UNIVERSIDADE ESTADUAL DE CAMPINAS

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"MODULAÇÃO HORMONAL DO CRESCIMENTO PROSTÁTICO EM DIFERENTES ETAPAS DO DESENVOLVIMENTO"

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"Muchos años después, frente al pelotón de fusilamiento, el coronel Aureliano Buendía había de recordar aquella tarde remota en que su padre lo llevó a conocer el hielo. Macondo era entonces una aldea de veinte casas de barro y cañabrava construida a la orilla de un río de aguas diáfanas que se precipitaban por un lecho de piedras pulidas, blancas y enormes como huevos prehistóricos. El mundo era tan reciente, que muchas cosas carecían de nombre, y para mencionarlas había que señalarlas con el dedo.

(…)

Cien años de soledad (Fragmento)

Gabriel García Márquez

Para

Victor Julio e Lilia Ines

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SUMÁRIO

Resumo

RESUMO

O controle do crescimento e da função prostática é baseado, numa complexa rede de interações de hormônios esteróides e seus receptores e num intrincado mecanismo de interconversão entre eles. A fisiologia e o crescimento prostático são dependentes também de hormônios peptídicos e fatores de crescimento, além de interações epitélio-estroma. O desenvolvimento prostático é caracterizado por uma etapa de indução pré-natal, uma janela de ramificação e citodiferenciação pós-natal, e um período de crescimento iniciado na puberdade, dada a produção testicular de testosterona (T). A conversão da testosterona para diidrotestosterona (DHT), pela enzima 5-α-redutase, é importante na origem embrionária da glândula. Esta dependência androgênica é também demonstrada no adulto, onde a privação androgênica leva à marcante regressão prostática.

Ações importantes na fisiologia prostática de outros hormônios diferentes dos andrógenos como a progesterona e estrógeno vêm sendo esclarecidas recentemente. Particularmente revelou-se a ação do estrógeno como um fator regulador durante o desenvolvimento normal da glândula, mas também, fator associado com a alta incidência de patologias durante envelhecimento, período no qual a alteração da relação estrógeno-testosterona surge como conseqüência da queda da concentração de andrógenos. Complicando esta situação, tem-se a ação de enzimas como a aromatase, e de outras envolvidas com a transformação da T e da DHT em compostos sem atividade androgênica, como a 3β-hidroxi-esteróide desidrogenase (3β-HSD) e a 3β-adiol-hidroxilase (CYP7B1) que junto com a coordenada expressão temporal-espacial dos receptores de hormônios esteróides fornecem o equilíbrio necessário para o normal desenvolvimento prostático.

Este trabalho reúne importantes resultados sobre a ação de desreguladores endócrinos em diferentes etapas do desenvolvimento prostático. Utilizando um modelo *in vivo* foram reproduzidos vários eventos de exposição a desreguladores endócrinos em diferentes idades. Dos resultados, pode-se concluir que:

1. as substâncias químicas podem ter efeitos totalmente diferentes se aplicadas na idade perinatal, no período pré e pós-púbere ou no adulto;

2. o momento de exposição às substâncias exógenas no organismo em desenvolvimento é decisivo para determinar seu caráter e seu potencial futuro e,

3. ainda que as exposições sejam durante as etapas iniciais do desenvolvimento, as manifestações podem somente ser observadas na maturidade.

ABSTRACT

Prostatic growth control and function are based on complex interactions between steroid hormones and their receptors and on intricate map of interconversion among them. Prostatic physiology and growth are also dependent of peptide hormones and growth factors, besides epithelium-estroma interactions. Prostatic development is characterized by a stage of fetal induction, a branching window and one phase of differentiation and growth period during the puberty, which is stimulated by the increasing testosterone (T) levels. Conversion of T to dihydrotestosterone (DHT), catalyzed by the 5α-reductase enzyme, is important for the embryonic origin of the gland. This androgenic dependence is also demonstrated in the adult, as androgen deprivation promotes prostatic regression. Important roles of non-androgenic hormones have been described more recently. In particular, estrogen was revealed as an important regulator of the normal development of the gland, which is also associated with the high incidence of pathologies in the elderly. Other factors, such as the existence of enzymes like aromatase, involved in the conversion of T in its inactive metabolites, as the 3-hidroxi-steroid dehydrogenase (3-HSD) and to 3-adiol-hidroxilase (CYP7B1) that supply the necessary balance for the normal prostatic development with the coordinate temporal-space expression of the receptors. This work presents important results about the action of endocrine disruptors in different stages of prostatic development under different hormonal environments using the endocrine disruptors. The main results are:

1. Endocrine disruptors produce different effects depending on the period of administration;
2. the time of exposition to the exogenous drugs is decisive to determine its effects and,
3. exposure during initial stages of the development may cause effects observed only during sexual maturity.

Introdução

INTRODUÇÃO

MORFOLOGIA DA PRÓSTATA VENTRAL DE RATOS

A glândula prostática se encontra localizada na base da bexiga urinaria, onde aparece intimamente associada com a uretra. Esta é classificada como glândula exócrina, cuja atividade biológica exige o acúmulo lento e ocasionalmente a expulsão rápida de pequenos volumes de fluido; para estes requerimentos, a arquitetura geral prostática caracteriza um órgão muscular com espaços grandes de armazenamento e com considerável capacidade secretora [1]. Desta forma, ela é composta por um conjunto de estruturas túbulo-alveolares, nas quais as estruturas epiteliais encontram-se envolvidas por um estroma [2].

Ao contrário da próstata humana, a dos roedores encontra-se dividida em lobos, cada um com características morfológicas e padrões de ramificação próprios. Especificamente, o lobo ventral consiste de oito conjuntos de ductos que se originam a partir da uretra como uma simples estrutura tubular que se ramifica distalmente [3].

Neste conjunto de ductos é possível distinguir três regiões morfológica e funcionalmente distintas, denominadas respectivamente, distal, intermediária e proximal, de acordo com sua posição em relação à uretra [4, 5]. Na região distal, são encontradas células epiteliais colunares alta com atividade proliferativa, circundada por células musculares lisas que formam uma camada esparsa e descontínua, associada a um número grande de fibroblastos [6]. Na região intermediária, as células epiteliais também são colunares altas, apresentando características de células secretoras, mas sem atividade proliferativa. Nesta região, a camada de células musculares lisas é fina e contínua. Na região proximal, as células epiteliais são cúbicas e baixas, sendo freqüente a presença de células apoptóticas. As células musculares lisas formam uma camada espessa. Tanto na região intermediária como na proximal, o tecido fibroso está presente no espaço entre os ductos e, ocasionalmente, intercalando a camada de células musculares lisas.

Compartimento epitelial prostático

O epitélio prostático é formado por vários tipos celulares, diferenciados entre si por marcadores específicos. As células epiteliais luminares expressam citoqueratina (Ck) 8 e 18 junto com o antígeno prostático específico (PSA) e o receptor de andrógeno (AR). As células luminais basais menos freqüentes possuem como marcadores as Cks 5 e 14, CD44 e Bcl-2. Acredita-se

que estas células epiteliais são originadas a partir de células progenitoras comuns que expressam um grupo de marcadores intermediários Cks 5, 14, 8, 18, caracteristicamente a Ck 19 [7] e estando também relacionadas com a expressão de integrinas [8]. Estas células progenitoras encontram-se localizadas na região proximal dos ductos prostáticos migrando distalmente, sendo reguladas negativamente na ausência de estímulos proliferativos por diferentes fatores de crescimento, entre eles o TGFβ [9].

A atividade das células epiteliais é fortemente influenciada pelos componentes estromais, e as diferenças fenotípicas das células epiteliais encontradas ao longo dos ductos prostáticos parecem estar relacionadas à distribuição diferenciada dos fibroblastos e das células musculares lisas [4, 6, 10].

Em humanos, as células epiteliais secretoras prostáticas são separadas da membrana basal e do estroma por uma camada de células basais [1]. Em murinos, há menos células basais e a camada formada por elas é descontinua [11].

Outro tipo celular presente no epitélio prostático são as células neuroendócrinas, as quais estão dispersas entre as camadas basal e luminal [12]. Estas são caracterizadas pela independência androgênica [13] e são consideradas células totalmente diferenciadas sem capacidade proliferativa. A função exata destas células não está totalmente estabelecida, mas acredita-se que elas atuam no crescimento e diferenciação da glândula prostática, sendo também implicadas na origem de neoplasias [14].

Compartimento Estromal

Entre as células epiteliais e o estroma encontra-se a membrana basal. Esta estrutura é extremamente importante no controle das atividades celulares e, principalmente, na manutenção da fisiologia das células epiteliais [15]. Composta principalmente de colágeno tipo IV e laminina, a membrana basal é essencial para a manutenção do fenótipo diferenciado e secretor das células epiteliais glandulares [16]. Os principais componentes das membranas basais foram detectados na próstata humana normal e nas membranas basais dos carcinomas com diferentes graus de diferenciação tumoral, com exceção do colágeno do tipo VII, que está ausente dos ductos neoplásicos [17].

Após a privação de andrógenos, e seguido dos eventos apoptóticos apresenta-se à perda de adesão da célula à membrana basal, as quais se tornam extremamente pregueadas e laminadas contendo moléculas intactas de laminina, mesmo após 21 dias de castração [18].

Por outro lado, foi observado que a laminina [19] está presente uniformemente na membrana basal ao longo do sistema de ductos prostáticos, enquanto o colágeno tipo IV é encontrado na membrana basal da região distal e intermediária, mas praticamente ausente na região proximal. Eles também demonstraram que o processo de involução prostática inicia-se com a perda ou redução do colágeno tipo IV, nas diferentes regiões dos ductos, durante os primeiros dias após castração, enquanto a laminina permanece intacta. Após o período de pico de apoptose (3^o e 4^o dia após a castração), o colágeno tipo IV volta a ser encontrado na membrana basal dos ductos. Os autores sugerem associação direta entre o aumento da apoptose e a perda de colágeno tipo IV.

As células musculares lisas (CML) representam 22% da área total da próstata humana [20], predominando ao redor dos ductos, onde se encontram em íntimo contato com a membrana basal das células epiteliais. Já na próstata ventral de ratos, as CML ocupam cerca de 5% do volume total da glândula e cerca de 14% do estroma [21]. As CML têm ação preponderante nos mecanismos de estimulação parácrina, especialmente sobre o epitélio [22], e provavelmente também sobre as demais células estromais.

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

A matriz extracelular do estroma, além de apresentar os componentes de membrana basal das células musculares lisas, é formada também por fibras de colágeno tipo I e tipo III, as quais sofrem um extenso rearranjo na próstata em regressão. Este rearranjo é caracterizado por um aspecto pregueado das fibras ao redor dos ductos e está intimamente associado às funções assumidas pelas células musculares lisas após a castração [23].

Microfibrilas de colágeno tipo VI e fibras do sistema elástico também são encontradas no estroma prostático e apresentam modificações durante a involução prostática. Estes componentes

parecem estar envolvidos no controle de alguns aspectos do comportamento celular e na manutenção da integridade estrutural do órgão [18, 24].

DESENVOLVIMENTO PROSTÁTICO

Formação dos ductos genitais

No estágio ambisexual, aproximadamente no décimo dia de vida intrauterina (VIU) em camundongos e na sétima semana em humanos, as gônadas em ambos os sexos apresentam-se com aspecto idêntico. Nesta fase o desenvolvimento do fenótipo masculino é dado pela expressão do gene SRY para o fator determinante do testículo (TDF, *testis-determining factor*) localizado no cromossomo Y. Até então os embriões indiferenciados possuem dois pares de ductos genitais; os ductos mesonéfricos (de Wolff) que são precursores do sistema reprodutor masculino, e os paramesonéfricos (de Müller) que originarão estruturas do sistema reprodutor feminino [25].

Nos machos, as células de Sertoli dos testículos fetais produzem a substância inibidora de Müller (MIS) em torno da sexta semana de VIU, já a produção de testosterona começa uma semana depois pelas células Leydig, que estimulam os ductos mesonéfricos a formarem os ductos genitais masculinos. A MIS, por sua vez, induz a regressão dos ductos paramesonéfricos [26].

A parte proximal dos ductos mesonéfricos torna-se altamente contorcida e forma o epidídimo, enquanto que, na parte distal, os ductos mesonéfricos adquirem um espesso revestimento de músculo liso tornando-se o ducto deferente. Finalmente, uma evaginação lateral da extremidade caudal dá origem à vesícula seminal [26].

Indução prostática

Nas fases iniciais do desenvolvimento embrionário a região caudal do intestino primitivo sofre uma divisão que origina o canal anal e o seio urogenital (SUG) este último, localizado ventralmente, é formado por uma região central epitelial e uma lateral mesenquimal e sofrerá uma série de eventos morfológicos, que determinarão a formação da glândula prostática [27, 28]. A indução prostática normal é controlada através da sinalização parácrina do mesênquima sobre o epitélio, após o estímulo androgênico [29]. Desta forma, o mesênquima do SUG é capaz de originar brotos epiteliais prostáticos em combinação com o epitélio de bexiga ou de vagina, sendo que o epitélio do SUG em combinação com o mesênquima de bexiga ou de vagina carece desta capacidade [30]. Presente inicialmente no mesênquima, o receptor de andrógeno (AR) é essencial

para a diferenciação prostática e, na sua ausência ou na de andrógenos, o desenvolvimento é comprometido [29, 31 – 35].

Interessantemente a indução prostática não é um evento genético-dependente [36]. Quando o SUG feminino é estimulado androgenicamente no período adequado, desenvolve cordões epiteliais similares aos encontrados no SUG de embriões masculinos [37]. Após o estímulo androgênico a camada de músculo liso disposta entre o epitélio e o mesênquima do SUG é modulada negativamente, o que permite o estabelecimento de sinais parácrinos intercelulares necessários para uma adequada indução prostática [38].

A sinalização intercelular estimula o surgimento de cordões epiteliais em direção ao mesênquima adjacente [37, 39] que em humanos acontece ao redor da décima semana de VIU, sendo que após três semanas, consistem de aproximadamente 70 ductos principais, alguns em processo de citodiferenciação [29]. A diferenciação do epitélio prostático acompanha a maturação do estroma prostático. Andrógenos atuam sobre os ARs no mesênquima urogenital (UGM) para induzir a proliferação epitelial, ramificação ductal e citodiferenciação nos subtipos celulares basal e luminal [29, 40]. Por sua vez, o epitélio prostático em desenvolvimento direciona os padrões de diferenciação do músculo liso prostático [15, 41]. Nem o epitélio prostático nem o músculo liso prostático são capazes de se desenvolver na ausência do outro tecido [30, 42], o que acontece como resultado de sinalização intercelular entre o mesênquima e o epitélio do seio urogenital [31 – 33].

Nos roedores, no momento do nascimento a próstata é rudimentar e passa por um extensivo processo de ramificação e de diferenciação celular durante as duas primeiras semanas de vida [43].

Novas evidências sugerem que as interações epiteliais-mesenquimais durante a organogênese prostática envolvem várias famílias de moléculas altamente conservadas, incluindo a s*onic hedgehog* (*Shh*) [44 – 47], proteínas morfogenéticas do osso (do inglês, *Bone morphogenetic protein*) [48], fatores de crescimento dos fibroblastos (FGF 7 e FGF10) [41], fator de crescimento transformante (TGFβ) [49], a *Notch-Delta membrane molecule* [50, 51], vários genes *homeobox genes*, da família NK, especificamente o *Nkx3.1* [52] e da família Hox, especificamente, *Hoxa-13 e Hoxd-13* [53], (Tabela 1).

Tabela 1. *Moléculas envolvidas no processo de morfogênese ductal da próstata.*

MOLÉCULAS	ATIVIDADE RELACIONADA
Shh	Iniciação da formação e diferenciação dos ductos [27]
BMP	Regulação da ramificação [3].
Hoxa-13 e Hoxd-13	Morfogênese ductal prostática [53].
Notch-1	Expressão associada com as células epiteliais progenitoras prostáticas [50].
Nkx3.1	Diferenciação epitelial (células epiteliais luminais e basais) [52].
FGF7 e FGF10	Ação mitogênica sobre o epitélio durante o desenvolvimento prostático [41].
TGFβ	Estimula proliferação epitelial na região distal e atua como fator regulador na região proximal prostática [54].

Shh: Sonic Hedgehog protein, BMP: Proteínas morfogenéticas do osso, FGF: fator de crescimento dos fibroblastos, TGF: fator de crescimento transformante.

Ainda que a expressão destes fatores decresça com a maturidade, ela permanece relativamente alta no período pós-natal, que é caracterizado pela formação de grande parte dos ductos principais e por uma intensa morfogênese ductal [48, 50, 53, 55] (Figura 1).

