. Dev Dyn 239(9):2386-2392.

Campos SGP, Zanetoni C, Scarano WR, Vilamaior PS, Taboga SR. 2008. Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. Int J Exp Pathol 89(1):13-24.

Cordeiro RS, Scarano WR, Campos SGP, Santos FC, Vilamaior PS, Góes RM, Taboga SR. 2008. Androgen receptor in the Mongolian gerbil ventral prostate: Evaluation during different phases of postnatal development and following androgen blockage. Micron 39:1312-1324.

Corradi LS, Campos SG, Santos FC, Vilamaior PS, Góes RM, Taboga SR. 2009. Long-term inhibition of 5-alpha reductase and aromatase changes the cellular and extracellular compartments in gerbil ventral prostate at different postnatal ages. Int J Exp Pathol 9(1):79-94.

Cunha GR. 2008. Mesenchymal-epithelial interactions: past, present, and future. Differentiation 76:578-586.

Cunha GR, Chung LW. 1981. Stromal-epithelial interactions-I. Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice. J Steroid Biochem 14(12):1317-1324.

Cunha GR, Hayward SW, Wang YZ, Ricke WA. 2003. Role of the stromal microenvironment in carcinogenesis of the prostate. Int J Cancer 107:1-10.

Ellem SJ, Risbridger GP. 2009. The dual, opposing roles of estrogen in the prostate. Ann N Y Acad Sci 1155:174–86.

Fiala JC. 2005. Reconstruct: a free editor for serial section microscopy: J Microsc 218(Part1):52-61.

Huang Z, Hurley PJ, Simons BW, Marchionni L, Berman DM, Ross AE, Schaeffer EM. 2012. Sox9 is required for prostate development and prostate cancer initiation. Oncotarget 3(6):651-663.

McPherson SJ, Ellem SJ, Risbridger GP. 2008. Estrogen-regulated development and differentiation of the prostate. Differentiation 76:660-670.

Meeks J, Schaeffer EM. 2011. Genetic regulation of prostate development. J Androl 32(3):210-217.

Morani A, Warner M, Gustafsson J-Å. 2008. Biological functions and clinical implications of oestrogen receptors alfa and beta in epithelial tissues. J Intern Med 264(2):128-142.

Ousset M, Keymeulen AV, Bouvencourt G, Sharma N, Achouri Y, Simons BD, Blanpain C. 2012. Multipotent and unipotent progenitors contribute to prostate postnatal development. Nat Cell Biol 14(11):1131-1138.

Perez APS, Biancardi MF, Góes RM, dos Santos FA, Taboga SR. 2011. Exposure to ethinylestradiol during prenatal development and postnatal supplementation with testosterone causes morphophysiological alterations in the prostate of male and female adult gerbils. Int J Exp Pathol 92(2):121-130.

Perez APS, Biancardi MF, Vilamaior PSL, Góes RM, Santos FC, Taboga SR. 2012. Microscopic comparative study of the exposure effects of testosterone cypionate and ethinylestradiol during prenatal life on the prostatic tissue of adult gerbils. Microsc Res Tech 75(8):1084-1092.

Prins GS, Putz O. 2008. Molecular signaling pathways that regulate prostate gland development. Differentiation 76:641-659.

Shaeffer EM, Marchionni L, Huang Z, Simons B, Blackman A, Yu W, Parmigiani G, Berman DM. (2008) Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer. Oncogene 27:7180-7191.

Takeda H, Mizuno T, Lasnitzki I. 1985. Autoradiographic studies of androgen binding sites in the rat urogenital sinus and postnatal prostate. J Endocrinol 104(1):87-92.

Thomson AA. 2008. Mesenchymal mechanisms in prostate organogenesis. Differentiation 76:587-598.

Thomson AA, Marker PC. 2006. Branching morphogenesis in the prostate gland and seminal vesicles. Differentiation 74:382-392.

Thomson AA, Timms BG, Barton L, Cunha GR, Grace OC. 2002. The role of smooth muscle in regulating prostatic induction. Development 129:1905-1912.

Timms BG. 2008. Prostate development: a historical perspective. Differentiation 76:565-577.

Timms BG, Hofkamp L. 2011. Prostate development and growth in benign prostatic hyperplasia. Differentiation 82:173-183.

Timms BG, Mohs TJ, Didio, LJA. 1994. Ductal budding and branching patterns in the developing prostate. J Urol 151(5):1427-1432.

Wilson JD. 2011. The critical role of androgens in prostate development. Endocrinol Metab Clin N Am 40:577-590.

Wu X, Xu K, Zhang L, Deng Y, Lee P, Shapiro E, Monaco M, Makarenkova HP, Li J, Lepor H, Grishina I . 2011. Differentiation of the ductal epithelium and smooth muscle in the prostate gland are regulated by the Notch/PTEN-dependent mechanism. Dev Biol 356:337-349.

Figure Legends

Figure 1. Histology of sequential budding events in male gerbil prostate. Histological sections of E20–P1 groups were stained with hematoxylin-eosin, showing the complete pattern of the early prostatic developmental period. (A, B, C) Transverse section of the UGS in the E20 group, showing the lack of buds arising from the UR. (D, E, F) UGS of the E21 group, showing the beginning of the budding process, evidenced by the formation of small epithelial invaginations. (G–L) Sequential periods of bud elongation characterized by a fast epithelial proliferation towards the VMP. (M, N, O) UGS of the E24 group, showing penetration of the buds inside the VMP. (P, Q, R) The first prenatal day (P1 group) is characterized by the beginning of the branching morphogenesis process; branching at the tip of the buds can be observed. VMP (ventral mesenchymal pad); PM (periurethral mesenchyme); UR (urethral epithelium); BR (branching); arrows (ventral prostatic buds); large

arrows (smooth muscle layer); arrowhead (cell division); dashed lines (budding and/or branching); insert (area that is shown at higher magnification in the next image). (A, D, G, J, M, P – scale bar: 50 μm; B, E, H, K, N, Q – scale bar: 20 μm; C, F, I, L, O, R – scale bar: 10 μm).

Figure 2. Three-dimensional reconstruction of the UGS of all experimental groups. (A) Section of the UGS of the E20 group showing the absence of buds arising from the UR. (B) UGS of the E21 group, showing the presence of the first buds arising from the UR. (C, D) UGS of the E22 and E23 groups, showing the progression of bud elongation towards the VMP. (E) UGS of the E24 group, showing the moment when the buds reach the VMP. (F) Representation of the beginning of branching morphogenesis characterized by the presence of small branches arising from the buds. Structures in red (smooth muscle), green (urethral epithelium), dark blue (urethra lumen), light blue (VMP), orange (prostatic buds). VMP (ventral mesenchymal pad); SML (smooth muscle layer); UR (urethral epithelium); BM (bladder muscle); VBD (ventral buds); DLBD (dorsolateral buds); large arrow (buds reaching the VMP in the E24 group); Insert (beginning of the branching process). Scale bar: 200 μm.

Figure 3. Immunofluorescence for smooth muscle α-actin of all experimental groups, showing the influence of the smooth muscle layer during the budding process. (A–D) Presence of the smooth muscle encircling the entire urethral perimeter in sections lacking ventral prostatic buds. These regions precede the gap between the smooth muscle of the bladder and the smooth muscle of the urethra. (E–J) UGS sections of the E22, E23, and E24 groups, showing the pattern of budding in which the buds growth in a "V" shape, crossing the gaps of the smooth muscle layer, towards the VMP. (K, L) Urogenital tract of the P1 group, showing a bud turning from the SML. VMP (ventral mesenchymal pad); PM (periurethral mesenchyme); UR (urethral epithelium); V (ventral); D (dorsal); arrows (gaps of the smooth muscle layer); large arrow (ventral bud penetrating the VMP); dashed lines (buds). (A, C, E, G, I, K – scale bar: 100 μm; B, D, F, H, J, L – scale bar: 50 μm).

Figure 4. Pattern of bud growth showing a V-shaped elongation towards the VMP. (A, B) Sections stained with HandE showing the directionality of bud elongation. (C) Three-dimensional reconstruction of the E23 group, showing the lack of buds at the center of the urethral epithelium and the presence of buds on opposite sides. (D) Schematic representation of the V-shaped elongation of the buds, which is stimulated by a complex cascade of paracrine factors coming from the PM and VMP. VMP (ventral mesenchymal pad); SML (smooth muscle layer); PM (periurethral mesenchyme); UR (urethral epithelium); white arrow (region lacking in ventral buds); PS (paracrine signaling). (A, B – scale bar: 100 μm; C – scale bar: 200 μm).

Figure 5. Immunohistochemical assay showing the expression of the AR, ERα, and PCNA either in the periurethral mesenchyme (PM) or in the epithelial compartment (urethral epithelium and buds) in the UGS of the E23 group. (A, B) The AR shows stronger expression of this receptor in the PM than in the buds. (C, D) ERα shows similar levels of expression in both the PM and the epithelial compartment. (E, F) PCNA shows intense cell proliferation in both the PM and the epithelial compartment. Note the stronger staining in the basal layer of the UR than in the higher layers. PM (periurethral mesenchyme); UR (urethral epithelium); arrows (positive mesenchymal cells); large arrows (ventral prostatic buds); small arrows (positive epithelial cells).

Figure 6. Immunohistochemical assay showing the expression of the AR and ERα in the VMP of the E23 and P1 groups. (A, B) The AR is expressed in the PM but is absent inside the bud. (C, D) ERα shows levels of expression in both the PM and the epithelial compartment. Arrows (positive mesenchymal cells); large arrows (positive epithelial cells).