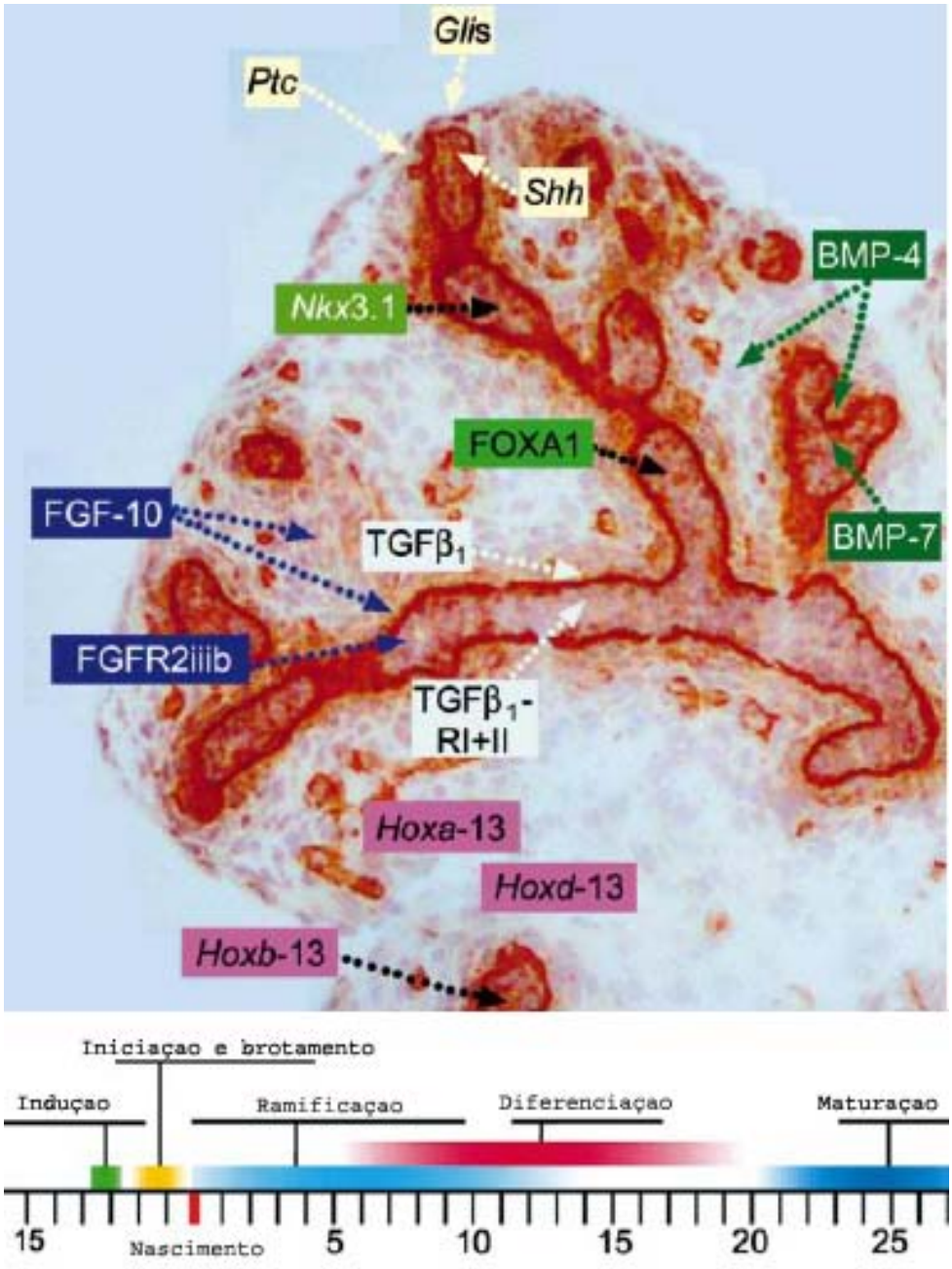

Figura 1.: *Localização espaço-temporal de genes relacionados ao desenvolvimento prostático. Acima, secção transversal da próstata ventral no quinto dia de desenvolvimento, mostrando a localização epitelial e mesenquimal da expressão gênica de moléculas envolvidas no desenvolvimento prostático. Na parte inferior, as etapas do desenvolvimento prostático são mostradas num gradiente de expressão temporal (Dias).* ***FGF:*** *fator de crescimento dos fibroblastos,* ***TGF:*** *Fator de crescimento transformante,* ***FGFR:*** *receptor do fator de crescimento dos fibroblastos,* ***BMP:*** *Proteínas ósseas morfogenéticas. Reproduzido de Huang* [52] .

EXPRESSÃO DOS RECEPTORES DE HORMÔNIOS ESTERÓIDES

Assim como para as demais estruturas sexuais masculinas, a estimulação por andrógenos é absolutamente necessária para o desenvolvimento da próstata [29]. Embora a testosterona seja o principal andrógeno produzido pelos testículos, é a diidrotestosterona (DHT), produto da sua redução, a responsável pela morfogênese prostática [56]. A DHT é produzida no SUG pela ação da enzima 5α-redutase [57].

A produção de andrógenos pelos testículos do feto começa antes e continua durante a morfogênese prostática [58]. Neste mesmo período os testículos fetais atingem seu nível máximo de produção de andrógenos, o qual declina suavemente na 18ª semana e permanece basalmente estável até a puberdade [33, 58]. O processo em camundongos acontece no 17º dia e, em ratos, entre o 18° e o 20° dia de VIU. Nas duas espécies, os lobos ventrais surgem a partir de ductos principais originados na região ventral do SUG [42].

As respostas celulares aos andrógenos sistêmicos são mediadas pela interação do receptor de andrógeno (AR) e seus ligantes (T e DHT). Estes fatores são determinantes para que exista uma completa indução prostática. Assim, alterações na expressão do gene do AR comprometem o desenvolvimento prostático [59]. Por outro lado, se a ação da enzima 5 α-redutase é a afetada; a conversão de T em DHT é comprometida e, mesmo com o direcionamento do SUG para a formação de um tecido prostático, este se apresenta rudimentar e pouco desenvolvido.

A interação de andrógenos e estrógenos é demonstrada pela freqüente co-localização dos ER e AR tanto nas células epiteliais como nas estromais, sugerindo uma regulação fina entre concentrações hormonais e expressão de receptores.

Em condições normais, a próstata ventral de roedores adultos possui a expressão do AR na totalidade dos núcleos das células epiteliais luminais e, aproximadamente, na metade das células epiteliais basais. As células interacinares, especialmente as CML são também AR positivas, porém o padrão de intensidade é inferior ao apresentado pelas células epiteliais [60]. Ao longo dos ductos prostáticos, entre a região proximal e distal, não existe uma diferença significativa da expressão do AR, o que sugere que as diferentes respostas que envolvem hormônios esteróides são medidas indiretamente por fatores parácrinos e não pela expressão regional diferenciada do AR [61]. Em relação aos receptores de estrógeno, expressão baixa do receptor alfa (ERα) têm sido reportada no compartimento estromal e nas células basais epiteliais

da próstata normal humana [62], enquanto o receptor beta (ERβ) é expresso no núcleo das células epiteliais, inclusive em algumas células estromais próximas aos ácinos [63].

Nas fases pré-natais, a expressão diferenciada no estroma e no epitélio dos receptores indica uma ação espaço-temporal coordenada dos hormônios esteróides. Inicialmente, a expressão do AR na próstata humana acontece ao redor da 11ª semana de VIU no estroma, sendo detectada nos cordões epiteliais, posteriormente ao redor da 16ª semana de VIU com uma contínua expressão estromal. Pouco antes do nascimento a expressão do AR no compartimento epitelial decresce, sendo localizada principalmente no estroma, em especial, nas células musculares lisas. Duas semanas após do nascimento, no DPN 15, a expressão do AR reaparece no epitélio [64].

A expressão do ERβ aparece na 13ª semana de VIU aumentando junto com a concentração de estrógeno durante a metade do período gestacional. Contrariamente, o ERα, em humanos, parece ter pouca participação nos eventos iniciais do desenvolvimento prostático, já que a expressão acontece só no DPN 30, quando é intensamente expresso nas células da região periacinar [42].

Entre outros, a separação feto-placenta resulta em uma diminuição nas concentrações séricas de estrógeno, progesterona, gonadotrofina coriônica humana (hCG) e hormônio lactogênico placentário (hPL) [26]. Também é apresentado um aumento nos níveis de hormônio luteinizante (LH), que nos machos estimula a produção de testosterona promovendo um pico nos primeiros dias após do nascimento que influencia eventos relacionados à morfogênese prostática [65]. Este período é também de sensibilidade prostática e a administração de testosterona acelera o crescimento da próstata, levando-a a atingir precocemente o crescimento máximo [66]. Uma situação contrária, a castração, inibe o crescimento e o desenvolvimento da próstata durante a puberdade, efeito que pode ser revertido com a administração de testosterona [29, 65].

De acordo com o estágio do seu desenvolvimento, a próstata responde de forma diferente ao estímulo hormonal [30]. No período neonatal, o estímulo hormonal induz principalmente ramificação ductal [66]. Num estágio mais avançado, na puberdade, as concentrações androgênicas elevadas estimulam principalmente o aumento de peso seco da glândula, acompanhado de um pequeno aumento das ramificações ductais [31].

Na puberdade apresenta-se o início do crescimento prostático, que é caracterizado por um aumento do peso seco da próstata mais do que, pelo aumento no número das ramificações ductais [31, 67] que é característico da próstata em desenvolvimento [30].

EIXO HIPOTÁLAMO – HIPÓFISE – GÔNADA

O controle neuroendócrino do metabolismo, do crescimento e de certos aspectos da reprodução é mediado por uma combinação de sistemas neurais e endócrinos centrados no eixo hipotálamo-hipófise [68].

A hipófise é constituída por um lobo anterior (adeno-hipófise), um lobo intermediário e um lobo posterior (neuro-hipófise) [68]. A secreção de gonadotrofinas (LH e FSH) é feita pelos gonadotrofos, células especializadas da pituitária constituindo de 7% a 15% do total de células da hipófise anterior, encontradas desde os primeiros estágios do desenvolvimento [69].

O LH estimula a ovogênese na fêmea e a espermatogênese no macho. Neste, sua principal ação ocorre pelo estímulo da produção de testosterona pelas células de Leydig. A ação do FSH envolve a síntese de estrógenos nas fêmeas pela ação da enzima aromatase e nos machos, regula a maturação dos espermatozóides a partir das espermatides [68].

Os receptores de LH e FSH pertencem à família de receptores associados à proteína G os quais dividem homologia de 50% no domínio extracelular e 80% no domínio transmenbrana. A associação destes com seus ligantes ativa a *adenilato-ciclase* o que leva à formação de AMP cíclico, que se liga à subunidade reguladora da proteína-quinase ativando sua subunidade catalítica. Esta última, por meio de várias vias, estimula as enzimas da biossíntese de estrógeno e testosterona. O sinal é terminado pela endocitose e degradação do complexo ligante-receptor [70].

Como já mencionado, os andrógenos são requeridos para o crescimento normal e atividades funcionais da próstata. No homem, os principais andrógenos circulantes são a androstenediona e a T, sendo esta última produzida, na sua maior parte (cerca de 95%), pelos testículos. As glândulas adrenais contribuem com menos de 5% na produção dos esteróides sexuais [71], sendo regulada pelo hormônio adrenocorticotrófico (ACTH). Nos dois tecidos existem pelo menos duas vias biossintéticas a partir da pregnenolona; a via predominante é a δ-5, que resulta na produção de androstenediona e testosterona; já a via δ-4, menos proeminente, leva à síntese de deidroepiandrosterona (DHEA) e androstenediol. Uma vez sintetizada, a maior parte da DHEA é inativada via sulfatação, enquanto uma pequena fração é convertida em

androstenediona e, a seguir, em T, nos tecidos periféricos e na próstata [72]. Em homens saudáveis, os andrógenos adrenais contribuem ligeiramente na função prostática normal, embora em estados tumorais prostáticos a contribuição da adrenal pode ser suficiente para promover o crescimento prostático, principalmente após orquiectomia [73].

Estima-se que apenas 2 a 3% da T encontra-se disponível em sua forma livre, sendo que o restante encontra-se ligado a proteínas séricas como a SHBG (do inglês, *Sex Hormone-Binding Globulin*), albumina e globulina de ligação com corticoesteróide [74]. Entre as três proteínas, a SHBG possui a maior afinidade pela T.

ENDOCRINOLOGIA PROSTÁTICA

A conversão de T no mais potente andrógeno intracelular, a 5α-diidrotestosterona (DHT), dá-se pela ação da enzima 5α-redutase (Figura 2). Dois tipos de 5α-redutase foram identificados. A 5α-redutase tipo I encontra-se na maioria dos tecidos, enquanto a 5α-redutase tipo II, que é codificada pelo gene SRD5A2, localizado no cromossomo 2p23 [75], predomina nos tecidos genitais, incluindo a próstata. Quando a finasterida, inibidor específico da 5α-redutase tipo II, foi administrada a homens para o tratamento de hiperplasia prostática benigna (BPH) as concentrações séricas de DHT diminuíram cerca 70%; enquanto o conteúdo prostático diminuiu de 85 a 90% [76]. A quantidade remanescente de DHT na próstata pode ser atribuída à ação da isoenzima tipo I.

A DHT intracelular é rapidamente metabolizada em uma reação reversível para 3α, 17β-androstenediol (3α-diol), ou para 3β, 17β-androstenediol (3βAdiol) (Figura 2). A enzima responsável por esta via de inativação é a 3β-hidroxiesteróide desidrogenase Tipo II (3β-HSD). O 3βAdiol é finalmente convertido irreversivelmente em esteróides inativos hidrossolúveis, pela da ação da 3β Adiol Hidroxilase (CYP7B1) (Figura 2) [77].

O estudo da ação da CYP7B1 na próstata permite obter novas apreciações sobre o uso de alguns medicamentos. A finasterida é um medicamento usado no tratamento da BPH, atuando no bloqueio da 5α-redutase Tipo II.

A princípio, a ação da finasterida bloqueia a produção do potente andrógeno DHT levando a uma diminuição do crescimento prostático. Considerado a ação da CYP7B1, a finasterida atuaria prevenindo a formação do 3βAdiol e removendo sua ação antiproliferativa por

bloquear o ERβ. Além disto, a inibição da 5α-redutase Tipo II causa acúmulo de testosterona aumentando a expressão de AR na próstata [78].

A aromatase pode converter o excesso de testosterona em estrógeno que agiria sobre os receptores ERα e ERβ (Figura 2). Isto poderia resultar em crescimento displásico da próstata. No entanto, não existem estudos que forneçam informação precisa sobre o padrão de expressão de algumas enzimas consideradas chave no metabolismo prostático, como a aromatase, 5α redutase, CYP7B1, 3β HSD na próstata e que procurem averiguar sua modulação pelos andrógenos.

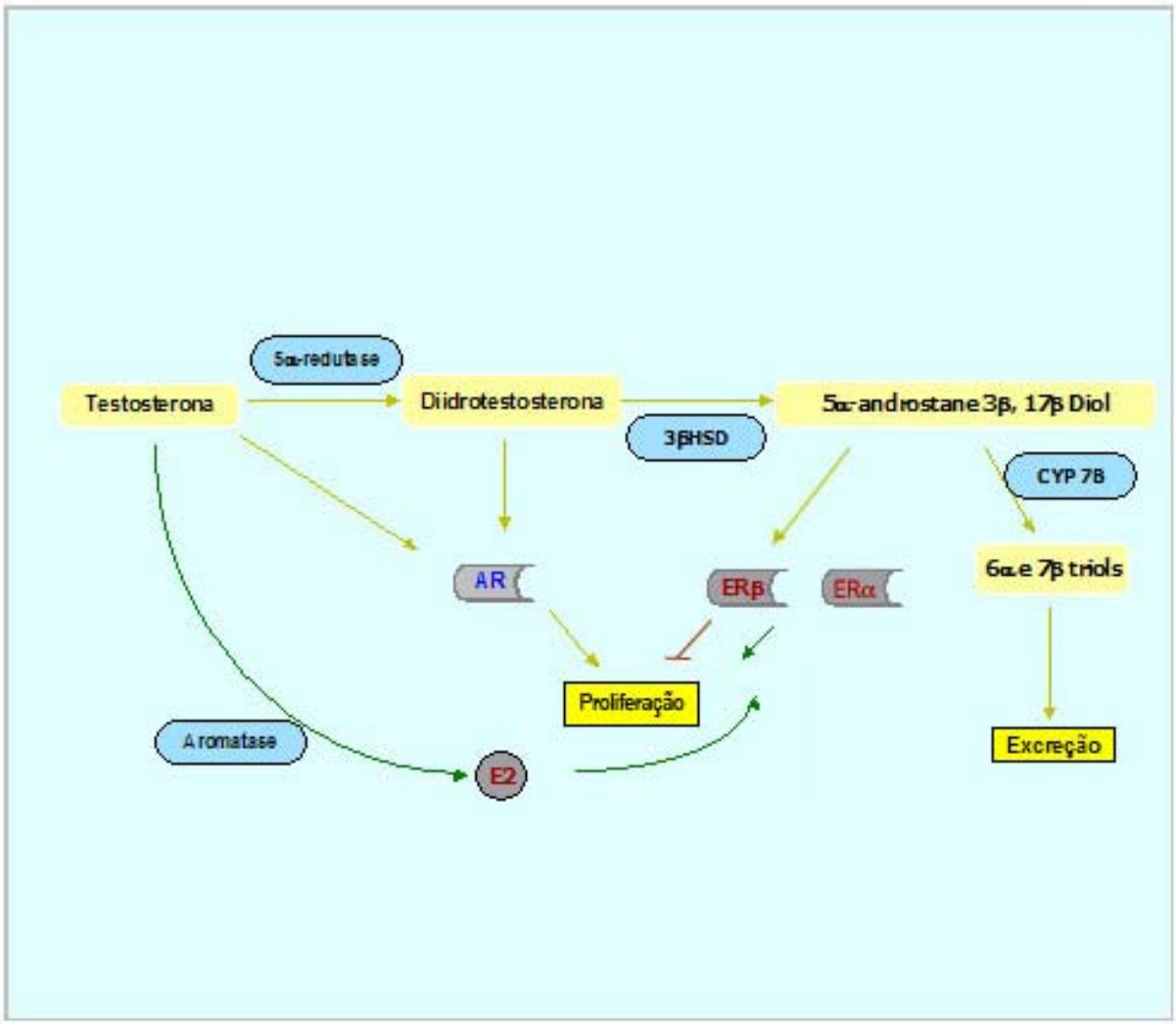

Figura 2. Relações entre andrógenos e estrógenos na regulação do crescimento prostático e seus inibidores. A testosterona tem papel fundamental na fisiologia prostática. No órgão, ela é convertida em diidrotestosterona (DHT) pela enzima 5α-redutase. Tanto a testosterona quanto a DHT ligam-se aos receptores de andrógeno, promovendo a proliferação das células epiteliais e/ou manutenção do seu estado diferenciado. Sua ação sobre as células estromais é variável, dependendo do tipo celular considerado. A DHT é normalmente convertida a 5α-androstane 3b, 17 b diol, pela enzima 3β-hidroxiesteroide desidrogenase Tipo II (3bHSD). O 5α-androstane 3b, 17 b diol liga-se ao receptor de estrógenos, competindo com o estrógeno circulante e impedindo sua atividade proliferativa. O excesso de 5α-androstane 3b, 17 b diol é processado pela enzima 3βAdiol hidroxilase (CYP7B1) a 6a e 7 b triols, que são eliminados. Nos machos, o estrógeno circulante origina-se da ação da enzima aromatase sobre a testosterona. AR = Receptor de andrógeno; ERα = Receptor de estrógeno subtipo β ERβ = Receptor de estrógeno subtipo β; E2 = Estrogéno; → = Ação do E_2 sob seus receptores; → = Metabolismo da testosterona; → = Vias inibidas.

ESTRÓGENOS E PRÓSTATA

Ação do estrógeno na próstata ventral de ratos

Por meio de um estudo pioneiro [79] foi demonstrada a dependência androgênica nos processos de indução, regulação e crescimento da próstata. A partir destas pesquisas iniciais o conhecimento dos processos cresceu consideravelmente, principalmente orientado às vias metabólicas dos andrógenos, especificamente as que incluem a redução de testosterona (T) em diidrotestosterona (DHT).

Esta visão tem sido também reforçada pelos conceitos tradicionais que fazem uma forte separação entre hormônios "masculinos" e "femininos" para os andrógenos e estrógenos, respectivamente. Com tudo isto, a presença das duas formas de receptor de estrógeno (ERα e ERβ) no trato reprodutor masculino - testículos, ductos eferentes, epidídimo, próstata, glândulas bulbo uretrais e vesícula seminal - sugere ação direta dos estrógenos neste órgãos [80].

Os modelos recentes de camundongos transgênicos (Tabela 2), relacionados direta ou indiretamente com o metabolismo dos estrógenos, demonstraram que se a ação dos andrógenos é importante na regulação prostática, os estrógenos estão sutilmente envolvidos com o controle de diferentes processos durante a indução, desenvolvimento e função da próstata.

Tabela 2. *Modelos de camundongos geneticamente modificados associados com as vias metabólicas de estrógeno e fenótipos conseqüentes destas alterações.*

MODELO	ALTERAÇÃO	FENÓTIPO PROSTÁTICO
αERKO [81].	Deleção do ERα	Aumentada com a idade
βERKO [82].	Deleção do ERβ	Normal, hiperplasia
AROM+ [83].	OE do gene cyp19	Rudimentar
ArKO [84].	Disrupção do gene cyp19	Aumentada, hiperplasia

*ER**α**=receptor de estrógeno alfa, ER**β**=receptor de estrógeno beta. OE=Superexpressão.*

A presença dos receptores para estrógeno parece ser responsável por parte das alterações que sofre a próstata frente ao tratamento com estrógenos (E_2). A ação do E_2 ocorre

principalmente sobre o eixo hipotálamo-hipófise, causando atrofia testicular e queda na produção de T [85]. Entretanto, o estudo da cinética de apoptose na próstata ventral demonstra que o E_2 causa uma antecipação da apoptose das células epiteliais prostáticas, atingido no segundo dia após o início do tratamento, enquanto o pico causado pela castração ocorre no terceiro dia [86].

O passo final da biossíntese do estrógeno a partir da testosterona é catalisado pela enzima aromatase citocromo p450, enzima envolvida na transformação irreversível de andrógenos em estrógenos e que está presente no retículo endoplasmático de numerosos tecidos [84, 87].

Nos humanos, a p450 aromatase é o produto do gene Cyp19, o qual pertence à família do citocromo p450, família que possui mais de 500 membros [88]. Este complexo enzimático é composto por duas proteínas; a NADPH-citocromo p450 redutase e a citocromo p450 aromatase, nesta última encontra-se o grupo heme e o sitio ativo de ligação aos esteróides [89].

Recentemente foi confirmada a presença de várias enzimas envolvidas no metabolismo no estrógeno, incluindo a aromatase, nas células epiteliais e estromais da próstata humana, o que sugere um modelo de auto-regulação, já que estes tipos celulares também expressam o receptor de estrógeno, especialmente do tipo β. [90]. O aumento no conteúdo de E_2 são reportados na próstata na 16ª semana de VIU. Ao contrário dos andrógenos, o E_2 aumenta até o final da gestação quando cai abruptamente devido à falta da síntese maternal e placental [91].

Durante a gestação dos roedores, os fetos masculinos e femininos são dispostos aleatoriamente no útero materno mantendo um íntimo contato entre eles. Isto permite um fluxo de hormônios esteróides por um mecanismo de drenagem venosa entre fetos adjacentes [92]. A exposição hormonal para cada feto varia de acordo com o sexo dos fetos adjacentes. Assim, os fetos situados entre fêmeas (denominados 2F) são influenciados estrogênicamente, enquanto os fetos localizados entre machos (denominados 2M) estão sob influência androgênica [34] (Figura 3). A exposição hormonal durante a fase fetal influencia o desenvolvimento neural e sexual, condicionando as respostas aos estímulos hormonais na fase adulta [93 – 96].

Nas fêmeas, a exposição elevada aos estrógenos decorrente da posição intra-uterina estimula a ocorrência precoce do primeiro ciclo estral, fato associado com a primeira ovulação pós-pubertal [97]. A hipótese e comprovação de que a próstata, por ter seu desenvolvimento sob o comando da interação entre estrógenos e andrógenos, seria influenciada pela disposição intra-uterina veio do trabalho de Timms [34]. Ele encontrou diferenças na organogênese da glândula dos

machos 2F, quando comparados com machos menos expostos aos estrógenos (2M ou FM), observando nos primeiros um aumento nas áreas dos brotos prostáticos que originam os diferentes lobos. O aumento de estrógeno circulante nos machos 2F, aproximadamente 30% mais alto se comparado com animais 2M, seria o responsável por sensibilizar a resposta das células prostáticas aos andrógenos por meio de dois mecanismos possíveis: 1) produzindo um aumento do número de receptores de andrógeno (aproximadamente três vezes) e 2) gerando uma maior afinidade dos receptores de andrógeno pelos seus ligantes [96].

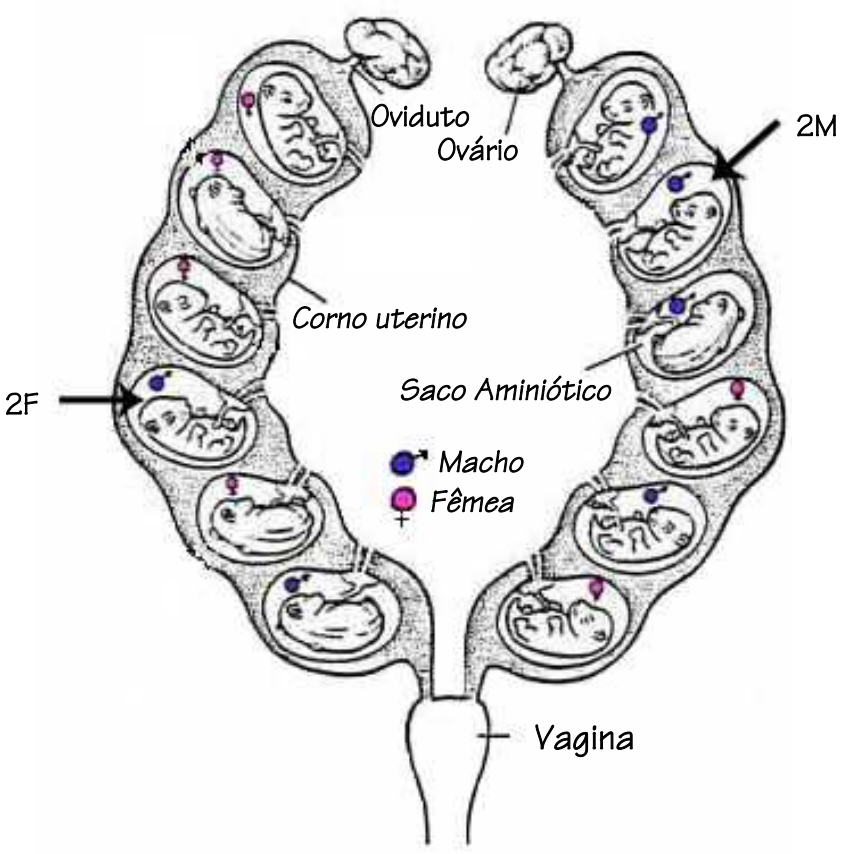

Figura 3.: *Distribuição dos fetos nos cornos uterinos durante a gestação. Os fetos em cada corno são classificados de acordo com sua proximidade a posição caudal. MM (2M) e FM são fetos com influência masculina; MF e FF (2F) são fetos com influência feminina; M0 e F0 são fetos localizados proximalmente ao cervix.(Reproduzido de Hernández-Tristán e colaboradores)* [94].

Imprinting hormonal – estrogênico

Ainda que a morfogênese da glândula prostática de roedores seja iniciada durante a fase fetal, a maior parte do seu crescimento e desenvolvimento ocorre no período neonatal [98]. Está bem estabelecido que, nesta fase, alguns eventos podem ser "memorizados" pelas células em órgãos, em um fenômeno denominado *imprinting*. Foi revelada [35] a existência de uma expressão sincronizada, temporal e espacial, do receptor de estrógeno α (ERα) e ERβ nas células epiteliais e estromais da próstata ventral. Este padrão junto ao sinal estrogênico, é necessário para o desenvolvimento da próstata em camundongos. Com o uso de animais geneticamente modificados para os receptores de estrógeno ERα e ERβ ele demonstrou que o ERα mas não o ERβ é necessário para a existência do *imprinting* neonatal durante as primeiras semanas de vida.

Estes resultados confirmam o conceito preestabelecido de um equilíbrio entre os dois receptores. Os sinais proliferativos e de diferenciação dos ligantes estrogênicos são mediados pelo ERα enquanto os sinais anti-proliferativos acontecem mediados pelo ERβ quando ligado ao 3βAdiol, um metabólito do DHT, como já observado [99].

As ações inibitórias de estrógeno acontecem principalmente pelos seguintes mecanismos: 1) supressão de gonadotrofinas (especialmente secreção de LH); 2) prejuízo no desenvolvimento das células de Leydig, o que leva a uma inadequada produção de testosterona; 3) supressão da secreção do fator de crescimento semelhante à insulina (IGF) pelas células de Leydig, fator que está associado à descida dos testículos a partir da cavidade abdominal, predispondo conseqüentemente a criptorquidia [100].

Os receptores de estrógeno (ERs) ligados a hormônios esteróides podem regular a transcrição gênica pela ligação direta aos elementos responsivos estrogênicos (EREs) de genes alvos. Esta ligação é intermediada por uma série de proteínas que atuam como coativadoras dos receptores esteróides (do inglês: *steroid receptor coactivators).* Esta ligação, posteriormente, leva à associação do complexo CBP/p300, coativador transcripcional, que possui atividade acetiltransferase estabilizando o inicio da transcrição gênica [101, 102].

A especificidade das respostas celulares pode ser devida principalmente à concentração das isoformas de ERs e coativadores nos tecidos e células, já que a função destes coativadores é especifica [103]. Alterações na expressão destes coativadores durante o período de exposição aos hormônios esteróides pode participar nos mecanismos do *imprinting* estrogênico [104], os quais

promovem respostas diferenciadas na idade adulta, que dependendo do tipo de evento pode levar à predisposição a patologias prostáticas [105].

Objetivos

OBJETIVOS

Este trabalho teve por objetivo estudar aspectos da regulação e dependência hormonal da glândula prostática em diferentes fases do desenvolvimento, assim como demonstrar alterações nas respostas celulares da glândula como conseqüência da alteração do ambiente hormonal produzido por diferentes desreguladores endócrinos.

Artigos

ARTIGOS

O presente trabalho deu origem aos artigos:

- Pubertal and adult prostate response to postnatal exposure to different drugs affecting endocrine regulation.

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- Post-pubertal and adult prostate response to prepubertal exposure to different hormonal enviroments.

- The Aromatase inhibitor letrozole down regulates androgen receptor in rat ventral prostate.

Pubertal and adult prostate response to postnatal exposure to different drugs affecting endocrine regulation

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Short title: **Prostate response to endocrine disruptors**

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Abstract

In order to obtain new insights into how neonatal exposure to exogenous drugs affects the physiology of the ventral prostate, prepubertal, pubertal and adult rats were exposed to different drugs acting on the metabolism of steroid hormones during the first week of postnatal life. Finasteride (an inhibitor of 5α-reductase), letrozole (an inhibitor of aromatase), tamoxifen (a partial estrogen antagonist), and a supraphysiological dose of estrogen were administered. Tamoxifen and letrozole resulted in decreased prostate weight gain up to day 21 after birth. In contrast, estrogen caused an increase of the luminal and stromal compartments. Changes in cell proliferation kinetics were detected after treatment with letrozole and estrogen. Androgen receptor (AR) was not dramatically influenced by the different treatments; however, different patterns of AR staining were observed after the different treatments, with a notably stronger staining by day 91 of age in estrogen-treated animals. We conclude that exposure to endocrine disruptors during the first week of life significantly affects prostate development and growth but shows little effect on prostate morphology in mature animals. However, this early exposure alters the prostatic response during the first contact with high levels of steroid hormones that occurs during puberty.

INTRODUCTION

Delivery of the placenta results in a decreases in fetal blood levels of estrogens, progesterone, human chorionic gonadotropin (hCG), and human placental lactogen. In male mammals, after a transient fall in testosterone levels as the hCG stimulus declines, pituitary LH secretion rebounds and there is a surge of plasma testosterone that persists at significant levels for several weeks (Mann *et al*., 1989). A perinatal testosterone surge in males results in adults levels of testosterone that permanently alter androgen sensitivity (Naslund & Coffey, 1986).

Although morphogenesis of the prostate gland starts during the fetal phase, growth and development mainly take place during the neonatal period when the gland consists of three to four pairs of solid cords of epithelial cells budding from the urogenital sinus (Woodham *et al*., 2003). These prostatic stem cells are potential targets for the testosterone provided by the postpartum surge, with the immature murine prostate undergoing ductal branching morphogenesis, extensive growth and differentiation of epithelial and stromal cells under the continuous influence of androgens (Hayward & Cunha, 2000).

The prostate responds differently to hormonal stimuli depending on the developmental stage (Hayward & Cunha, 2000). During the neonatal period, the hormonal stimulus mainly induces ductal ramification (Berry & Isaacs, 1984). Later in puberty, increasing androgen levels stimulate glandular growth accompanied by a rather small increase in ramifications (Sugimura *et al*., 1986).

Perinatal exposure to different compounds with hormonal activity during this specific sensitive window promotes inadequate responses to hormones in adult life and this effect has been associated with a predisposition to prostatic diseases (Stoker *et al*., 1999; Risbridger *et al*., 2005).

The aim of the present study was to determine the effects of exposure to exogenous drugs during the first few days after birth on the growth of the ventral prostate.

MATERIALS AND METHODS

After acclimatization under controlled conditions (lights on from 7:00 to 19:00 h, temperature 20-24^{o}C), virgin female Wistar rats were mated within their own colony. Vaginal smears were inspected daily and the morning on which spermatozoa were found was considered to be the first day of pregnancy. The pregnant females were divided into five groups according to the treatment administered to their pups, as described below. The experiments were carried out according to the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Experimentation of UNICAMP (protocol number 592-1). After growth until postnatal days 21, 42 and 91, treated animals and controls were weighed and killed by cervical dislocation. Blood samples were obtained by cardiac puncture and the ventral prostate and testes were dissected out and weighed before fixation.

Experimental groups

I. Inhibition of dihydrotestosterone (DHT) formation. To determine the capacity of early exposure to DHT to modulate postnatal prostate growth, finasteride (Merck & Co., Inc., Whitehouse Station, NJ, USA) was injected from postnatal days 1 to 5 at the dose of 50 mg/kg using 10% ethanol in triolein as vehicle (25 μL volume) (Rittmaster *et al*., 1991).