Figure 1

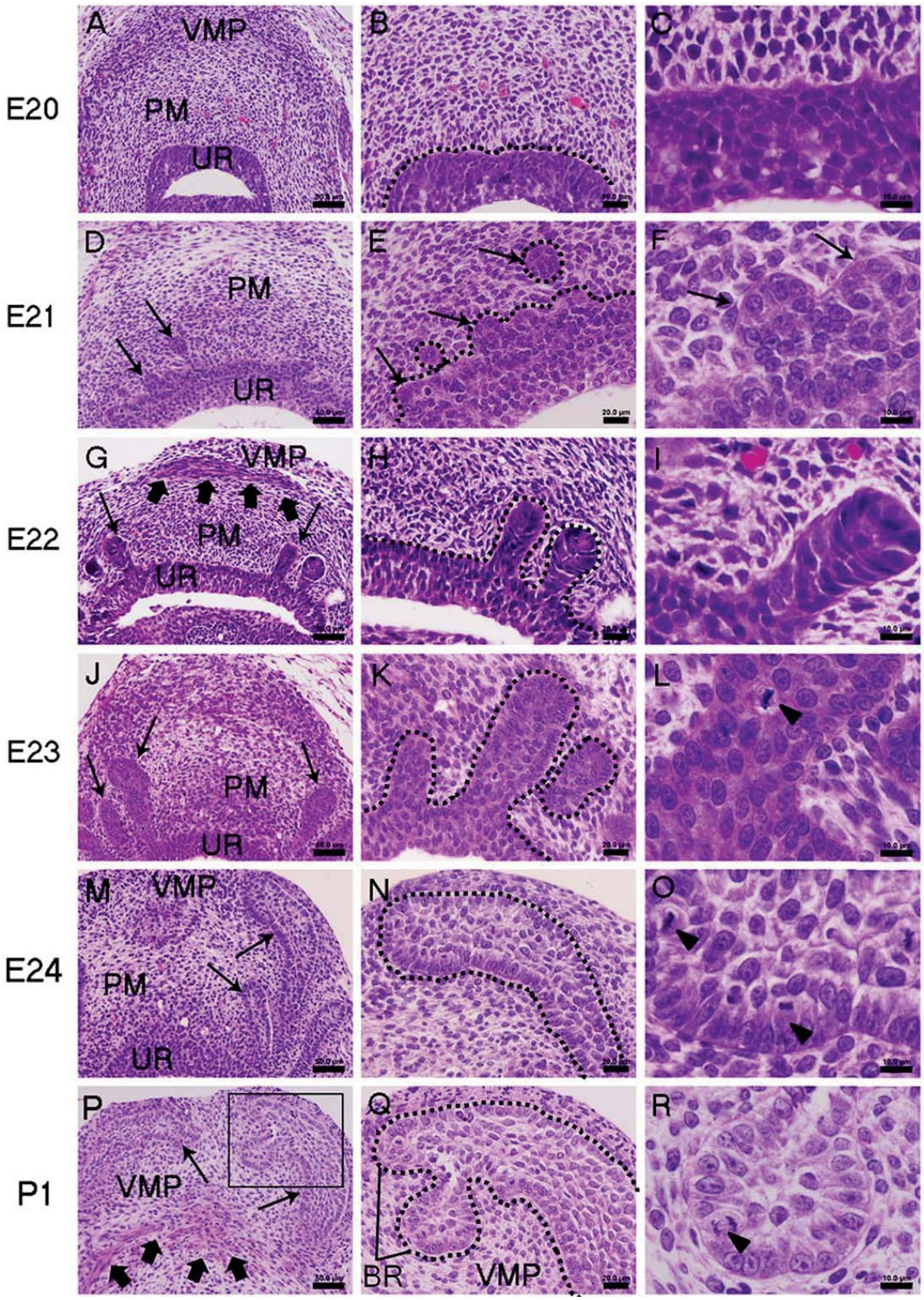

E20
E21
E22
E23
E24
P1
A
VMP
PM
UR
B
C
D
PM
UR
E
F
G
VMP
PM
UR
H
I
J
PM
UR
K
L
M
VMP
PM
UR
N
O
P
VMP
Q
BR
VMP
R

Figure 2

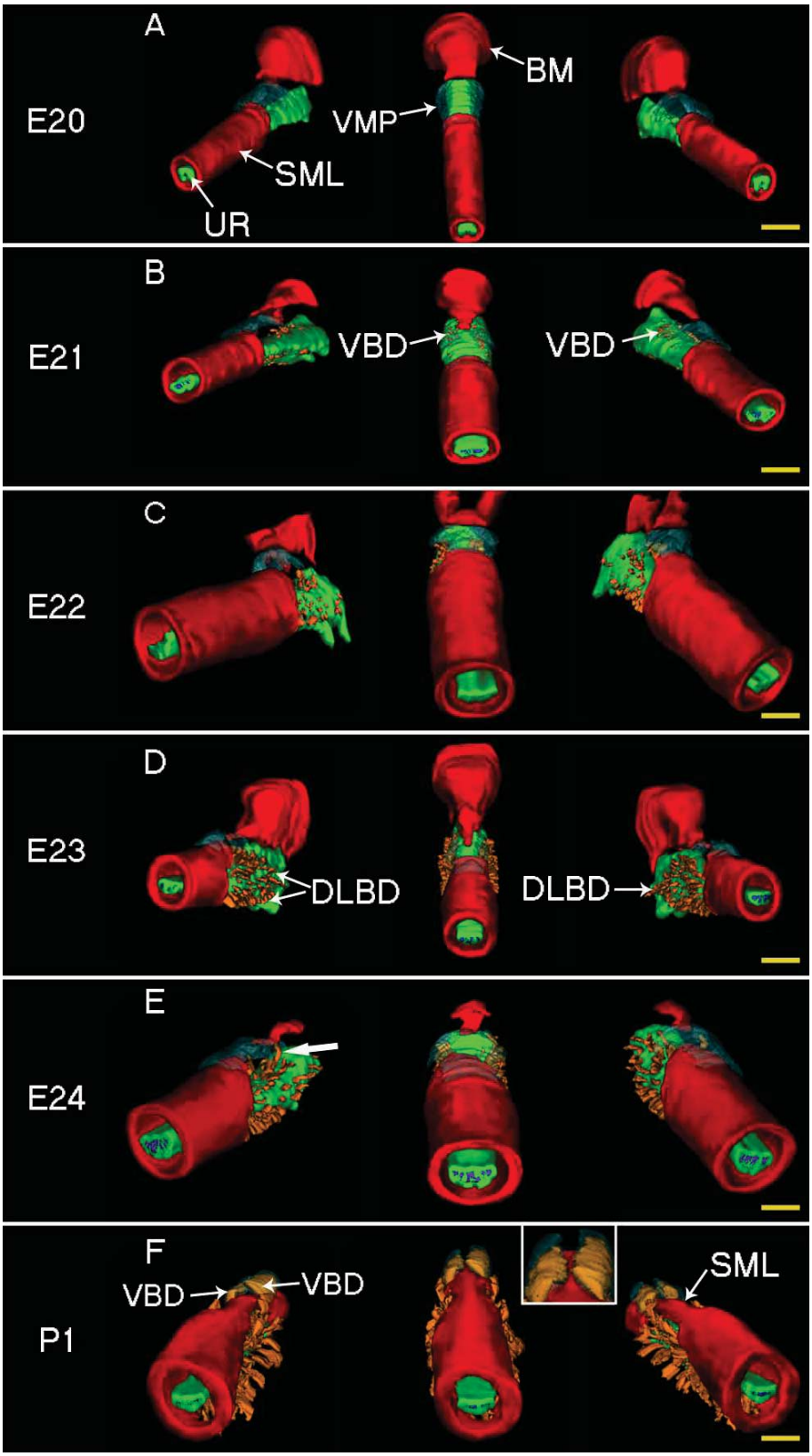

A
E20
BM
VMP
SML
UR
B
E21
VBD
VBD
C
E22
D
E23
DLBD
DLBD
E
E24
F
P1
VBD
VBD
SML

Figure 3

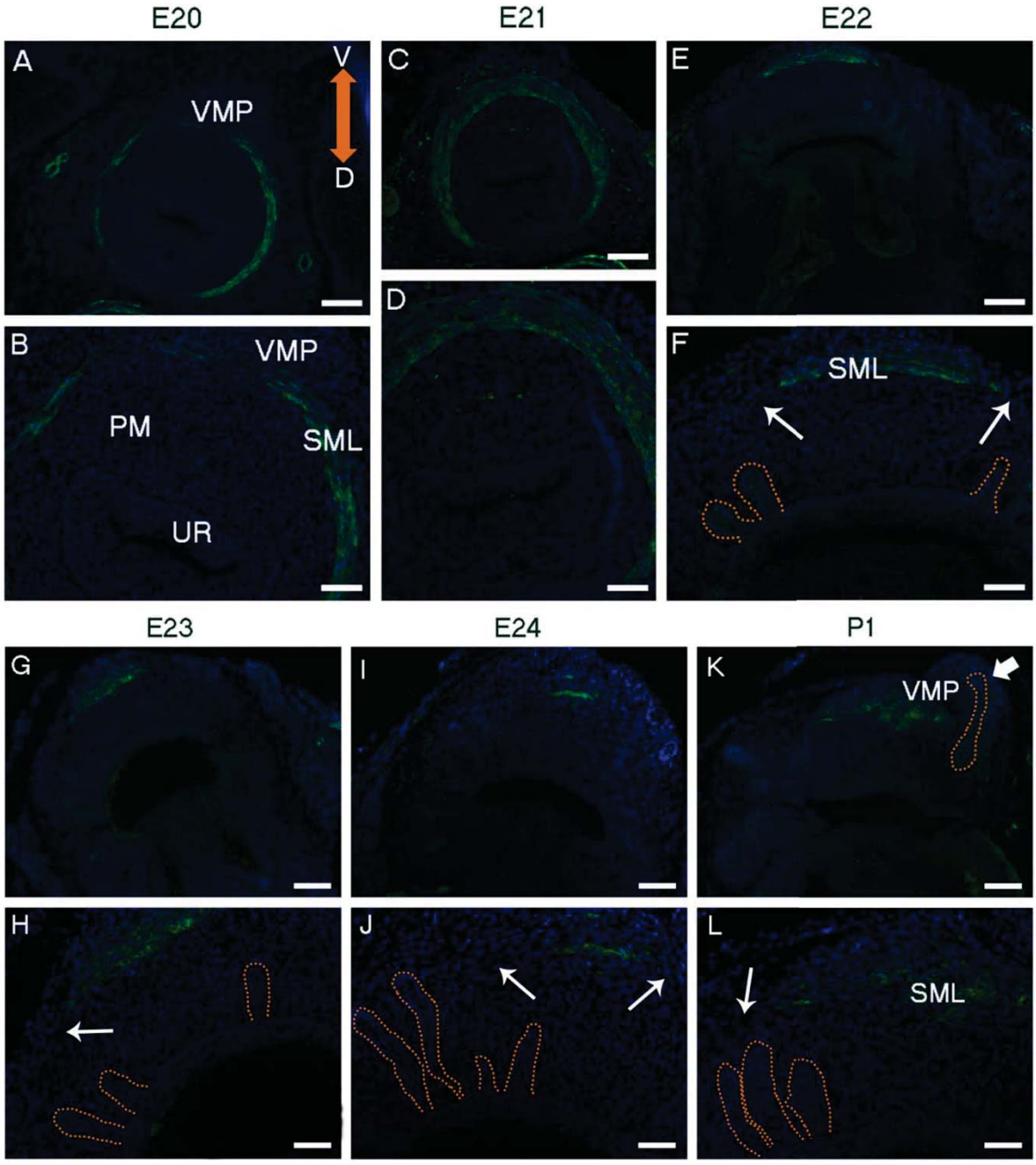

E20
E21
E22
A
V
VMP
D
C
E
B
VMP
PM
SML
UR
D
F
SML
E23
E24
P1
G
I
K
VMP
H
J
L
SML

Figure 4

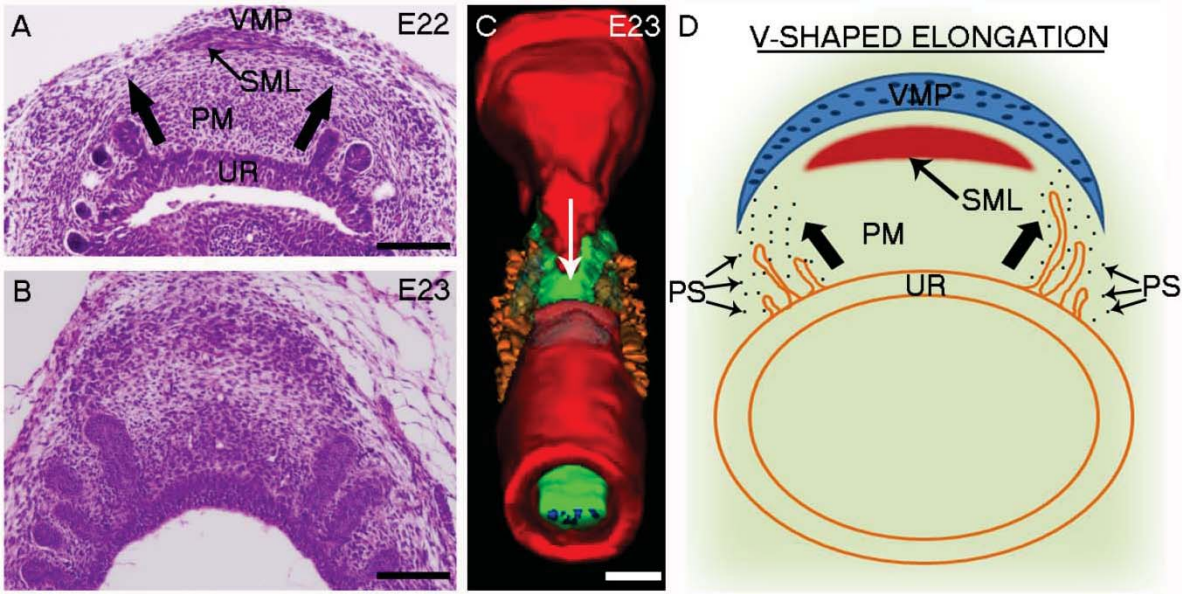

A
VMP
E22
SML
PM
UR
B
E23
C
E23
D
V-SHAPED ELONGATION
VMP
SML
PM
UR
PS
PS

Figure 5

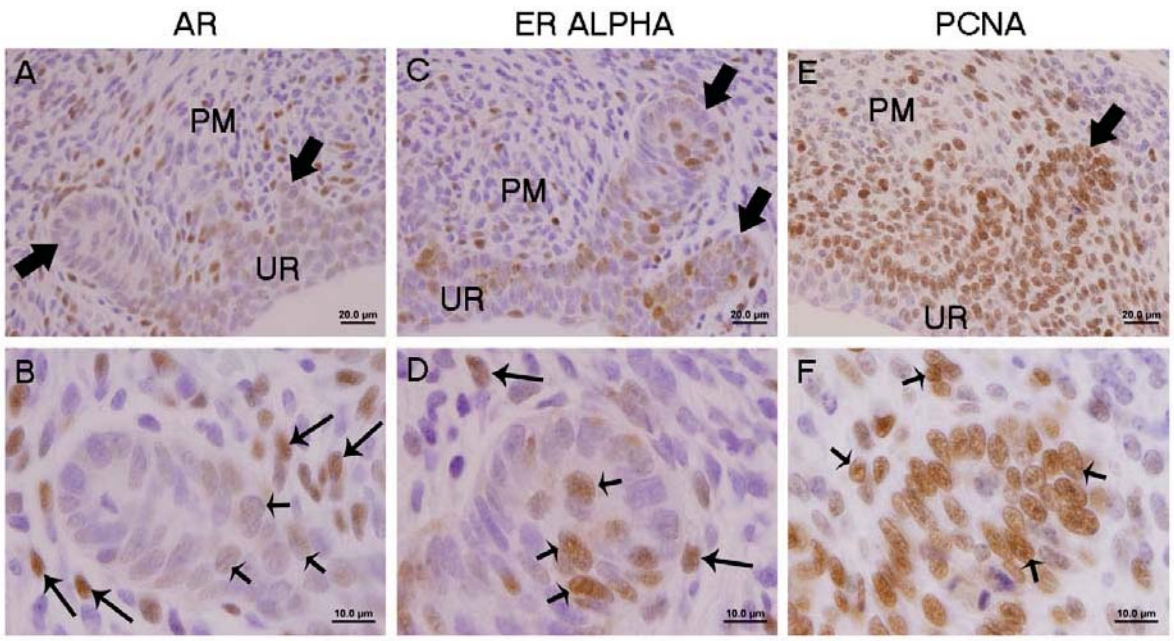
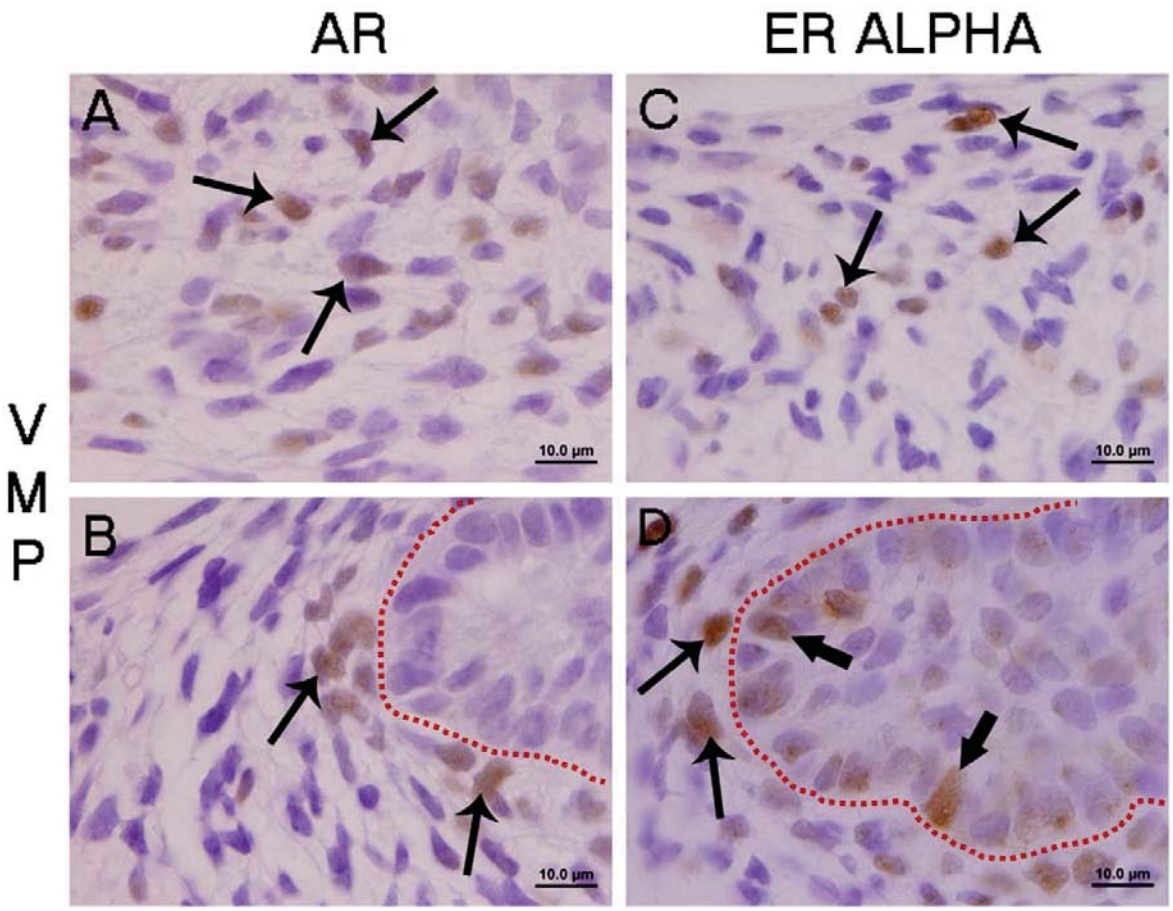

AR
ER ALPHA
PCNA
A
PM
UR
20.0 µm
C
PM
UR
20.0 µm
E
PM
UR
20.0 µm
B
10.0 µm
D
10.0 µm
F
10.0 µm

Figure 6



1. A exposição à testosterona durante a vida pré-natal causou a masculinização das fêmeas, fato evidenciado pelo aumento da distância anogenital, ausência de abertura vaginal e desenvolvimento de tecido prostático ectópico ao redor da vagina. No entanto, fêmeas expostas à testosterona durante a puberdade não apresentaram nenhum sinal de masculinização. Estas evidências mostram que o período de desenvolvimento intrauterino é uma fase muito mais sensível a androgenização das fêmeas do que a puberdade.

2. Os resultados mostraram desdobramentos diferentes no que diz respeito aos grupos experimentais de machos e fêmeas. Ao passo que a exposição das fêmeas durante a fase pré-natal tenha resultado no desenvolvimento de malformações do sistema reprodutor e de lesões prostáticas durante a vida senil, nenhuma evidência de malformações do sistema reprodutor foram verificadas nos machos.

3. Os resultados do presente trabalho reforçam as evidências sobre os efeitos da exposição androgênica anormal sobre o desenvolvimento da próstata. Além disso, este trabalho mostra que com o avançar da idade os efeitos da exposição androgênica anormal durante a gestação se acentuam tanto em machos quanto em fêmeas, indicando os potenciais riscos permanentes que o desbalanço hormonal durante a vida pré-natal pode causar no que diz respeito à saúde prostática.

4. No que diz respeito ao acometimento por hiperplasia adenomatosa associada à inflamação, estes resultados sugerem que as fêmeas são mais suscetíveis à exposição androgênica durante a fase pré-natal, enquanto que os machos além da exposição pré-natal precisaram de uma exposição androgênica adicional durante a puberdade para desenvolverem lesões prostáticas deste tipo.

5. Os resultados mostraram uma intensa alteração dos elementos estromais nas próstatas das fêmeas expostas à testosterona durante a vida pré-natal e puberal, principalmente no

que diz respeito ao aumento da camada de musculatura lisa, rearranjo de colágeno e espessamento das fibras elásticas.

6. De forma geral, todas as próstatas dos animais expostos à testosterona durante a gestação, tanto de machos quanto de fêmeas, apresentaram focos inflamatórios. Estas evidências sugerem uma associação entre o surgimento de lesões prostáticas do tipo hiperplasia adenomatosa e os eventos que dirigem os processos inflamatórios.

7. O processo de brotamento da próstata ventral do gerbilo da Mongólia se inicia entre o 20º e o 21º dia da vida pré-natal. A partir de então, os brotos passam por um intenso processo de elongação durante os próximos dias que antecedem o nascimento.

8. A morfogênese de ramificação do lobo ventral do gerbilo tem início no primeiro dia de vida pós-natal, momento que podem ser visualizadas os primeiras ramificações dos brotos que atingiram o VMP.

9. A camada de musculatura lisa (SML) parece agir como uma barreira à elongação dos brotos prostáticos, o que faz com que os mesmos se direcionem para regiões do mesênquima periuretral onde a SML esteja ausente.

10. A elongação dos brotos prostáticos ventrais segue um padrão de direcionalidade em forma de "V", o que sugere um mecanismo específico de sinalização nesta região.

11. Os receptores nucleares AR e ERα apresentam-se diferentemente distribuídos durante a organogênese prostática do lobo ventral. Enquanto o ERα se encontra expresso tanto no UGM quanto nas células epiteliais dos brotos, o AR se expressa preferencialmente em células do UGM. Estas evidências mostram que a testosterona influencia o brotamento prostático de forma indireta, pois age sobre as células do UGM que expressam AR. Estas, por sua vez, ativam vias de sinalização parácrina as quais atuam sobre as células epiteliais.

12. Também foi possível identificar a marcação de algumas células do VMP para AR e ERα, as quais apresentaram um padrão semelhante ao encontrado para o mesênquima periuretral. Além disso, os brotos que atingiram o mesênquima tinham imunomarcação positiva somente para ERα, fato que sugere uma não ativação da via do AR nas células dos brotos prostáticos.

Adams CE, Norris MI. 1973. Observations on reproduction in the Mongolian gerbil, *Meriones unguiculatus*. J Reprod Fert, 33:185-188.

Almeida CCD, Pinheiro PFF, Segatelli TM, Martinez M, Padovani CR, Martinez FE. 2001. Estrous cycle, anatomy and histology of the uterine tube of the Mongolian gerbil (*Meriones unguiculatus*). Rev Chil Anat, 19(2):191-196.

Barfield MA, Beeman EA. 1968. The oestrous cycle in the Mongolian gerbil, *Meriones unguiculatus*. J Reprod Fert, 17:247-251.

Biancardi MF, Perez APS, Góes RM, Santos FCA, Vilamaior PSL, Taboga SR. 2012. Prenatal testosterone exposure as a model for the study of endocrine-disrupting chemicals on the gerbil prostate. Exp Biol Med, 237:1298-1309.

Biancardi MF, Santos FCA, Madi-Ravazzi L, Góes RM, Vilamaior PSL, Felisbino SL, Taboga, SR. 2010. Testosterone promotes an anabolic increase in the rat female prostate (Skene's paraurethral gland) which acquires a male ventral prostate phenotype. 293(12):2163-75.

Bigsby R, Chapin RE, Daston GP, Davis BJ, Gorski J, Gray LE, Howdeshell KL, Zoeller T, Vom Saal FS. 1999. Evaluating the effects of endocrine disruptors on endocrine function during development. Environ Health Perspect, 107:613-618.

Black BE, Paschal BM. 2004. Intranuclear organization and function of the androgen receptor. Trends Endocrinol Metab, 15(9):411-417.

Cunha GR, Hayward SW, Wang YZ, Ricke W. 2003. Role of the stromal microenvironment in carcinogenesis of the prostate. Int J Cancer, 107:1-10.

Cunha GR. 2008. Mesenchymal-epithelial interactions: past, present, and future. Differentiation, 76:578-586.

Cunha GR. 2008. Mesenchymal–epithelial interactions: past, present, and future. Differentiation, 76:578-856.

Cunha TS, Cunha NS, Moura MJCS, Marcondes FK. 2004. Esteróides anabólicos e a sua relação com a prática desportiva. Rev Bras C Farm, 40(2): 165-179.

Fochi RA, Perez APS, Bianchi CV, Rochel SS, Góes RM, Vilamaior PSL, Taboga SR, Santos FCA. 2008. Hormonal oscillations during the estrous cycle influence the

morphophysiology of the gerbil (*Meriones unguiculatus*) female prostate (Skene Paraurethral Glands). Biol Reprod, 79:1084-1091.

Gardner WA. 1995. Hypothesis: the prenatal origins of prostate cancer. Hum Pathol, 26:1291-1292.

Hotchkiss AK, Furr J, Makynen EA, Ankley GT, Gray Jr LE. 2007a. In utero exposure to the environmental androgen trenbolone masculinizes female Sprague-Dawley rats. Toxicol Lett, 174:31-41.

Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenberg JG, Gray Jr LE. 2007b. Pré-natal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol Sci, 96(2):335-345.

Kirby RS, Christmas TJ, Brawer, M. 1996. Prostate Cancer. 1 ed. London, England: Mosby, 170 p.

Kosik K. 2011. Dialética do Concreto. 2.ed. São Paulo, Paz e Terra.

Mahoney JJ, Witschi E. 1947. Genetics of the female prostate in rats. Genetics, 32:369-378.

McNeal JE. 1981. The zonal anatomy of the prostate. Prostate, 2:35-49.

McPherson SJ, Ellem SJ, Risbridger GP. 2008. Estrogen-regulated development and differentiation of the prostate. Differentiation, 76:660-670.

Meireles JCR, Oliveira SV, Costa-Neto AO, Cerqueira EMM. 2013. Genotoxic and cytotoxic effects of testosterone cypionate (Deposteron®). Mutat Res, 753(2):72-5.

Nelson DL, Cox MM. 2000. Lehninger principles of biochemistry. 3 ed. New York, EUA: Worth Publishers, 1152 p.

Nicholson TM, Ricke WA. 2011. Androgens and estrogens in benign prostatic hyperplasia: Past, present and future. Differentiation, 82:184-199.

Nieschlag E, Behre HM. 1998. Testosterone: action, deficiency, substitution. 2 ed. Berlin, Germany: Springer-Verlag, 594p.

Nishino N, Totsukawa K. 1996. Study on the estrous cycle in the Mongolian gerbil (*Meriones unguiculatus*). Exp Anim, 45(3):283-288.

Norris ML, Adams CE. 1972. Incidence of cystic ovaries and reproductive performance in the Mongolian gerbil, *Meriones unguicuatus*. Lab Anim, 6(3):337-342.

Norris ML, Adams CE. 1981. Time of mating and associated changes in the vaginal smear of the post-parturient Mongolian gerbil (*Meriones unguiculatus*). Lab Anim, 15(2):193-198.

Orlando EF, Kolok AS, Binzcik GA, Gates JL, Horton MK, Lambright CS, Gray Jr LE, Soto AM, Guillette Jr LJ. 2004. Endocrine-disrupting effects of castle feedlot effluente on an sentinel species, the Fathead minnow. Environ Health Perspect, 112(3):353-358.