II. Inhibition of estrogen (E_2) formation. Exposure to physiological levels of estrogens by aromatization of testosterone was inhibited with letrozole (1 mg/kg; Novartis AG, Basel, Switzerland) dissolved in 25 μL of a 2.5% aqueous solution of carboxymethylcellulose and injected subcutaneously (s.c.) daily from postnatal days 1 to 5 (Tobin & Canny, 1998).

III. Blockade of estrogen receptors (ERs). ERs were blocked by the administration of tamoxifen (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 10 mg/kg dissolved in corn oil (Sigma) and injected s.c. daily from postnatal days 1 to 5 (Stoker *et al*., 1999).

IV. Supraphysiological levels of estrogen. The effect of a hyperestrogenic environment on the early postnatal ventral prostate of rats was investigated by the administration of 17β-estradiol (0.15 mg/kg; Sigma) dissolved in 25 μL corn oil and injected s.c. on days 1, 3 and 5 (Putz *et al*., 2001).

Hormone measurement

Serum levels of testosterone, estradiol (E_2), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were estimated by RIA. Blood samples were obtained by cardiac puncture immediately before death. Serum was separated by centrifugation and stored at -20^{o}C for subsequent hormone assays. Testosterone and E_2 concentrations were measured in duplicate in serum samples using Coat-a-Count kits (Diagnostic Products, Los Angeles, CA, USA).

Plasma LH and FSH were measured by double-antibody radioimmunoassay using specific kits provided from the National Hormone and Peptide Program (NIH / NIDDK, USA). All samples in the same experiment were measured in the same assay. The lowest detectable amount of $LHRP_3$ standard was 0.05 ng/ml and the intra-assay coefficient of variation was 4%. The lowest detectable amount of FSHRP2 standard was 0.2 ng/ml and the intra-assay coefficient of variation was 3.2 %.

Histology

The ventral prostate was immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 h. The samples were then washed, partially dehydrated, and embedded in Leica historesin. Two-micrometer sections were obtained and stained with

hematoxylin and eosin (Behmer *et al*., 1976). For immunohistochemistry, fixed samples were embedded in paraffin after dehydration in an increasing ethanol series and clearing in xylene.

Stereological analysis

For stereological analysis, five microscopic fields of the hematoxylin/eosin-stained sections obtained from five animals of each group were photographed with a Zeiss Axioskop microscope (Jena, Germany) and the Weibel multipurpose test grid (120 points, 60 test lines) was used for systematic field sampling (Huttunen *et al*., 1982).

The following parameters were evaluated based on the measurement of area-volume densities (percentage of tissue volume occupied by a defined tissue compartment): epithelium, stroma, lumen and smooth muscle cells. For the estimation of volume fractions, the number of grid points falling in each tissue compartment was counted. The volume of the ventral prostate served as reference volume. For approximation, the specific gravity of the prostate tissue was assumed to be 1.0 (DeKlerk & Coffey 1978; Huttunen *et al*., 1982).

Immunohistochemical staining

Paraffin-embedded sections (6 μm) were dewaxed and rehydrated. Antigens were retrieved by boiling the sections in 10 mM citrate buffer, pH 6.0, three times for 5 min in a microwave oven. The cooled sections were incubated in 1% H_2O_2 for 15 min to block endogenous peroxidase. Nonspecific binding was blocked by incubating the sections in 3% BSA for 1 h at room temperature. The sections were then incubated with polyclonal anti-androgen receptor (AR) antibody (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal anti-Ki67 antibody (clone MIB5; DakoCytomation Inc., Carpinteria, CA, USA) as primary antibody, both diluted 1:100 in 1% BSA at 4°C. Negative controls were only incubated with 1% BSA lacking the primary antibody. The EasyPath ABComplex/HRP kit (Novocastra Laboratories, Newcastle, UK) was used to visualize the bound antibodies according to manufacturer instructions. The sections were incubated in appropriate secondary antibody

solutions for 30 min, followed by washing for 10 min with PBS and incubation in avidin and biotinylated HRP solution for 30 min. After washing in PBS, peroxidase activity was detected with 3, 3'-diaminobenzidine tetrahydrochloride as chromogenic substrate. Sections were lightly counterstained with methyl green, dehydrated in an increasing ethanol series and xylene, and mounted in Entellan (Merck, Darmstadt, Germany).

Ki67-positive cell counts

Proliferative indices were obtained by counting Ki67-positive and Ki67-negative (methyl green-stained) nuclei at 400x magnification. Three immunostained sections per treatment were used and separate counts were performed for the epithelial and stromal compartments of each gland. Approximately 2,000 cells were counted for each treatment. The number of positive cells is expressed as the percentage of total cells of a given cell type (luminal and basal epithelial cells and stromal cells).

RESULTS

Body and organ weight

Table 1 shows the mean body weight of the animals after the different treatments. Only E_2-treated and tamoxifen-treated animals allowed to grow until the adult period (91 days) presented a significative difference in body weight when compared to the control group. E_2 caused a reduction of body weight by the 42nd day and an increase by the 91st day.

In intact animals, testis weight was 0.30 ± 0, 1.42 ± 0.03 and 3.35 ± 0.04 g at 21, 42 and 91 days of age, respectively. Testis weight was not affected by any of the treatments (Table 1).

Prostate and relative prostate weight

Prostate weight did not differ significantly compared to the control group (Table 1). Relative weight, estimated by dividing prostate weight by body weight, was lower in the finasteride, tamoxifen and letrozole groups. This difference was only observed in 21-day-old

animals (Table 1). No difference in relative weight was observed for the other age groups when compared to the control group.

Serum hormone levels

Hormone concentrations in 21-day-old control animals were 14.8 ± 0.9 ng/dL for testosterone, 84.2 ± 3 pg/mL for E_2, 2.4 ± 0.2 ng/mL for LH, and 4.1 ± 0.4 ng/mL for FSH. The administration of E_2, letrozole and finasteride reduced circulating levels of estradiol. No effect on hormone levels was observed for the other treatment, irrespective of age (Table 2). The following hormone levels were observed in 42-day-old rats: 174.5 ± 5.1 ng testosterone/dL, 18.7 ± 1.7 pg estradiol/mL, 3.1 ± 0.2 ng LH/mL, and 15.6 ± 0.6 ng FSH/mL. The reduction in testosterone levels caused by letrozole was the only variation observed at the 42nd day.

In 91-day-old animals, hormone levels were 318.4 ± 23.2 ng/dL for testosterone, 18.8 ± 1.1 pg/mL for estradiol, 4.1 ± 0.3 ng/mL for LH, and 9.9 ± 0.5 ng/mL for FSH. No significant difference was observed between treatments and controls, except for a decrease in the LH concentration (Table 2).

Histological analysis

Histological analysis of the rat ventral prostate of 21-day-old animals revealed a polarized columnar epithelium interspersed with basal cells (Fig. 1A). Stromal cells were usually spindle shaped and loosely organized around epithelial structures (Fig. 1A). Estradiol treatment resulted in a multilayer epithelium in some regions (Fig. 1B). The epithelial cell nuclei appeared more compact and presented an irregular outline. No change in volume density was observed for the non-muscular stroma and luminal compartment, but there was a decrease in epithelial volume density and a significant increase in smooth muscle cell volume density. A significant increase in the absolute volume of the luminal and stromal (non-muscular, smooth muscle cells) compartments was also found (Fig. 3B). No qualitative light microscopic changes were observed

in finasteride-treated rats (Fig. 1C). However, stereological analysis showed a significant reduction in the volume densities of the stromal and epithelial compartments, and significant increases of the lumen and of the smooth muscle cell compartment (Fig. 3A). The absolute volume of the stromal and epithelial compartments was found to be reduced (Fig. 3B), whereas no changes in the luminal and smooth muscle cell compartments were observed.

In tamoxifen-treated rats (Fig. 1D), presence of apoptotic figures were observed for the epithelial compartment and there was a clearly visible increase in the volume density of smooth muscle cells, whereas the other compartments remained unchanged (Fig. 3A). In contrast, the absolute volume of the lumen, epithelium and stromal compartments showed a significant reduction, whereas the smooth muscle cell compartment was unchanged (Fig. 3B).

The normal structure of the ventral prostate previously described for controls was preserved in letrozole-treated animals (Fig. 1E). Despite an apparent normal histological structure, abortive mitoses were found in the epithelium (Fig. 1E, arrow). There was no change in volume density. The absolute volume of the epithelium, lumen and stroma was found to be reduced, but there was no change in the smooth muscle cell compartment (Fig. 3B).

The ventral prostate gland of 42-day-old animals contained acini lined with a single layer of tall columnar cells, while the ducts were lined with cuboidal cells, with the distal region preserving the characteristic infolding and tall columnar epithelium (Fig. 2A).

Neonatal treatment with estrogen did not alter the morphological features observed in control rats (Fig. 2B). Although stereology showed a relative decrease of the epithelial compartment, absolute volume remained unchanged (Fig. 3C and D).

Neonatal exposure to finasteride did not affect the morphological features of the rat ventral prostate (Fig. 2C). No effect was observed on relative or absolute volume of the tissue compartments, except for an increase in relative luminal volume (Fig. 3C).

Administration of tamoxifen affected the height of the glandular epithelium, which was consistently smaller (Fig. 2D). There was a proportional increase in the relative volume occupied by the lumen (Fig. 3C), although the absolute volume did not differ from that of vehicle-treated rats. Apoptotic figures were frequent in the epithelium (Fig. 2D, detail).

Treatment with letrozole caused no alterations in the morphological features of the rat ventral prostate (Fig. 2E). However, reduced relative (Fig. 3C) and absolute volumes (Fig. 3D) were observed for the epithelial compartment, contrasting with the presence of mitotic cells (Fig. 2E). The stromal compartment presented an increased relative volume.

The mature prostate of 91-day-old control rats was characterized by a continuous layer of tall epithelial luminal cells, a discontinuous layer of basal cells, and a well-defined layer of stromal components, with distinguishable smooth muscle cells and fibroblasts (Fig. 2F).

The luminal cells were well defined and a pale stained region corresponding to the Golgi complex suggested continuous secretory activity (arrows, Fig. 2F). Stereological analysis showed that the epithelium and lumen were the predominant compartments of the gland, occupying most of its volume density (Fig. 3E).

The most evident effect of treatment with E_2 was observed in the stromal compartment, with changes in the distribution of stromal cells (Fig. 2G) and in the relationship between epithelial and smooth muscle cells (detail, Fig. 2G).

Neonatal treatment with finasteride at the dose used caused no dramatic alterations in the epithelial or stromal compartment of adult rats, but stromal cells presented higher densities in some regions of the gland (Fig. 2H), with an apparent disruption of the normal distribution of stromal cells along the ductal system. Despite the above mentioned changes, no alteration in volume density or absolute volume of the tissue compartments was observed in early adulthood (Fig. 3E, F).

No changes in tissue organization of the ventral prostate were observed for adult rats treated with tamoxifen during the perinatal period (Fig. 2I), except for the presence of apoptotic cells in the distal region of the ductal system (Fig. 2J).

In letrozole-treated rats, stromal cell number were found to be increased in some regions (Fig. 2K); however, no changes were observed in the epithelial compartment.

Immunohistochemical analysis of AR

In intact rats, AR staining was localized exclusively in the nucleus of epithelial and stromal cells (Fig. 4A). AR staining appeared non-uniform between cells within the same duct, with the staining intensity in luminal cells ranging from very strong to moderate. Despite this variation, luminal cells showed intense staining for AR than basal cells (arrow).

Treatment with E_2 slightly reduced AR staining intensity, with nuclear staining ranging from absent to intense (Fig. 4B). In finasteride-treated rats, the nuclear staining was rather uniform (Fig. 4C), in contrast to the reduced staining intensity observed in stromal cells, although staining was preserved in some smooth muscle cells. Tamoxifen treatment did not caused alteration in the staining of luminal epithelial cells (Fig 4D). Letrozole treatment did not alter the epithelial staining for AR, with epithelial cells presenting homogeneous staining intensities. The same was observed for stromal cells (Fig. 4E).

The ventral prostate of 42-day-old rats presented strong but slightly variable staining in the nuclei of epithelial and stromal cells (Fig. 4F). Neonatal treatment with E_2 did not alter the staining pattern previously described for control rats (Fig. 4G). Finasteride treatment caused a marked reduction in the staining intensity of AR-positive cells compared to the control group (Fig. 4H). However, this effect was not uniform and some regions preserved the strong staining observed for controls. Tamoxifen treatment caused a slight reduction in the staining of luminal epithelial cells, but presented strong staining in the nuclei of stromal cells (Fig. 4I). Letrozole had no effect on AR staining (Fig. 4J).

In 91-day-old control rats, AR staining appeared in the nuclei of epithelial and stromal cells (Fig. 4K), with the latter presenting weaker staining than the former. After neonatal treatment with E_2, AR staining was more intense than in controls. Proliferating cells were AR positive (Fig. 4L). In 91-day-old finasteride-treated rats, intense nuclear staining for AR was observed in the epithelium, whereas luminal cells presented different staining intensities (Fig. 4M). Figure 4N shows the results of AR staining in the prostate of tamoxifen-treated animals. No difference was observed between epithelial cells and controls. Neonatal treatment with letrozole had no effect on AR staining in adult rats (Fig. 4O).

Ki67 immunostaining

Ki67 expression was mainly observed in luminal cells of control, 21-day-old (Fig. 5A), and 42-day-old rats (Fig. 5B). In contrast, proliferating activity was reduced in the luminal cells of adult rats (Fig. 5C). Basal cells were not reactive to Ki67 in 21- and 91-day-old animals, in contrast to 42-day-old animals (Fig. 5B) which presented Ki67-positive, proliferating basal cells. Stromal cells presenting Ki67 staining were more frequent in 21-day-old animals (Fig. 5A).

Neonatal E_2 treatment resulted in a more intense proliferative activity of luminal cells in 42-day-old animals (Fig. 5A and D). However, no difference in proliferation rates were observed between 21-day-old and 91-day-old animals when compared to the control group. Proliferative activity was observed in basal cells of 21-day-old animals, but the rate did not differ from controls. No proliferation was seen in the basal cell compartment of 42- and 91-day-old animals, similarly to controls. In contrast, a larger number of Ki67-positive cells was observed in the stroma of 42-day-old animals, with lower activity in 21- and 91-day-old animals compared to controls. Finasteride caused no difference in proliferative activity compared to controls. Letrozole promoted an increase in the proliferative activity of epithelial cells in the prostate gland of 21-day-old (Fig. 5E) and 91-day-old animals (Fig. 5F). Basal and stromal cells did not differ from

the control group. Tamoxifen caused no alteration in the proliferation rate of prostate cells. However, proliferative activity was observed in the different ductal regions.

DISCUSSION

Prostate organogenesis from the fetal stages to the prepubertal period offers a unique opportunity to characterize the transition from a proliferative state to a relatively quiescent gland, with growth following overall body weight gain (Vilamaior *et al*., 2006). The present study demonstrated that a tunable balance between steroid hormones is necessary for postnatal development of the ventral prostate and that the interference with this normal process leads to altered growth patterns not only during development and puberty, but also in adulthood.

The correct temporal-spatial sequence of the action of estrogen is necessary for normal development, and drugs interfering with this action such as tamoxifen or letrozole were found to affect absolute prostate weight by day 21 after birth, although these drugs exert their effects differently, with tamoxifen blocking ERs and letrozole inhibiting the action of aromatase action which, in turn, blocks E_2 synthesis.

It has been well documented that perinatal exposure to low doses of E_2 stimulates prostate growth in adult life (Putz *et al*., 2001). In the present study, this effect was noted in 21-day-old rats. However, the strongest effect of neonatal estrogen consisted of promoting a higher proliferative index in 42-day-old rats, a period characterized by the increasing expression of a subset of genes that are upregulated by testosterone at the onset of puberty (Dhanasekaran *et al*., 2005). A higher proliferative index of basal cells was also observed and this event might be associated with the subsequent development of squamous epithelial metaplasia (Merk *et al*., 1986). We hypothesized that neonatal estrogen caused androgen hypersensitivity through the increased expression of AR and probably a higher affinity of the AR-androgen complex (Nonneman *et al*., 1992), conditions

that are associated with the post-castration onset of androgen-independent prostate cancer (Feldman & Feldman 2001).

An unexpected result was the decrease in prostate weight observed in letrozole-treated animals. Previous reports have shown that a deficient action of aromatase and the consequent absence of estrogen cause enlargement of the prostate (Fisher *et al*., 1998). However, the proliferative index showed a loss of the inhibitory effect of estrogen in luminal epithelial cells as demonstrated by the inhibition of aromatase with letrozole. Both events, weight loss and increased proliferative index, probably resulted in aberrant prostate growth. An important sign of this aberrant growth was the high frequency of abortive mitoses observed by day 21 after birth, which was associated with a reduced absolute volume of the epithelial compartment in 42-day-old animals. The proliferative effect of neonatal letrozole on epithelial cells was also observed later in adult 91-day-old rats.

Finasteride is widely used in the treatment of benign prostatic hyperplasia in mature men, since it causes a rapid fall in DHT levels within the first eight hours after administration (Kaplan, 2001). In the present study, neonatal treatment with finasteride caused no alteration in testosterone or LH levels, even in 21-day-old animals. However, by blocking 5α-reductase and subsequently the formation of DHT during the neonatal period, finasteride decreased prostate weight and epithelial and stromal compartment volumes in 21-day-old animals. DHT is very important for virilization of the urogenital sinus and for induction of the male external genitalia, because it is a more potent androgen than testosterone and serves to amplify androgenic stimulation when testosterone levels are low (Killian *et al*., 2003). This effect was not preserved in later stages. There are two probable explanations for this observation. First, it is possible that DHT, although necessary for their differentiation, is not an important trophic hormone for prostate in postnatal life, in contrast to its fundamental importance during early prostate development (George & Peterson, 1988). Second, neonatal treatment with finasteride might have long-lasting effects that manifest by

day 21 after birth, but does not affect prostatic imprinting that may affect later growth during puberty.

The *in vitro* and *in vivo* effects of tamoxifen are controversial because of the direct and side effects of the drug (White, 1999). Although the primary mechanism of action of tamoxifen is believed to be the inhibition of the ER, some data regarding the action of tamoxifen indicate that additional non-ER-mediated mechanisms do exist (Mandlekar & Kong 2001). The overall decrease of the compartments observed in tamoxifen-treated animals shows that a transient neonatal block of ER by tamoxifen caused a reduction of prostate weight. However, there was no decrease in the absolute volume of the smooth muscle cell compartment, indicating a possible upregulation of smooth muscle cells by tamoxifen (Bruengger *et al*., 1983).

During the pubertal phase (starting 7 weeks after birth), the prostate shows an active period of growth (Vilamaior et al., 2006). Only estrogen and letrozole treatments affected prostate weight and histological organization, indicating that estrogen imprinting is a major factor in prostate homeostasis.

While some results such as reduced prostate weight were common for both letrozole and tamoxifen treatment, others are contrasting such as the active proliferation of luminal cells during the prepubertal period and in adult life as a result of neonatal letrozole treatment. This finding suggests that antagonizing ERs and blocking estrogen production cause different responses and that the action of estrogen is complex and promotes permanent changes in the ability to respond to androgen stimulation.

Previous studies have shown the effects of low doses of different hormonal disruptors during the postnatal period that alter prostate physiology in adulthood (Corbier *et al*., 1995; Putz *et al*., 2001).

Reports have shown the occurrence of sequential developmental events along the first postnatal weeks, which are tightly associated with hormone action. These hormonal stimuli

produce different dynamics in cell proliferation and tissue organization until the gland reaches the physiologically active adult pattern.

We conclude that the response of the ventral prostate gland to exposure to hormonal disruptors during postnatal life is more dramatic during the prepubertal period than in adult life and that the prostate response to a pubertal testosterone surge is altered by neonatal treatment with estrogen drugs.

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Figure legends

Figure 1. H&E-stained sections of the ventral prostate from 21-day-old rats treated with different drugs during the neonatal period. (A) Histological aspect of the prostate from a control rat showing the acini (A) surrounded by the stroma (ST). The glandular epithelium consists of tall columnar luminal cells (arrows) and triangular basal cells (arrowhead) (925x). (B) In the prostate of E_2-treated animals, some cells with proliferative activity (arrows) and normal glandular epithelium were seen (925x). (C) After finasteride treatment, both acini (A) and stroma (ST) preserve the histological aspects of control animals (370x). (D) In tamoxifen-treated animals, the prostatic epithelium shows apoptotic figures containing a typically fragmented nucleus (arrow). Flat muscular cells predominate in the stroma (SMC) (925x). (E) In letrozole-treated rats, the histological structure is normal and the control structure is preserved (arrows) (370x). Bars = 20 μm (A, B, D) and 60 μm (C, E).

Figure 2. H&E-stained sections of the ventral prostate from 42-day-old rats after neonatal treatment with different drugs. (A) Prostate of control animals. The epithelium consists of tall columnar luminal cells surrounded by a thin layer of smooth muscle cells (370x and 925x, respectively). (B) In the ventral prostate of E_2-treated animals, the histological pattern is similar to that of control animals (370x). (C) The prostate of finasteride-treated animals presents normal glandular structures, with tall columnar epithelium surrounded by smooth muscle cells (SMC). (D) In tamoxifen-treated rats, the prostate presents a high density of apoptotic cells (arrows) (370x and 925X, respectively). (E) In letrozole-treated animals, mitotic activity is present (arrow). The detail shows a normal epithelium in contact with smooth muscle cells (SMC) (370x and 925x, respectively).

In H&E-stained sections of the ventral prostate from 91-day-old animals treated with different drugs. (F) Control showing the characteristic tall columnar epithelium (arrow) with basal nuclei; a thin layer of smooth muscle cells is clearly visible (925x). (G) Intermediate region of the rat ventral prostate of E_2-treated animals showing an evident smooth muscle cell (arrow) (370x and 925x, respectively). (H) In finasteride-treated animals, the prostate presents normal glandular structures (925x). (I) In tamoxifen-treated rats, the prostate presents the morphological characteristics of a normal epithelium. An apoptotic body is observed (J) (925x). (K) Letrozole treatment did not affect normal prostate histology. The detail highlights an organized layer of smooth muscle cells (SMC) (370x and 925x, respectively). Bars = 20 μm (A, B, C, D, E, G, K) and 60 μm (F, H, I, J).

Figure 3. Volume density (A, C, E) and absolute volume (B, D, and F) of the different prostatic compartments (epithelium, lumen, stroma, and smooth muscle cells) determined by stereology in 21-, 42- and 91-day-old rats after neonatal treatment with different drugs. Control, estrogen, finasteride, tamoxifen, and letrozole. Data are reported as the mean ± SEM (n = 5). *$p \leq 0.05$. NMS= non-muscular stroma, SMC = smooth muscle cell.

Figure 4. Immunohistochemical localization of the androgen receptor in the ventral prostate after neonatal treatment with different drugs. (A-E) in 21-day-old rats; (A) sections of the prostate of control rats showing a detail of the epithelium with intense nuclear staining. Stromal cells also present positive staining (arrow). (B) E_2-treated animals show AR-positive staining (arrows). (C) Intermediate region of finasteride-treated animals presenting intense staining. (D) Sections of the prostate from tamoxifen-treated animals. Note that the nuclear staining was less intense compared to controls. (E) In letrozole-treated animals, the prostate preserves the nuclear staining pattern. (Fig. A-E 925x).

In 42-day-old rats, micrograph showing general staining in the epithelium and stroma of the prostate of control rats (F). Intense staining was clearly visible in stromal (arrows) and epithelial cells. In E_2-treated rats, differences in staining depending on the prostatic region and different intensities of nuclear staining (G) could be observed. Section obtained from finasteride-treated animals positive epithelial staining (H). Sections obtained from tamoxifen-treated animals showing nuclear staining (I). AR-positive staining in sections from letrozole-treated animals (J). (Fig. 4F-J 925x).

In 91-day-old rats after neonatal treatment with different drugs. (K) Control showing positive staining in the luminal epithelial cells. E_2 treatment did not change the staining pattern of luminal cells (L). Different intensities of nuclear AR staining are observed in finasteride-treated animals (M). In tamoxifen-treated rats, a decrease in AR staining could be observed in some regions (N). In letrozole-treated animals, the staining intensity was not altered when compares to the control (O). (Fig. 4K-O 925x).

Figure 5. Ki67 immunostaining in postnatal prostates after neonatal treatment with different drugs. The graphs depict the percentage of positively stained cells in the luminal (A) and stromal (B and C) compartments. (A) Percentage of positively stained luminal cells in 21, 42 and 91-day-old animals after treatment with E_2, finasteride, tamoxifen and letrozole. (B) Percentage of positively stained basal cells in 21, 42 and 91-day-old animals after treatment with E_2, finasteride, tamoxifen and letrozole. (C) Percentage of positively stained stromal cells in 21, 42 and 91-day-old animals after treatment with E_2, finasteride, tamoxifen and letrozole. Note that the highest percentage of labeled cells in all prostate compartments was observed in 42-day-old animals treated with E_2 (D). Note also that treatment with letrozole stimulated the proliferative activity of luminal cells in 21 (E) and 91-day-old animals (F). (D-F 925x).

Table 1. *Body, prostate, and testis weight of adult rats after treatment with different drugs. Values are the mean± SEM (n=5).*

Postnatal day	Group	Body weight (g)	Prostate (mg)	Relative prostate weight	Testis (g)
21	Control	42.0 ± 0.70	29.4 ± 0.20*	0.071± 0.00	0.30 ± 0.00
	E_2	49.4 ± 0.40*	39.6 ± 0.20*	0.080 ± 0.00	0.31 ± 0.00
	Finasteride	44.2 ± 0.30	25.6 ± 0.70	0.058 ± 0.00*	0.30 ± 0.00
	Tamoxifen	49.6 ± 0.60*	16.6 ± 3.30*	0.033 ± 0.00*	0.30 ± 0.00
	Letrozole	46.6 ± 1.30	22.2 ±1.00*	0.047 ± 0.00*	0.30 ± 0.00
42	Control	137.6 ± 2.60	86. 0 ± 1.30	0.062 ± 0.00	1.42 ± 0.03
	E_2	105.4 ± 1.40*	79.0 ± 3.00	0.075 ± 0.00	1.24 ± 0.05
	Finasteride	155.4 ± 0.40	77.4 ± 1.70	0.049 ± 0.00	1.65 ± 0.03
	Tamoxifen	126.2 ± 4.85	82.6 ± 2.20	0.067 ± 0.00	1.60 ± 0.05
	Letrozole	147.6 ± 1.80	76.2 ± 3.30	0.052 ± 0.00	1.64 ± 0.05
91	Control	334.3 ± 5.20	311.5 ± 8.40	0.093 ± 0.00	3.35 ± 0.04
	E_2	369.8 ± 4.80*	308.0 ± 8.51	0.083 ± 0.00	3.30 ± 0.07
	Finasteride	333.8 ± 4.80	255.8 ± 12.10	0.076 ± 0.00	3.53 ± 0.07
	Tamoxifen	322.6 ± 4.70	284.8 ± 5.40	0.088 ± 0.00	3.34 ± 0.06
	Letrozole	326.3 ± 5.70	348.2 ± 11.70*	0.107 ± 0.00	3.24 ± 0.05

* = $P< 0.05$, as compared to the control

Table 2. *Serum testosterone, estradiol, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels of adult rats after treatment with different drugs. Values are the mean ± SEM (n=5).*

Postnatal day	Group	Testosterone (ng/dL)	Estradiol (pg/mL)	LH (ng/mL)	FSH (ng/mL)
21	Control	14.8± 0.90	84.2 ±3.00	2.4 ± 0.20	4.1 ± 0.40
	E_2	15.7±1.80	42.9 ± 3.60*	1.0 ± 0.14	5.5 ± 0.50
	Finasteride	12.9 ± 2.50	14.9 ± 2.90*	1.2 ± 0.30	17.0 ± 3.20
	Tamoxifen	13.6 ± 2.10	30.2 ± 3.40*	1.23 ± 0.04	5.6 ± 0.90
	Letrozole	14.0 ± 1.00	65.1 ± 3.70	1.3 ± 0.30	2.6 ± 0.20
42	Control	174.5 ± 5.10	18.7 ± 1.70	3.1 ± 0.20	15.6 ± 0.60
	E_2	128.5 ± 4.90	17.6 ± 2.30	4.3 ± 0.01	8.8 ± 1.60
	Finasteride	69.0 ± 14.30*	8.0 ± 2.20	4.0 ± 1.00	13.3 ± 0.40
	Tamoxifen	142.9 ± 14.20	10.2 ± 1.50	4.2 ± 0.80	13.2 ± 2.10
	Letrozole	123.5 ± 6.30	49.6 ± 15.70	5.6 ± 1.40	8.8 ± 1.50
91	Control	318.4 ± 23.20	18.8 ± 1.10	4.1 ± 0.30	9.9 ± 0.50
	E_2	287.6 ± 10.50	11.5 ± 1.40	4.7 ± 0.10	9.3 ± 0.50
	Finasteride	415.4 ± 25.40	13.4 ± 0.40	7.1 ± 0.80	9.9 ± 0.90
	Tamoxifen	205.6 ± 5.00	13.8 ± 0.20	3.0 ± 0.10	10.8 ± 1.00
	Letrozole	289.1 ± 44.10	21.3 ± 0.90	2.3 ± 0.60*	13 ± 0.80