Perez APS, Biancardi MF, Vilamaior PSL, Góes RM, Santos FCA, Taboga SR. 2012. Microscopic comparative study of the exposure effects of testosterone cypionate and ethinylestradiol during prenatal life on the prostatic tissue of adult gerbils. Microsc Res Tech, 75(8):1084-1092.

Price D. 1963. Comparative aspects of development and structure in the prostate. Natl Cancer Inst Monogr, 12:1–27.

Prins GS, Putz O. 2008. Molecular signaling pathways that regulate prostate gland development. Differentiation, 76:641-659.

Prins GS, Tang WL, Belmonte J, Ho SM. 2008. Perinatal Exposure to oestradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis. Basic Clin Pharmacol Toxicol, 102:134-138.

Prins GS, Tang WL, Belmonte J, Ho SM. 2008. Perinatal Exposure to oestradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis. Basic Clin Pharmacol Toxicol, 102:134-138.

Rochel SS, Bruni-Cardoso A, Taboga SR, Vilamaior PSL, Góes RM. 2007. Lobe identity in the Mongolian gerbil prostatic complex: a new rodent model for prostate study. Anat Rec (Roboken), 290:1233-1247.

Santos FCA, Leite RP, Custódio AMG, Carvalho KP, Monteiro-Leal LH, Santos AB, Góes RM, Carvalho HF, Taboga SR. 2006. Testosterone stimulates growth and secretory activity of the adult female prostate of the gerbil (Meriones unguiculatus). Biol Reprod, 75:370-379.

Shaeffer EM, Marchionni L, Huang Z, Simons B, Blackman A, Yu W, Parmigiani G, Berman DM. 2008. Androgen-induced programs for prostate epithelial growth and

invasion arise in embryogenesis and are reactivated in cancer. Oncogene, 27:7180-7191.

Shehata R. 1972. Female prostate in the house rat Rattus rattus. Acta anat, 83(3):426-34.

Shehata R. 1975. Female prostate in Arvicantihis niloticus and Meriones lybicus. Acta Anat, 92:513-523.

Shehata R. 1980. Female prostate and urethral glands in the home rat, Rattus norvegicus. Acta Anat, 107:286-288.

Singh J, Handelsman DJ. 1999. Imprinting by neonatal sex steroids on the structure and function of the mature mouse prostate. Biol Reprod, 61: 200-208.

Soder O. 2005. Perinatal imprinting by estrogen and adult prostate disease. Proc Natl Acad Sci USA, 102:1269-1270.

Staack A, Donjacour AA, Brody J, Cunha GR, Carroll P. 2003. Mouse urogenital development: a pratical approach. Differentiation, 71:402-413.

Takeda H, Mizuno T, Lasnitzki I. 1985. Autoradiographic studies of androgen binding sites in the rat urogenital sinus and postnatal prostate. J Endocrinol, 104(1):87-92.

Thomson AA, Timms BG, Barton L, Cunha GR, Grace OC. 2002. The role of smooth muscle in regulating prostatic induction. Development, 129:1905-1912.

Thomson AA. 2008. Branching Mesenchymal mechanisms in prostate organogenesis. Differentiation, 76:587-598.

Timms BG, Lee CW, Aumller G, Seitz J. 1995. Instructive induction of prostate growth and differentiation by defined urogenital sinus mesenchyme. Microsc Res Tech, 30(4):319-332.

Timms BG. 2008. Prostate development: a historical perspective. Differentiation, 76:565-577.

Toppari J. 2008. Environmental endocrine disrupters. Sex Dev, 2:260-267.

Wilhelm D, Koopman P. 2006. The makings of maleness towards an integrated view of male sexual development. Nat Genet, 7:620-631.

Wilson JD. 2011. The critical role of androgens in prostate development. Endocrinol Metab Clin N Am, 40:577-590.

Wimpissinger F, Stifter K, Grin W, Stackl W. 2007. The female prostate revisited: perineal ultrasound and biochemical studies of female ejaculate. J Sex Med, 4:1388-1393.

Wimpissinger F, Tscherney R, Stackl W. 2009. Magnetic resonance imaging of female prostate pathology. J Sex Med, 6:1704-1711.

Wu JT. 1974. Artificial insemination and induction of pregnancy in the Mongolian gerbil (*Meriones unguiculatus*). J Reprod Fert, 37:139-140.

Zaviačič M. 1999. The Female Prostate: From vestigial Skene's parauretral glands and ducts to woman's functional prostate. 1.ed. Bratislava, Slovakia: Slovack Academic Press, 171p.

VIII.1 Resultados preliminares referentes ao estudo dos efeitos da exposição pré-natal à testosterona sobre a organogênese prostática de gerbilos (*Meriones unguiculatus*) neonatos.

1. Propostas do estudo

Tendo em vista os resultados obtidos através dos estudos apresentados na presente tese, o nosso grupo tem dado continuidade nesta linha de pesquisa na tentativa de se buscar um maior entendimento dos possíveis mecanismos envolvidos com a alteração do desenvolvimento prostático em decorrência da interferência androgênica. Sendo assim, estes resultados, ainda parciais, aqui apresentados tem o objetivo de mostrar algumas evidências obtidas até o presente momento.

Logo, o objetivo deste estudo tem sido avaliar o padrão da morfogênese prostática em fêmeas neonatas (1 dia de idade) do gerbilo da Mongólia submetidas à exposição pré-natal à testosterona.

2. Material e Métodos

2.1. Animais

As fêmeas e machos de gerbilos (*Meriones unguiculatus*) utilizados neste experimento foram obtidos do Centro de Bioterismo da Universidade Estadual Paulista, campus de Botucatu (SP), e mantidos no biotério do Instituto de Biociências, Letras e Ciências Exatas da UNESP, campus de São José do Rio Preto (SP), em caixas de polietileno, com substrato de maravalha, sob condições controladas de luminosidade e temperatura média de 25°C, sendo fornecidas água filtrada e ração *"ad libitum"*. A água filtrada foi acondicionada em garrafas de vidro, para se evitar a interferência de disruptores endócrinos, como o bisfenol a, que liberados de garrafas plásticas.

2.2. Delineamento experimental

Neste experimento foram utilizadas 10 fêmeas virgens adultas e 10 machos adultos (90 a 120 dias de idade) para formar as matrizes (casais). Cada fêmea foi acondicionada com um macho da mesma idade para que ocorresse o acasalamento. Após a formação dos casais, os mesmos foram aleatoriamente divididos em 2 grupos experimentais, com 5 casais por grupo. Das 10 fêmeas grávidas provenientes destes casais, 5 foram mantidas como grupo controle e 5 foram tratadas com injeções subcutâneas de 500 µg de testosterona (T) diluída em 100 µL de óleo mineral nos 20º e 22º dias de gestação. Após o nascimento dos filhotes, os mesmos foram separados de acordo com o sexo e com o grupo experimental do qual faziam parte, como apresentado abaixo:

Grupo C (Controle/veículo): animais de 1 dia de idade provenientes de mães que não foram submetidas à manipulação hormonal.

Grupo TG (Testosterona na gestação): filhotes expostos durante a gestação a duas doses de 500 µg de T/animal nos 20º e 22º dias de gestação. No dia do nascimentos do filhotes, os mesmos foram sacrificados pela manhã para coleta dos fragmentos teciduais.

O cipionato de testosterona (Deposteron – Novaquímica/Sigma) foi diluído em óleo mineral (Nujol - Mantecorp). Cada injeção subcutânea continha um volume de 100 µL de solução (T + óleo).

Como o número de animais que nascem é variável entre os casais de gerbilos, a utilização de 5 casais por grupo experimental garantiu a obtenção de uma amostra satisfatória e representativa de cada grupo experimental empregado neste experimento. A figura 1 demonstra uma representação esquemática do delineamento experimental.