* = $P < 0.05$, as compared to the control

Figure 1

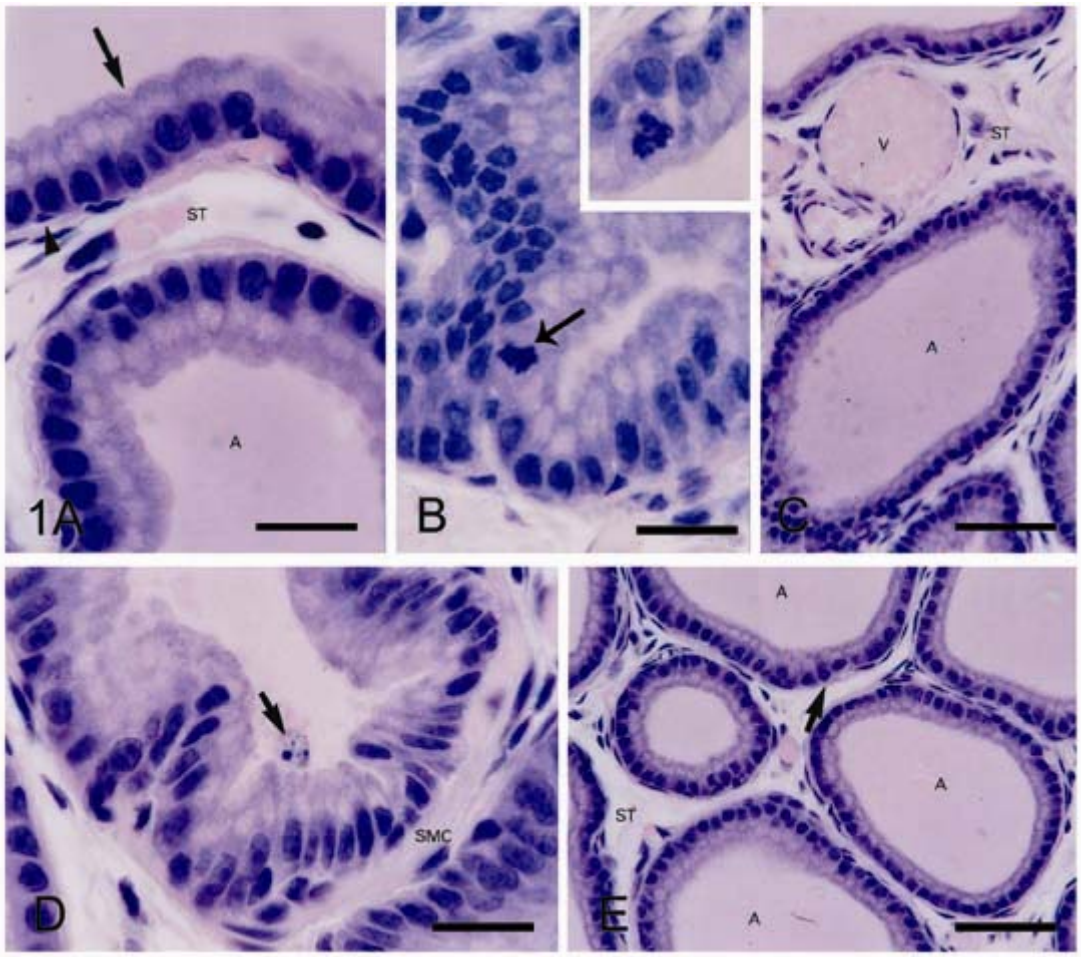


Figure 2

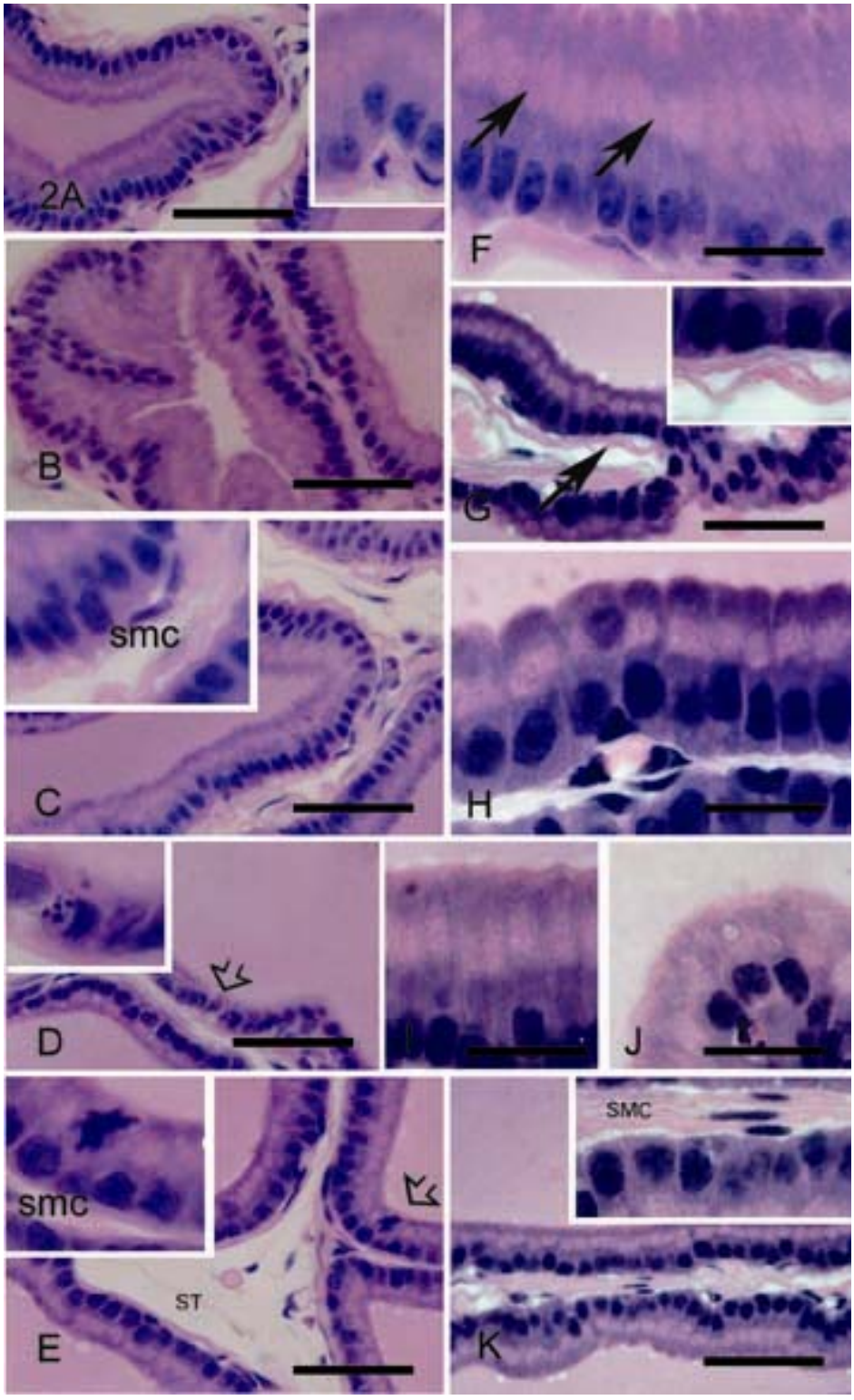


Figure 3

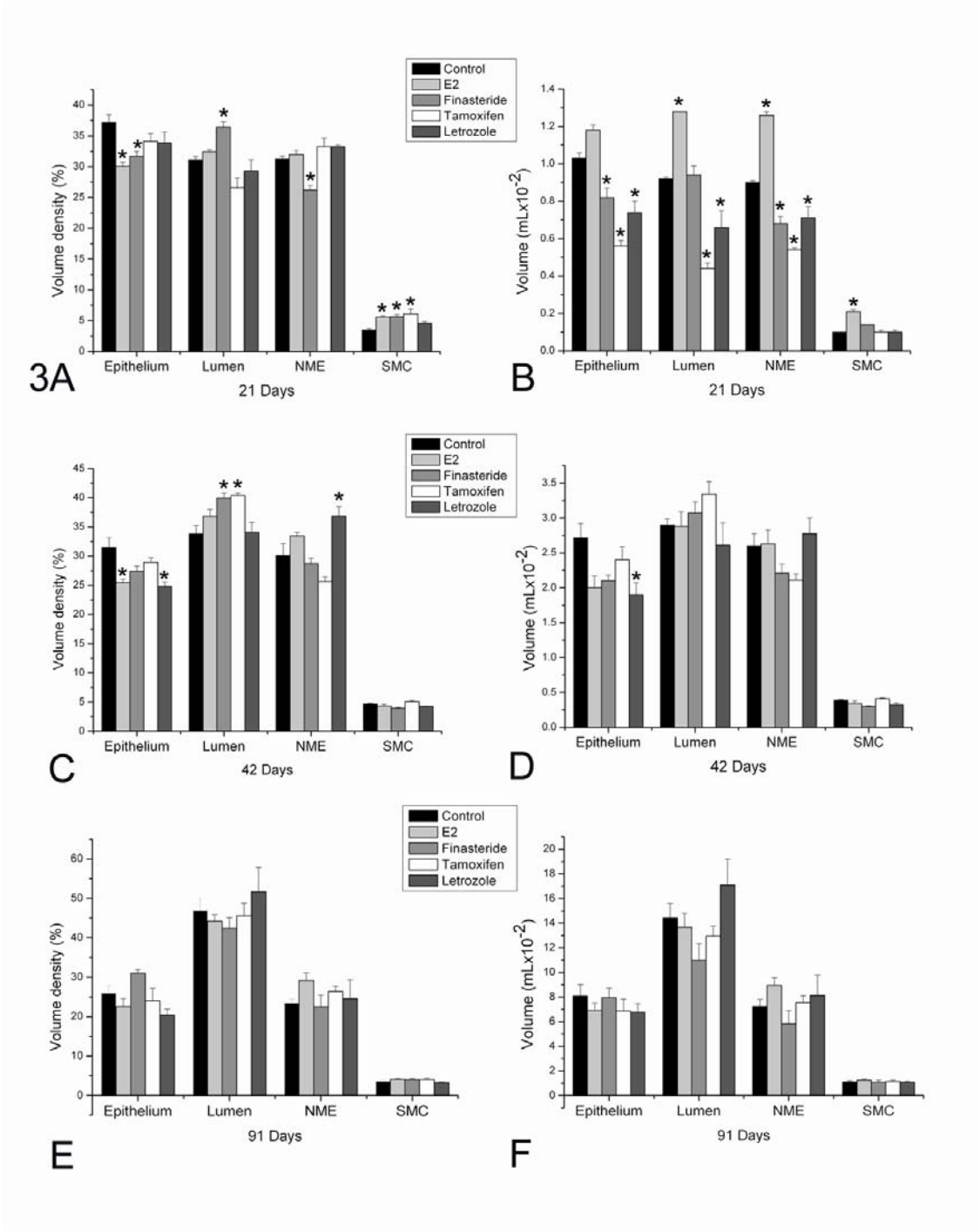

Control
E2
Finasteride
Tamoxifen
Letrozole
Volume density (%)
Volume (mLx10-2)
Epithelium
Lumen
NME
SMC
21 Days
42 Days
91 Days
3A
B
C
D
E
F

Figure 4

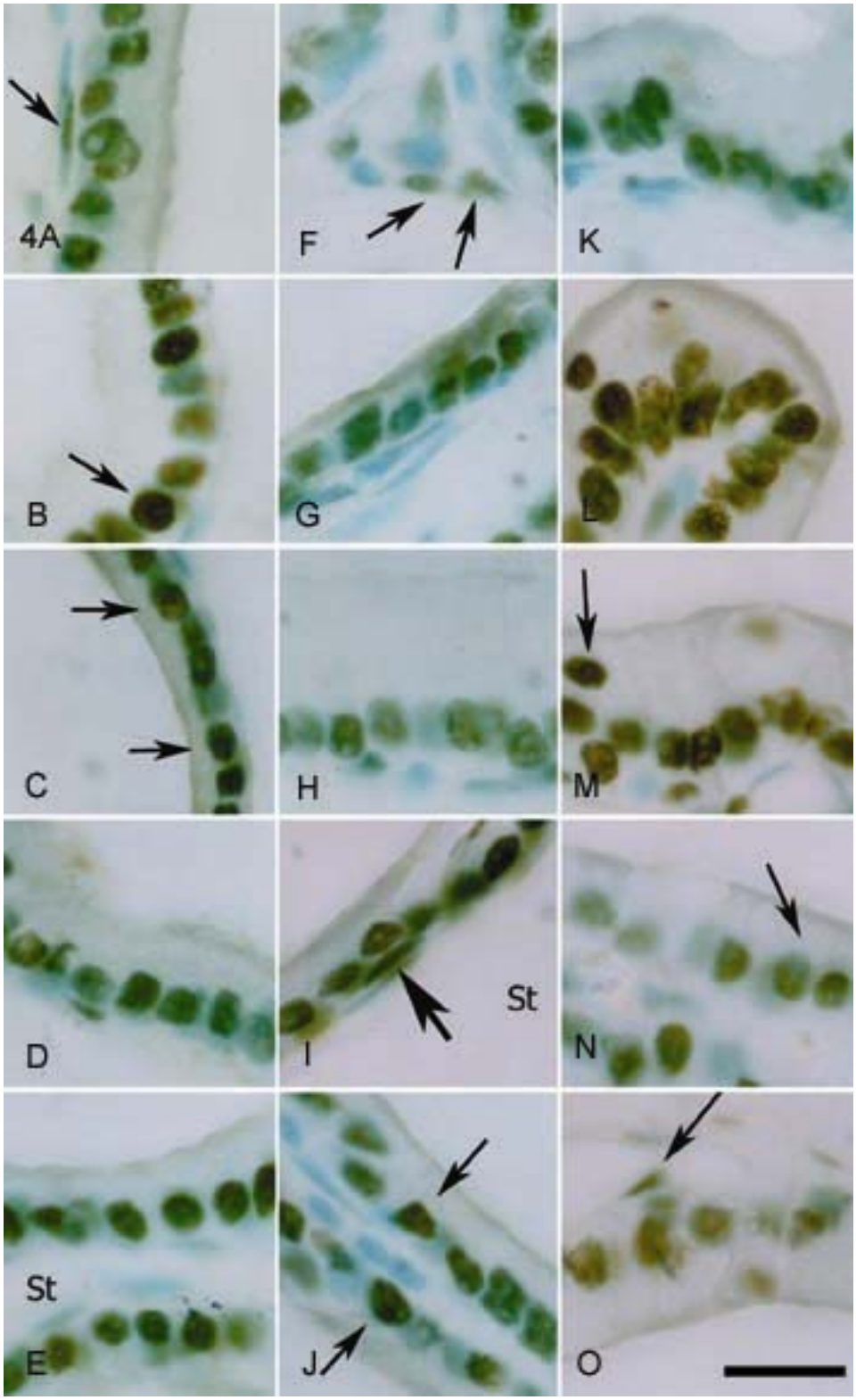
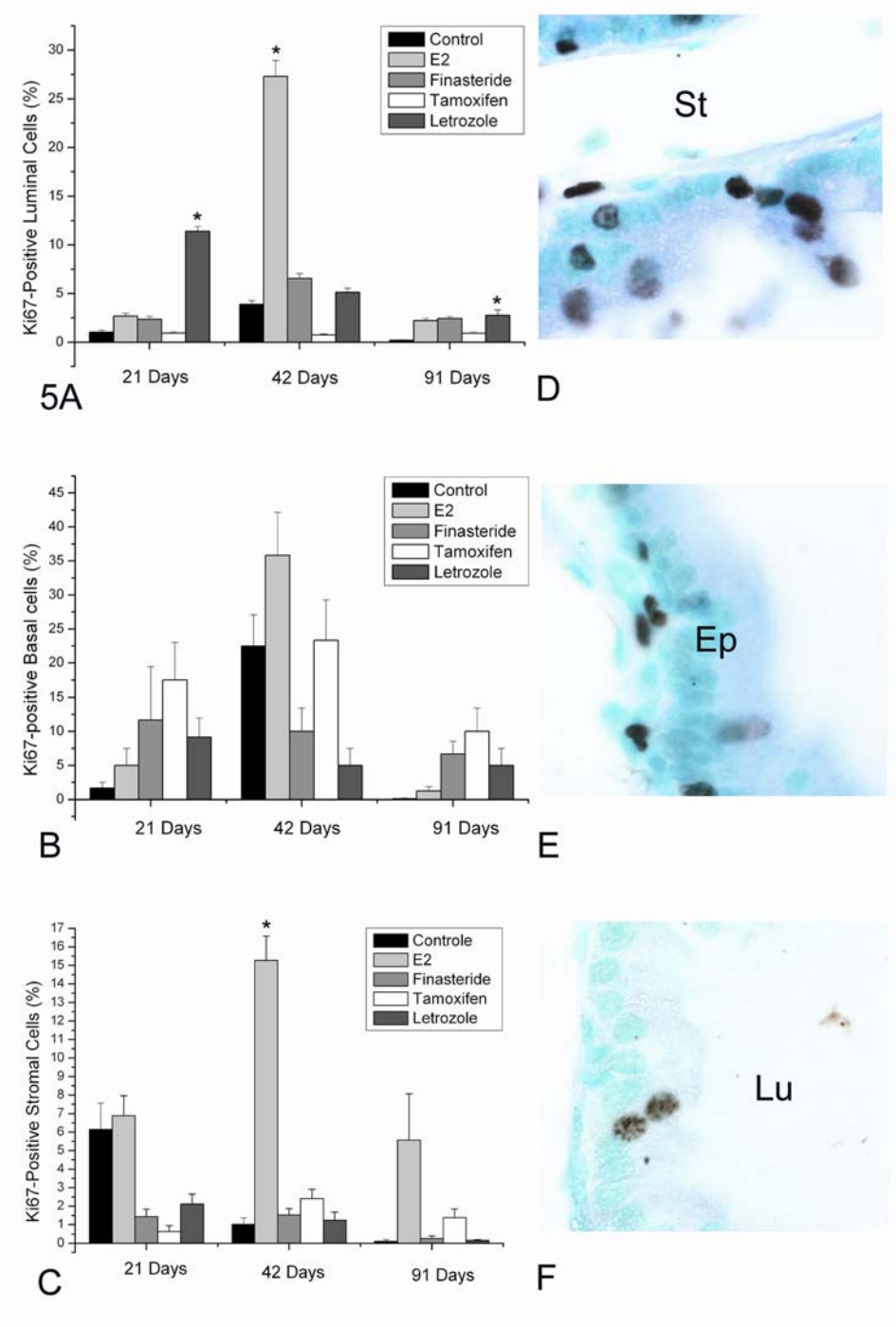


Figure 5


Control
E2
Finasteride
Tamoxifen
Letrozole
Ki67-Positive Luminal Cells (%)
21 Days
42 Days
91 Days
5A
St
D
Ki67-positive Basal cells (%)
B
Ep
E
Controle
Ki67-Positive Stromal Cells (%)
C
Lu
F

Post-pubertal and adult prostate response to prepubertal exposure to different hormonal enviroments.

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Abstract

The present study was carried out to examine the effects of exposure to different hormonal disruptors on early postnatal life on the juvenile phase of prostatic development. Twenty eight-day-old animals were submitted to different treatments (flutamide, testosterone, estradiol, tamoxifen and castration) aiming at establishing different hormonal environments. Analyses were done at the post-puberal phase (58-day-old) and in the adult (91 days-old). The HHG axis was affected by estrogen and testosterone. Although showing no effect on the HHG axis, flutamide and tamoxifen influenced tissue organization especially the luminal compartment reflecting their action on the secretory activity. The proliferating kinetics was affected by the different treatments especially at 58 days of treatment. No dramatic alteration in both androgen receptor intensity and distribution was detected. We conclude that the pre-puberal period is also sensitive to endocrine disruptors because the components of HHG axis are still immature and susceptible to disruption.

INTRODUCTION

The endocrine system has important functions in a multitude of physiological processes including embryogenesis, cellular differentiation, homeostasis and carcinogenesis [1].Steroid hormones have broad developmental and psychological effects even at the earliest stages of life. Moreover, testosterone has important effects throughout life, ranging from the obvious effects on pubertal development to the physiologic deregulation that contributes to male baldness pattern and to the genesis of prostatic hyperplasia [2].

In humans, after the third trimester of pregnancy the prostate gland enters a quiescent phase due to a drop in testosterone levels. This dormant phase persists until puberty when the testosterone level surges and the prostate doubles in size and acquires the ability to produce secretions [3]. Normal puberty is associated with the onset and progressive activation of the hypothalamic-pituitary-gonadal axis and the resultant development of secondary sexual characteristics. This is achieved by increases in gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), and the sex steroids testosterone and dihydrotestosterone (DHT), to control a wide variety of processes during puberty including prostate development [2].

The peri-natal exposure to different compounds with hormonal activity in this specific sensitive “window” promotes inadequate responses to hormones in adult life this effect has been associated with a predisposition of prostatic pathologies [4, 5]

It has been hypothesized that enviromental factors may contribute to alterations in the reproductive development, including the increased incidence of human male reproductive-tract abnormalities [6]. Hormone mimicking or blocking chemicals may cause significant impairment during this critical period and disproportionate alteration in normal sexual maturation [1]. In most cases, the links between these compounds and adverse effects in humans have not been established. In the present study, one has checked whether exposure to steroid hormones or drugs

affecting their function has implications in prostatic development when administered to prepubertal rats.

MATERIALS AND METHODS

After acclimatization under controlled conditions (lights on 0700-1900h, temperature 20-24 ^{o}C) immature male rats (28 d of age, weighing approximately 125-130 g) were treated with different drugs (see below). Testes, seminal vesicle and ventral prostate were excised and weighed. The experiments were carried out according to the Guide for Care and Use of Laboratory Animals and approved by the University's Committee for Ethics in Animal Experimentation of UNICAMP (Protocol number 592-1).

Treatments

I. Supraphysiological levels of estrogen: the effect of a hyperestrogenic environment during puberty ventral prostate of rats was investigated by the administration of 17β-Estradiol (Sigma Chemical Co., St. Louis, MO, U.S.A.) (0.15 mg/kg) [7].