3. Metodologia

3.1. Processamento histológico e análise morfológica

Fragmentos teciduais que abrangiam as regiões do seio urogenital de brotamento da próstata, com suas regiões correspondentes da bexiga, da uretra e da vagina, foram coletados no momento da morte dos animais. Os fragmentos foram fixados em metacarn

(60% - metanol, 30% - clorofórmio, 10 % - ácido acético), desidratados em série crescente de etanol, clarificados em xilol e então incluídos em Paraplast (Histosec, Merck). Os órgãos emblocados foram seccionados serialmente a 5µm e corados pela hematoxilina e eosina (HE). A documentação fotográfica foi realizada em um sistema de análise de imagens composto por um fotomicroscópio Olympus BX60 (Japão) acoplado ao software DP controller (Olympus) de análise de imagem.

4. Resultados parciais

4.1. Aspectos morfológicos

Através da análise morfológica geral, foi possível observar o padrão de brotamento a partir do epitélio uretral das fêmeas neonatas. Nas fêmeas do grupo C pode-se observar o padrão normal de disposição dos brotos no mesênquima periuretral (PM) ao redor da uretra (Fig. 2A, B). Além disso, não há crescimento de brotos em direção à parede lateral da vagina (Fig. 2A, B), assim como observado nas fêmeas que foram expostas à testosterona durante a gestação (grupo TG) (Fig. 2C, D). Além do desenvolvimento destes brotos atípicos ao redor da vagina (Fig. 2C), a uretra destas fêmeas tratadas também apresentou um grande número de brotamentos que proliferavam em todas as direções da circunferência uretral (Fig. 2D). Outro aspecto diferencial foi a presença de regiões com ausência da camada de musculatura lisa (SML) ao redor da porção dorsolateral da uretra das fêmeas do grupo TG (Fig. 2D).

5. Discussão

Embora ainda seja um assunto com pouco explorado, relatos que dizem respeito à origem pré-natal das doenças prostáticas podem ser encontrados na literatura científica (Gardner WA, 1995; Schaeffer et al., 2008). Além disso, trabalhos mostrando a semelhança dos programas de expressão gênica durante o desenvolvimento prostático e durante a instalação do câncer de próstata (Schaeffer et al., 2008), tem mostrado a importância de se

empregar o modelo de crescimento inicial da próstata como uma promissora metodologia para se entender a origem de doenças complexas como o câncer de próstata.

O artigo 1 desta presente tese mostrou que fêmeas de gerbilo expostas à testosterona durante o período fetal podem apresentar sérios desvios do desenvolvimento, como ausência de abertura vaginal, aumento da distância anogenital, desenvolvimento de hidrometrocolpos e formação de tecido prostático ectópico no trato reprodutor das fêmeas de gerbilos. Além disso, a próstata destas fêmeas foram afetadas por lesões do tipo hiperplasia adenomatosa, normalmente associadas à inflamação.

Embora ainda muito preliminares, os resultados obtidos com as fêmeas neonatas tem mostrado como a exposição à testosterona exógena pode alterar completamente o padrão da morfogênese prostática durante os momentos iniciais do desenvolvimento. As primeiras análises morfológicas mostraram como a exposição à testosterona altera completamente o padrão de brotamento da próstata nas fêmeas de gerbilo, causando alterações irreversíveis para o animal. Dentre estas alterações, o aumento no número de brotos que emergem do epitélio uretral e o aparecimento de tecido prostático ectópico ao redor da vagina foram as características mais destacáveis observadas nas fêmeas do grupo TG. Estes resultados esclarecem, de certa forma, como se dá origem embriológica do aparecimento de tecido prostático ectópico ao redor da parede da vagina de fêmeas de gerbilo velhas.

Além disso, nós acreditamos que a continuidade destes estudos poderão fornecer maiores evidências de qual a relação existente entre este desenvolvimento anormal e o aumento na predisposição para o desenvolvimento de lesões durante a vida senil, fato observado e discutido no artigo 1 desta presente tese.

Outros aspectos importantes também foram evidenciados, como a presença de regiões com interrupção da camada de musculatura lisa na porção dorsolateral em relação à uretra, aspecto não observados nas seções teciduais das fêmeas do grupo C. Existem evidências na literatura mostrando que a testosterona é fundamental para o controle da diferenciação da camada de musculatura lisa (Thomson et al., 2002). Além disso, a manutenção da indiferenciação da camada de musculatura lisa é essencial para que haja tempo suficiente dos brotos atingirem os respectivos mesênquimas para que possam

prosseguir no desenvolvimento dando sequência à morfogênese de ramificação (Prins e Putz, 2008).

Outro aspecto importante é a presença de tecidos mesenquimais laterais que provavelmente estão envolvidos na sinalização durante o brotamento, mas cujos mecanismos ainda são desconhecidos. Timms (2008) comenta brevemente sobre a existência de mesênquimas laterais na uretra de ratos, embora ainda pouco se conheça sobre a biologia dos mesmos.

De acordo com Thomson et al. (2002), o papel dos andrógenos durante a regulação da organogênese prostática é a de inativar um mecanismo que previne as interações ou sinalizações ente o epitélio uretral e o VMP. Esta ideia é muito interessante, pois como observado no grupo de fêmeas expostas à testosterona durante a vida fetal, houve um aumento considerável no número de brotos prostáticos emergindo a partir do epitélio uretral. Seguindo a proposta de Thomson et al. (2002), a testosterona pode ter causado a inativação dos mecanismos que previnem as sinalizações entre epitélio uretral e o VMP, o que levou à uma maior estimulação do processo de brotamento prostático.

Tendo em vista estes todos estes aspectos, nós acreditamos que a continuidade destes trabalhos poderão fornecer evidências mais esclarecedoras para a melhor compreensão dos mecanismos do desenvolvimento prostático tanto em condições normais quanto anormais. Desta maneira, assim como já proposto em trabalhos prévios da literatura (Schaeffer et al., 2008), nós acreditamos que o modelo de desenvolvimento inicial da próstata pode ser muito útil em estudos que visam uma melhor compreensão tanto dos mecanismos do desenvolvimento normal quanto dos que coordenam a instalação das doenças prostáticas.

6. Referências

Gardner WA. 1995. Hypothesis: the prenatal origins of prostate cancer. Hum Pathol, 26:1291-1292.

Prins GS, Putz O. 2008. Molecular signaling pathways that regulate prostate gland development. Differentiation, 76:641-659.

Shaeffer EM, Marchionni L, Huang Z, Simons B, Blackman A, Yu W, Parmigiani G, Berman DM. 2008. Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer. Oncogene, 27:7180-7191.

Thomson AA, Timms BG, Barton L, Cunha GR, Grace OC. 2002. The role of smooth muscle in regulating prostatic induction. Development, 129:1905-1912.

Timms BG. 2008. Prostate development: a historical perspective. Differentiation, 76:565-577.