II. Blockade of the estrogen receptor (ERs): the exposure to physiological level of estrogens was inhibited through the use of tamoxifen (Sigma Chemical Co) (10 mg/kg) [4].

I. Supraphysiological levels of testosterone: to determine the capacity of pubertal exposure to T in modulating the post-natal prostate growth, testosterone cipionate, was applied in the dosage of 5 mg/kg. [8].

III. Blockade of the androgen receptor (AR): the blockade of the ARs was attained with the use of flutamide (10 mg/Kg) (Sigma Chemical Co.) [1].

IV. Fall of serum levels of T and DHT: the effect of castration before puberty was investigated by the orchiectomy.

Treatments with E2, T, tamoxifen, and flutamide were done between 28 and 38 postnatal days. Control animals receive only corn oil (Sigma Chemical Co.) that was used as a vehicle, and the volume used was 1 mL/Kg body weight.

Hormone Measurement

Serum levels of testosterone (T), estradiol (E_2), luteinizing hormone (LH) and follicle stimulating hormone (FSH) were estimated by RIA. Blood samples were obtained by cardiac puncture immediately before death. The serum was separated by centrifugation and stored at -70 C for subsequent hormone assays. T and E_2 concentrations were measured in serum samples using Coat-a-count kits (Diagnostic Products, Los Angeles, CA, USA). Serum samples were assayed in duplicate.

Plasma LH and FSH were measured by double-antibody radioimmunoassay using specific kits provided from the National Hormone and Peptide Program (NIH / NIDDK, USA). All samples in the same experiment were measured in the same assay. The lowest detectable amount of $LHRP_3$ standard was 0.05 ng/ml and the intra-assay coefficient of variation was 4%. The lowest detectable amount of FSHRP2 standard was 0.2 ng/ml and the intra-assay coefficient of variation was 3,2 %.

Histology

The VP was immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr. Samples were then washed, partially dehydrated, and embedded in Leica historesin. Two micrometer sections were obtained and stained with hematoxylin and eosin [9]. For immunohistochemistry, fixed samples were embedded in paraffin, after dehydration with an ethanol series and clearing in xylene.

Stereological Analysis

For stereological purposes, five microscopical fields from the hematoxylin and eosin stained sections from five animals for each group were photographed with a Zeiss Axioskop

microscope (Jena, Germany) and the Weibel multipurpose graticule system (120 point, 60 test lines) [10] was utilized in a systematic field sampling. The following parameters were evaluated on the basis of area measurement-volume densities (percentage of tissue volume occupied by defined tissue compartment) of epithelium, stroma, lumen and smooth muscle cell. For the estimation of volume fractions, the number of counting grid points falling in each tissue compartment was counted. The volume of the VP served as the reference volume. For approximation it was assumed that the specific gravity of the prostate tissue was 1.0 [10, 11].

Immunohistochemical Staining

Paraffin-embedded sections (6 μm) of VP were dewaxed and rehydrated. Antigens were retrieved by boiling the sections in 10 mM citrate buffer, pH 6.0 3 times of 5 min in a microwave oven. The cooled sections were incubated in 1% H_2O_2 for 15 min to quench endogenous peroxidase. To block nonspecific binding, sections were incubated in 3% BSA for 1h at room temperature. Sections were then incubated with a polyclonal anti-AR (N-20) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA. U.S.A.) or a monoclonal anti-Ki67 (Clone-MIB5; DakoCytomation Inc., Carpenteria, CA, USA), both in 1:100 dilutions in 1% BSA at 4°C. Negative controls were incubated with only 1% BSA lacking the primary antibody. The EasyPath ABComplex/HRP kit (Novocastra. Laboratories, Newcastle, U.K) was used to visualize the bound antibodies, according to manufacturer's manual. The sections were incubated in appropriated secondary antibody solution for 30 min followed by washing for 10 min with PBS and incubation in Avidin and Biotinylated HRP solution for 30 min. After washing in PBS, peroxidase activity was revealed with 3, 3´diaminobenzidine tetrahydrochloride substrate. Sections were lightly counterstained with methyl green, dehydrated through ethanol series and xylene, and mounted in Entellan (Merck KGaA, Darmstadt, Germany).

Ki67-positive Cell Counts

Proliferative indices were assessed by counting Ki67-positive and Ki67-negative (methyl green-stained) nuclei at 400 x magnification. A total of three immuno-stained sections per treatment were used and separated counts were made for the epithelial and stromal compartments of each gland. Approximately 2,000 cells were counted for each treatment. The number of positive cells was expressed as the percentage of total cells of a given type (luminal and basal epithelial cells and stromal cells).

RESULTS

Body and organ weight

Treatments with the different drugs did not affect body weight, in contrast to castration which caused significant weight changes in adulthood (Table 1). Testis weight was not modified by treatment with the different drugs. There was a significant reduction in the seminal vesicle weight in castrate animals in adulthood, but not in the earlier stage.

The prostatic weight gain was compromised by E_2 and castration at the 58th days of age. Testosterone, on the other hand, promoted an increase in prostatic weight at this age (Fig. 1A). By the 91st day of age, the differences observed earlier were no longer seen, except for castration, which hampered prostatic development. The mean ventral prostate weighed about 217 mg in the control group. Castration caused a marked reduction in prostatic weight, while the other treatments had no effect.

Serum hormone levels

Table II summarizes the results for hormone measurements. The 58-day-old control rats showed a mean serum concentration of testosterone of 216.2 ng/dL. The other groups showed oscillations around this value but no significant difference was found. The testosterone levels in

castrated animals were bellow detection. The mean serum level of testosterone for the 91-day-old control rats was 302.6.

The serum level of estradiol for the 58-day-old control group was 32 pg/mL. Flutamide caused an increase in the levels of circulating estradiol, while the other treatments had no effect.

In the 58-day-old controls, the levels of LH and FSH were 3.36 and 7.72 ng/mL respectively. Only castration affected significantly those levels. In the 91-day-old animals, the levels of LH and FSH were 5.48 and 8.52 ng/mL respectively. Those values showed no difference from the controls in the different experimental groups.

Histological analysis

Pubertal phase (58 PND)

At the 58th day of age, the epithelial structures were well differentiated. Epithelial cells were polarized and surrounded by a well organized layer of stromal cells (Fig. 2A). The luminal compartment correponded to 50% of the volume of the gland, while the epithelium and stroma had 25% each (Fig. 3). The E2 treatement caused some regions of the epithelium to be shorter than the controls. Though E2 caused no alteration in tissue architecture, the absolute volume of the different tissue compartment was reduced, with the exception of the smooth muscle cells compartment.

After treatment with tamoxifen, the epithelial layer showed no alteration as compared to the controls, but the stroma appeared disorganized (Fig. 2C). No change was observed in the volume density of the different compartment. However, the absolute volume of the luminal compartment was reduced.

In the prostate of testosterone treated animals, proliferating cells were frequent (Fig. 2D). No change in the volume density was noted, indicating that the tissue organization was preserved. However, the absolute volume was increased for the different tissue compartments (Fig. 3).

Flutamide did not cause a modification of the prostatic histology, though an increase of the epithelium and a corresponding decrease in the lumen were detected by stereology. The absolute volume of the lumen was reduced.

The prostate in castrated animals, the epithelial structures were reduced and closer to each other (Fig. 2F). The distribution of luminal and basal cells were modified. The stromal cells showed spinous aspects. The volume density of the stromal was increased, and that of the lumen reduced. No change in the volume density of the epithelium was noted. The absolute volume of each compartment was reduced.

Adult phase (91 PND)

Figure 4 shows the morphological characteristics of the ventral prostate in the animals treated with different drugs in the pubertal stage. In the controls, the prostate shows a functional epithelium surrounded by an organized stroma. Some regions show proliferating cells (Fig. 4A). In the adult, the lumen occupies 47% of the prostatic volume, followed by the epithelium (~22%), non-muscular stroma (~20%) and the smooth muscle cells compartment (~3%) (Fig. 5).

E2 treatment caused no modification of the prostatic histology (Fig. 4B), except by a reduction in the lumen (Fig. 5C e D). Tamoxifen also had effect on neither the prostatic histology (Fig. 4C) nor on the contribution of the different tissue compartments to prostatic volume (Fig 5). Testosterone effects observed earlier were no longer detected (Fig. 5). Flutamide administration caused no change in prostatic histology (Fig. 4E), though an increase in the absolute volume was seen, reflecting an enlargement of the organ. Castration caused the acini to be atrophic and the stroma to be reorganized. The luminal compartment was reduced while the stroma occupied a relatively larger volume (Fig. 5).

Immunohistochemical analysis of AR

The ventral prostate showed a intense nuclear staining for the AR in the epithelial and stromal cells in the 58-day-old rats (Fig 6A). E_2 treatment caused a reduction in the staining

intensity, which was diffuse in some regions (Fig 6B). Tamoxifen did not change the epithelial pattern of AR staining, however stromal AR-positive cells were less frequently (Fig. 6C). Testosterone caused an increase in the AR staining intensity in the epithelial cells (Fig. 6D), while flutamide reduced this staining intensity (Fig. 6E-F). Castration caused a marked reduction in AR staining in the different prostatic regions (Figs. 6H-J).

At the 91 PND, the control prostate showed a strong staining for AR in the different cell types (Figs. 7A and E), E2 did not affect this staining pattern (Figs. 7B-7E). Tamoxifen caused a reduction in the staining intensity when compared to the controls (Figs. 7C and F).

Some regions of the prostate of testosterone-treated animals were less stained (Figs. 7H and K). Flutamide caused no change in the control pattern of AR staining (Figs 7I and J). Some staining was still observed in the castrated animals (Figs. 7J and M).

Ki67 immunostaining

During puberty, the luminal cells of the prostate in the control animals were frequently proliferative. Only flutamide did not diminish the number of proliferating luminal cells (Fig. 8A). The number of proliferating basal cells was diminished in the different treatments (Fig. 8C). No modification on the proliferative index of the stromal cells was observed (Fig. 8E).

In adulthood, the treatments with tamoxifen and flutamide caused increased on the number of proliferating cells (Fig. 8B). No effect was observed in the proliferative index of the basal cells (Fig. 8D). A negative effect on the proliferative index of the stromal cells was noted for the different treatments (Fig. 8F).

DISCUSSION

During puberty, there are hormonal changes that coordinate the transition to sexual maturity, including the growth and functional activation of the prostatic gland. In this period, the

prostate shows an active growth episode, resulting from the activation of a series of androgen driven genes [2]. This period is also characterized by a high level of cell proliferation in both epithelium and stroma. The epithelial basal cells present high proliferative activity during this period [12].

Cell proliferation kinetic was affect by the different treatments. The proliferation of epithelial luminal and basal cells were mainly affected. The different treatments employed here seem to affect the balance in the paracrine communication between the epithelium and stroma, thus affecting prostatic growth [13].

The treatment with testosterone did not interfere with the proliferation kinetics of luminal and stromal cells, but caused an inhibition of the proliferation of basal cells. In the rat ventral prostate, most of the luminal cells dye by apoptosis after androgen deprivation. The remaining androgen independent cells contain a high proportion of basal cells [14]. Restoration of the androgen stimuli caused the epithelium to recover. The cycle of prostatic regression-recover can be repeated many times, suggesting that the progenitor cells are androgen independent and have a high proliferative and regenerative capacity [15].

Only castration caused an effect on the body weight, causing a reduction in body mass. This is in accordance with previous results [16].

The prostatic epithelial and stromal cells are characterized by the strong expression of AR, which is responsible for its androgen dependency [17]. Even though none of treatments, but castration, had strong effect on the expression of the AR, differences were observed in staining intensity. This suggests that subtle changes took place in the androgen-androgen receptor complexes, which is known to be multifactorial [18]. The slight decrease in the expression of AR in the animal treated either with estradiol or tamoxifen indicates that the transactivation of the receptor might be compromised [19].This would contribute to the reduction in absolute volume

of the tissue compartments of the prostate in the animals treated with those drugs. Since AR expression is dependent on androgen, its expression is severely affected in castrated animals.

As the prostate undergoes a continuous process of growth which begins in the fetus and continues postnatally up to sexual maturity, prostatic growth is sensible to changes in the hormonal milieu [20].

In contrast to the early postnatal period, in which low doses of estrogen cause the prostate to be hyper responsive to androgens [7, 21] in the pre-pubertal phase the low estrogen dosing had an inhibitory effect. This might be associated to an indirect effect via the HPG axis. This would explain the diminished testis weight, which is consistent with an inhibitory effect of steroid hormones on the testicular germ cells, impairing spermatogenesis [22].

Testosterone administration, on the other hand, caused an increase on the weight of the gland with similar contribution from all different compartments, suggesting it anticipates puberty. The mechanism seems to be more complex, since the levels of testosterone were not kept high, as it takes place in puberty. Perhaps, exposing the prostate to a higher testosterone concentration, in a proper time window, activates a growth event which is mantained longer after the stimulus is removed.

Castration seriously hampered prostatic growth. It is though well known that prostatic regrowth takes place if the androgen stimulus is restored [23].

It was noticed that the alterations observed by the 58 PND were not maintained on later stages, except those attained by castration. This indicates that the prostatic changes observed during puberty did not compromise the physiology of the prostate in adulthood. However, the low level of testosterone after estradiol treatment as well as the increased proliferative indices observed in animals treated with estradiol and flutamide might result in complications later in life.

It is presently unknown how exposure to flutamide causes increases in the levels of circulating E2. It is possible that androgen receptor blockade causes an increase in the levels of available T which is aromatized to E2, increasing the levels of the latter.

The blockade of either the estrogen receptor (with tamoxifen) or the androgen receptor (with flutamide) affected prostatic growth. Even though no effect was seen on the epithelial and stromal compartments, the luminal compartment was reduced, suggesting that both signaling pathways regulated prostatic secretory activity.

This work brings about new data on the physiology of the immature prostate, suggesting that this period is also susceptible to hormonal disruption, with permanent changes in prostatic growth. This is interesting because exposure to hormonal disruptors during puberty might also predispose to prostatic diseases in later life.

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Table I. *Body, seminal vesicle and testis weight of adult rats after treatment with different drugs. Values are given as the mean ± S.E.M(n=6).*

PND	Treatment	Body Weight *(g)*	Seminal Vesicle weight (mg)	Testis Weight *(g)*
58	Control	240 ± 2.80	176.35 ± 25.23	2.89 ± 0.02
	Estrogen	211 ± 5.85	157.09 ± 32.36	1.88 ± 0.08*
	Tamoxifen	228.71 ± 2.53	171.41 ± 7.50	2.96 ± 0.05
	Testosterone	260.57 ± 4.40*	303.31 ± 7.50	1.73 ± 0.07*
	Flutamide	244.57 ± 4.27	196.13 ± 13.65	2.89 ± 0.05
	Castrated	248.17 ± 3.66*	16.27 ± 0.80*	_
91	Control	341.17 ± 4.26	318.48 ± 15.56	3.46 ± 0.03
	Estrogen	327.7 ± 7.10	283.8 ± 23.65	3.6 ± 0.01
	Tamoxifen	302.2 ± 5.45	317 ± 17.05	3.39 ± 0.03
	Testosterone	328 ± 4.38	337.4 ± 24.40	3.42 ± 0.03
	Flutamide	319 ± 5.95	307.68 ± 13.50	3.37 ± 0.01
	Castrated	292.67 ± 3.49*	8.28 ± 1.50*	_

* = $P < 0.05$, as compared to the control

Table II. *Serum Testosterone, Estradiol, Luteinizing hormone and Follicle stimulating hormone levels of adult rats after treatment with different drugs. Values are given as the mean ± S.E. (n=4).*

PND	Treatment	Testosterone (ng/dL)	Estradiol (pg/mL)	LH (ng/mL)	FSH (ng/mL)
58	Control	216.2 ± 14.55	32 ± 3.06	3.26 ± 0.05	7.72 ± 1.03
	Estrogen	96.10± 20.00	38.18 ± 2.55	1.36±0.27	11.82 ± 1.66
	Tamoxifen	203.60 ± 21.80	45.06 ± 1.45	3.44 ± 0.54	7.70 ± 0.51
	Testosterone	128.70 ± 21.05	55.52 ± 2.14	1.18 ± 0.20	4.80 ± 0.67
	Flutamide	178.70 ± 10.45	67.90 ± 8.35*	1.22 ± 0.11	10.26 ± 0.88
	Castrated	1.34 ± 0.00*	17.56 ± 0.74	44.54 ± 0.62*	27.70 ± 1.12*
91	Control	302.60 ± 25.64	26.13 ± 0.96	5.48 ± 0.54	8.52 ± 0.39
	Estrogen	171.5 ± 15.56	18.72 ± 1.48	3.02 ± 0.20	11.58 ± 0.80*
	Tamoxifen	329.10 ± 31.15	17.38 ± 0.57*	3.48 ± 0.36	10.30 ± 0.50
	Testosterone	204.50 ± 21.03	20.44 ± 1.22	3.86 ± 0.28	8.18 ± 1.01
	Flutamide	260.20 ± 25.00	18.91 ± 0.87	1.98 ± 0.26	5.50 ± 0.40
	Castrated	1.34 ± 0.00*	20.29 ± 1.16	55.17 ± 2.46*	24 ± 0.76*

* = $P < 0.05$, as compared to the control

Figure Legends

Figure 1. Relative prostatic weight prostate is presented as the ratio of prostatic to total body weight of 58-and (Fig. 1A) 91-day-old (Fig. 1B) animals after pre-pubertal treatment with different drugs. Relative prostate weight was decreased and increased, respectively in the E_2 and tamoxifen treatments. Absolute weight did not present significative alterations (inset). In 91-day-old animals, only the castrated group was significantly different from the control group. Control animals (CT), estrogen (EST), tamoxifen (TAM), testosterone (TEST), flutamide (FLU) and castrated (CAS). The absolute weight (inset) is presented for the different treatments (mean ± SEM; n = 5). * $p \leq 0.05$

Figure 2. H&E stained sections of the ventral prostate from 58-day-old rats treated in the pre-puberal period with different drugs. (2A) Histological aspect of the prostate from a control rat showing organized high columnar luminal cells. Detail shows a relation between smooth muscle stromal and epithelial cell compartment in the gland. (2B) Sections of the prostate of E_2-treated animals with evident decrease in the epithelial height (arrows), however some main regions conserves the normal architecture (Detail). (2C) In tamoxifen-treated rats the acini conserve (arrows) the histological aspects of controls animals, but an increased of the smooth muscle cell was observed. (2D) After treatment with testosterone the prostatic epithelium shows intense proliferative activity (arrows). (2E) Flutamide treatment did not alter morphological aspects of the gland. Detail showing a characteristic basal cell. (2F) Castration influenced dramatically the morphological aspects of the gland and the acini are atrophied and smooth muscle cells alter his morphological aspect (arrow).