7. Legendas

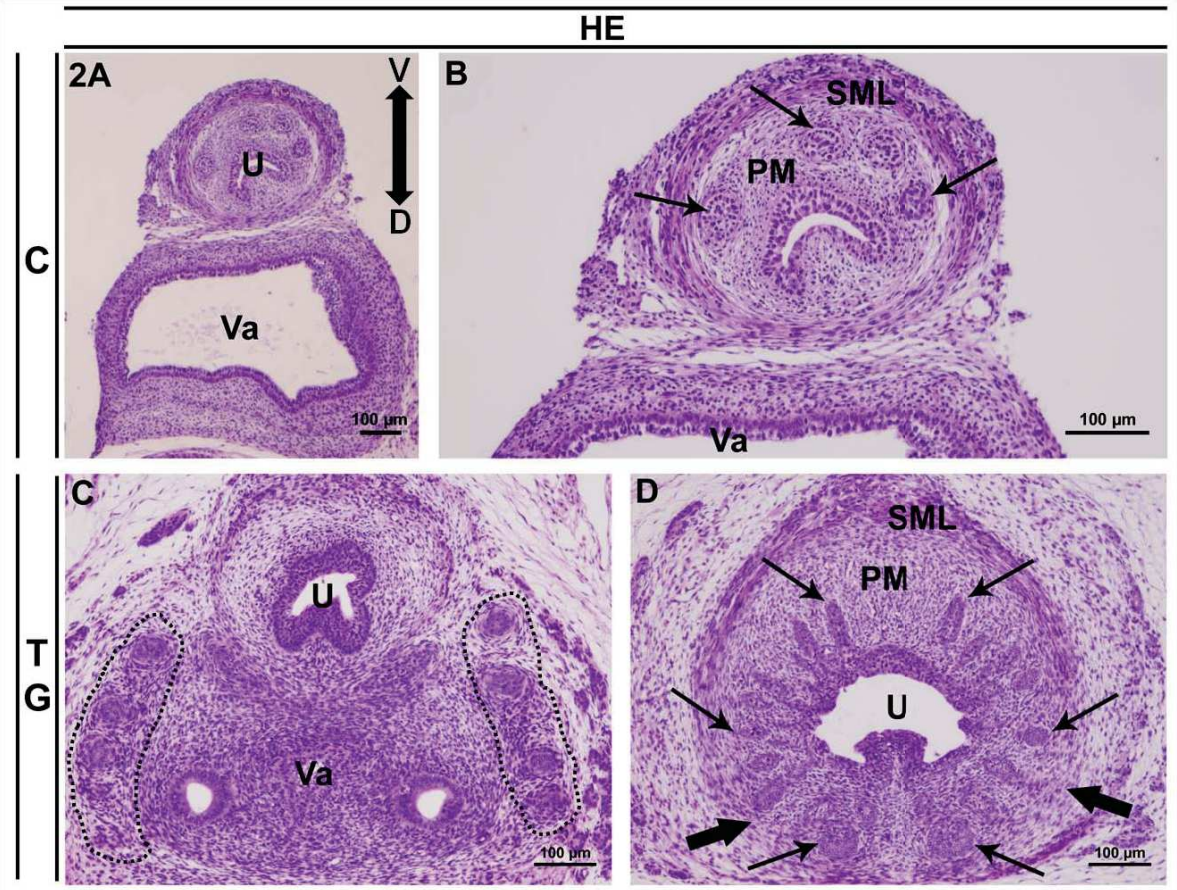
Figura 1. Representação esquemática do protocolo experimental empregado neste estudo. E0 representa o dia do cruzamento e P0 o dia do nascimento dos filhotes. E20 - E24 representa, consecutivamente, os dias do final do período gestacional no qual tem início os eventos da organogênese prostática. P1 representa o primeiro dia pós-natal, o qual corresponde ao dia de sacrifício dos filhotes.

Figura 2. Aspectos morfológicos do trato reprodutor das fêmeas corados com HE. (Fig. 2A, B) mostram o padrão de crescimento do brotos da próstata em fêmeas controle. Observe a ausência de brotos atípicos ao redor da vagina (Fig. 2A). (Fig. 2C, D) mostra a alteração no padrão de morfogênese de ramificação dos brotos prostáticos em fêmeas que foram expostas à testosterona exógena durante o período fetal. Observe a presença de brotos atípicos ao redor da vagina (Fig. 2C), além do aumento muito expressivo no número de brotos que surgem a partir do epitélio uretral em decorrência do estímulo androgênico (Fig. 2D). Na região dorsolateral é possível se verificar uma descontinuidade da camada de musculatura lisa que presente da região ventral até, aproximadamente, a porção média lateral da uretra. Uretra (U); Va (vagina); V (ventral); D (dorsal); PM (mesênquima periuretral); SML (camada de musculatura lisa); setas (brotos prostáticos); setas largas (regiões com interrupção da SML); pontilhado (região com brotos prostáticos atípicos).

Figura 1


PERÍODO GESTACIONAL
PERÍODO NEONATAL
E0
E20
E21
E22
E23
E24
(P0)
(P1)
DIA DO ACASALAMENTO
DIAS DO TRATAMENTO COM TESTOSTERONA
DIA DO NASCIMENTO DOS FILHOTES
DIA DO SACRIFÍCIO DOS FILHOTES
1

Figura 2


HE
C
2A
V
D
U
Va
100 µm
B
SML
PM
Va
100 µm
TG
C
U
Va
100 µm
D
SML
PM
U
100 µm

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **"Estudo da organogênese prostática normal e os efeitos da exposição androgênica intrauterina e puberal sobre a morfofisiologia da próstata de gerbilos senis"**:

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio - Comissão Interna de Biossegurança , projeto nº _____, Instituição: _____.

(X) CEUA - Comissão de Ética no Uso de Animais , projeto nº 021/09, Instituição: IBILCE/UNESP, São José do Rio Preto, SP.

( ) CEP - Comissão de Ética em Pesquisa, protocolo nº _____, Instituição: _____.

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

Aluno: Manoel Francisco Biancardi

Orientador: Sebastião Roberto Taboga

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

Carimbo e assinatura

Prof. Dr. ALEXANDRE LEITE RODRIGUES DE OLIVEIRA
Presidente da Comissão de Ética no Uso de Animais CEUA/UNICAMP

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

Carimbo e assinatura

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL IBILCE

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado ***"Avaliação da exposição androgênica intra-uterina e puberal como fator predisponente de lesões prostáticas em machos e fêmeas de gerbilos senis"*** (protocolo nº. 021/09 CEEA), sob responsabilidade do Prof. Dr. Sebastião Roberto Taboga, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética em Experimentação Animal, em reunião de 10/12/2009.

CERTIFICATE

UNESP / IBILCE Ethical Committee for Animal Research (CEEA) hereby certify that the scientific investigation entitled "***Exposure Assessment androgenic intrauterine and puberty as a predisposing factor of prostatic lesions in male and female gerbils senile***" (protocol nº. 0210/09 CEEA), on Sebastião Roberto Taboga responsibility, is in accordance with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and it was approved by the Committee of this Institute, on december 10th, 2009.

São José do Rio Preto, 10 de dezembro de 2009.

Profa. Dra. Rejane Maira Góes
Presidente da CEEA

Instituto de Biociências, Letras e Ciências Exatas – SAEPE
Rua Cristóvão Colombo, 2265 CEP 15054-000 São José do Rio Preto – SP - Brasil
Tel 17 221 2456 fax 17 221 2390
E-mail: bitonti@ibilce.unesp.br e ceea@ibilce.unesp.br

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – IBILCE/UNESP-CSJRP

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Organogênese do lobo ventral da próstata do gerbilo da Mangólia (*Meriones unguiculatus*) durante o período pré-natal"** (protocolo nº. 052/2011 CEUA), sob responsabilidade do Professor Doutor Sebastião Roberto Taboga, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais deste Instituto, em reunião de 29 de fevereiro de 2012.

CERTIFICATE

UNESP/IBILCE Ethical Committee for Animal Research (CEUA) hereby certify that the scientific investigation entitled **"Prostate ventrl lobe organogenesis of the Mongolian gerbil (*Meriones unguiculatus*) during the prenatal period"** (protocol nº. 052/2011 CEUA), on Sebastião Roberto Taboga responsibility, is in accordance with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and it was approved by the Committee of this Institute, on february, 29th, 2012.

São José do Rio Preto, 29 de fevereiro de 2012.

Profa. Dra. Rejane Maira Goes
Presidente da CEUA

Instituto de Biociências, Letras e Ciências Exatas – STAEPE
Rua Cristovão Colombo, 2265 - Jardim Nazareth
CEP 15054-000 São José do Rio Preto – SP - Brasil
Tel 17 221 2425 fax 17 221 2390 / 3221 2500
E-mail: bitonti@ibilce.unesp.br e ceua@ibilce.unesp.br