Figure 3. Volume density (A, C, E and G) and absolute volume (B, D, F and H), for the different prostatic compartments (epithelium, lumen, stroma, and smooth muscle cells) as determined by stereology in 58-day-old rats after pre-puberal treatment with different drugs. Control (CT), estrogen (EST), tamoxifen (TAM), testosterone (TEST), flutamide (FLUT) and castrated (CAS) (mean ± SEM) *$p \leq 0.05$.

Figure 4. H&E stained sections of the ventral prostate from 91-day-old rats treated in the pre-puberal period with different drugs. (4A) Morphological aspects of the prostate of control animals. Epithelium consists of high columnar luminal cells, enclosed by a fine layer of smooth muscle cells, proliferative activity was evident in the distal ductal regions (detail). (4B) In the ventral prostate of E_2-treated animals the histological pattern is similar to that of the control animals, but the layer of luminal cells have a irregular aspect (arrow). (4C) Tamoxifen-treated rats, with normal characteristic infolding of the distal ductal regions (detail). (4D) Testosterone-treated animals did not present alterations in the morphological aspects of the gland. (4E) Prostate of flutamide-treated rats did not show significative differences in the morphological characteristic when compared with control rats. (4F) The prostate of castrated rats shows an atrophic acini and expressive stromal compartment with significative increase of smooth muscle cells. Ep= Epithelium, Lu = lumen; smc = smooth muscle cell, St = stroma.

Figure 5. Volume density (5A, C, E and G) and absolute volume (5B, D, F and H), for the different prostatic compartments (epithelium, lumen, stroma, and smooth muscle cells) as determined by stereology in 91-day-old rats after pre-pubertal treatment with different drugs. Controls (CT), estrogen (EST), tamoxifen (TAM), testosterone (TEST), flutamide (FLUT) and castrated (CAS) (mean ± SEM) *$p \leq 0.05$.

Figure 6. AR expression in the ventral prostate of 58-day-old animals Wistar rats after pre-puberal treatment with different drugs. (6A) The control sections show detail of nuclear staining, detail of epithelium showing intense nuclear staining (arrow). (6B) In E_2-treated animals show decrease in the intensity of AR-positive staining. (6C) Sections of the prostate from tamoxifen-treated animals, the nuclear staining was less intense when compared to the controls but strongest when compared to E_2-treated animals. (6D) In testosterone treatment a strong AR-positive staining was observed. (6E-F) Flutamide treatment affects the AR-immunostaining in epithelial cells. (6G-I) After castration the AR-staining is significativelly diminished in the prostate epithelial cells. Ep= epithelium , St = stroma.

Figure 7. Immunohistochemical location of the androgen receptor in the ventral prostate of 91-day-old rats after pre-pubertal treatment with different drugs. (A) Low power micrograph shows general staining in the epithelium and stroma of the prostate in control rats. Intense staining was evident in the epithelial cells (D). After treatment with estrogen was not possible to observe differences in the staining when compared with control rats (B and E). Low power section of tamoxifen-treated animals shows a general staining pattern (C) in detail, epithelial staining (F). Sections of testosterone-treated animals showing nuclear staining (7H and K). In prostate sections of castrated animals a weak AR-staining is present in some nuclei of epithelial cells (7J – M). Lu = lumen; smc = smooth muscle cell, St = stroma.

Figure 8. Percentual of positive Ki67 immunostained cells in luminal (A -D) and stromal (E-F) compartments. Proliferating index of 58-day-old animals (A, C, E) and 91-day-old animals (B, D, F). Control (CT), estrogen (EST), tamoxifen (TAM), testosterone (TEST), flutamide (FLUT) and castrated (CAS). Note that the principal alterations were present in the 58-day-old animals (mean ± SEM) *$p \leq 0.05$

Figure 1

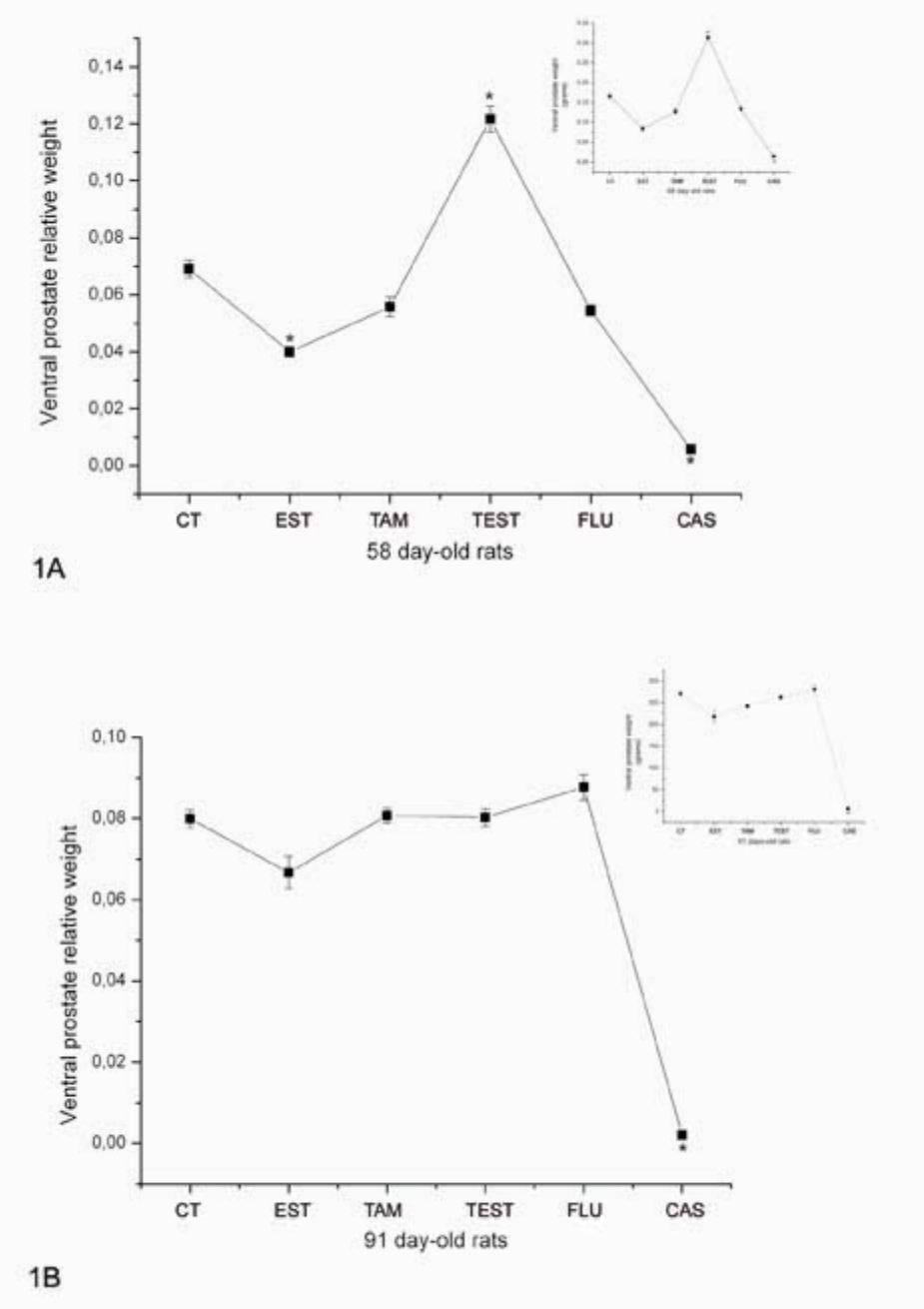

Ventral prostate relative weight
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
CT
EST
TAM
TEST
FLU
CAS
58 day-old rats
1A
Ventral prostate relative weight
0,10
0,08
0,06
0,04
0,02
0,00
CT
EST
TAM
TEST
FLU
CAS
91 day-old rats
1B

Figure 2

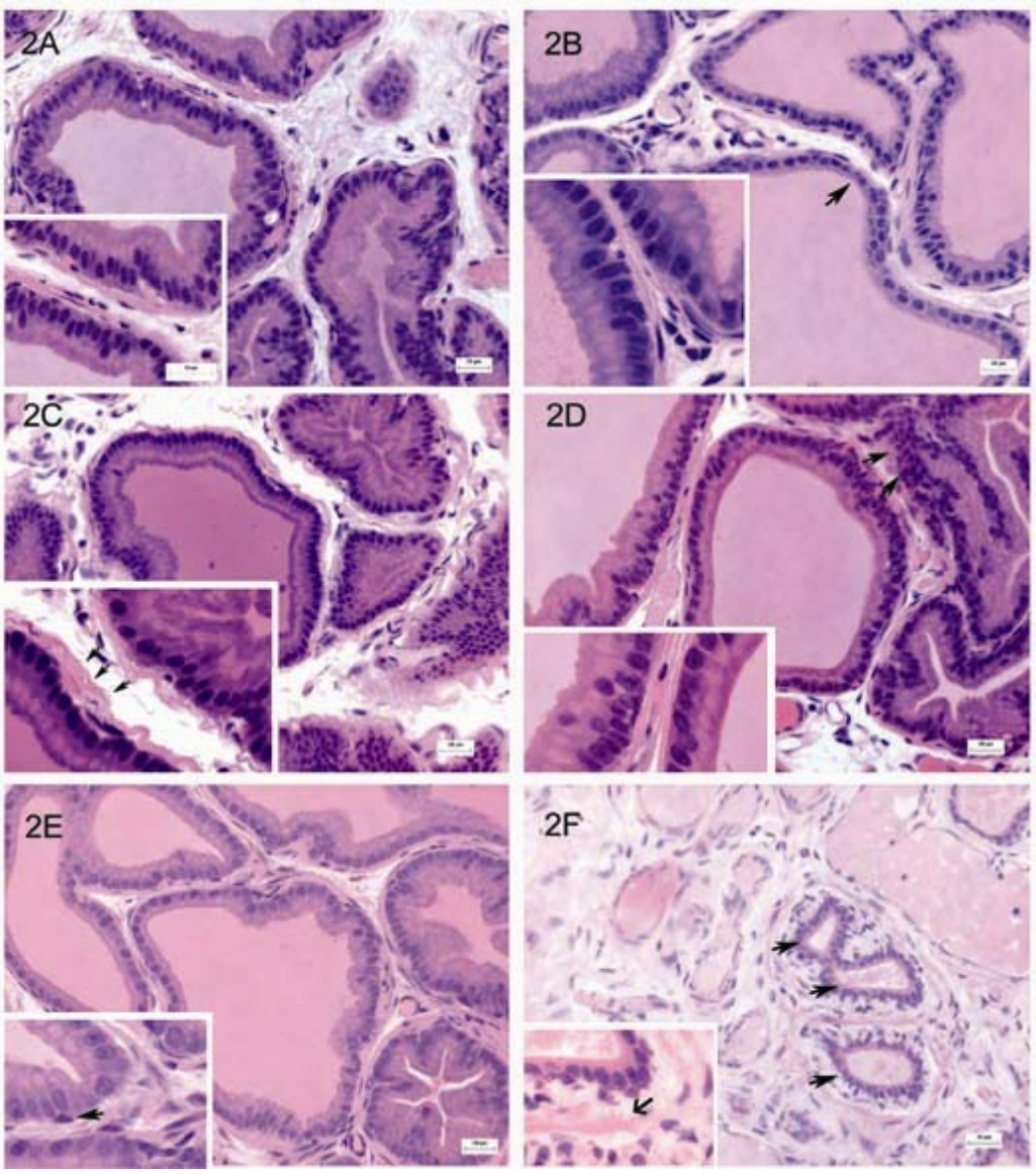


Figure 3

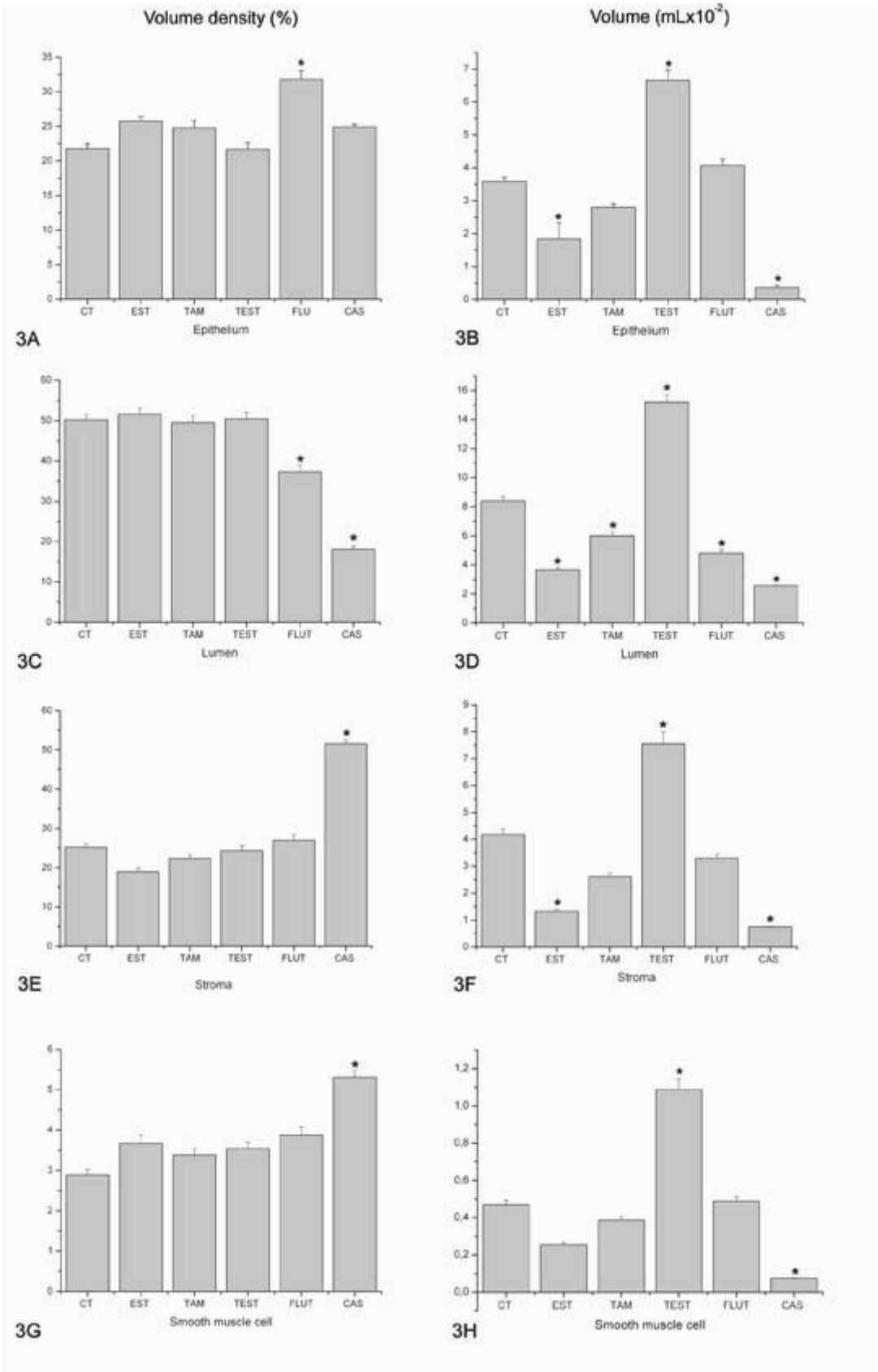

Volume density (%)
Volume (mLx10^{-2})
CT
EST
TAM
TEST
FLU
FLUT
CAS
Epithelium
3A
3B
Lumen
3C
3D
Stroma
3E
3F
Smooth muscle cell
3G
3H

Figure 4

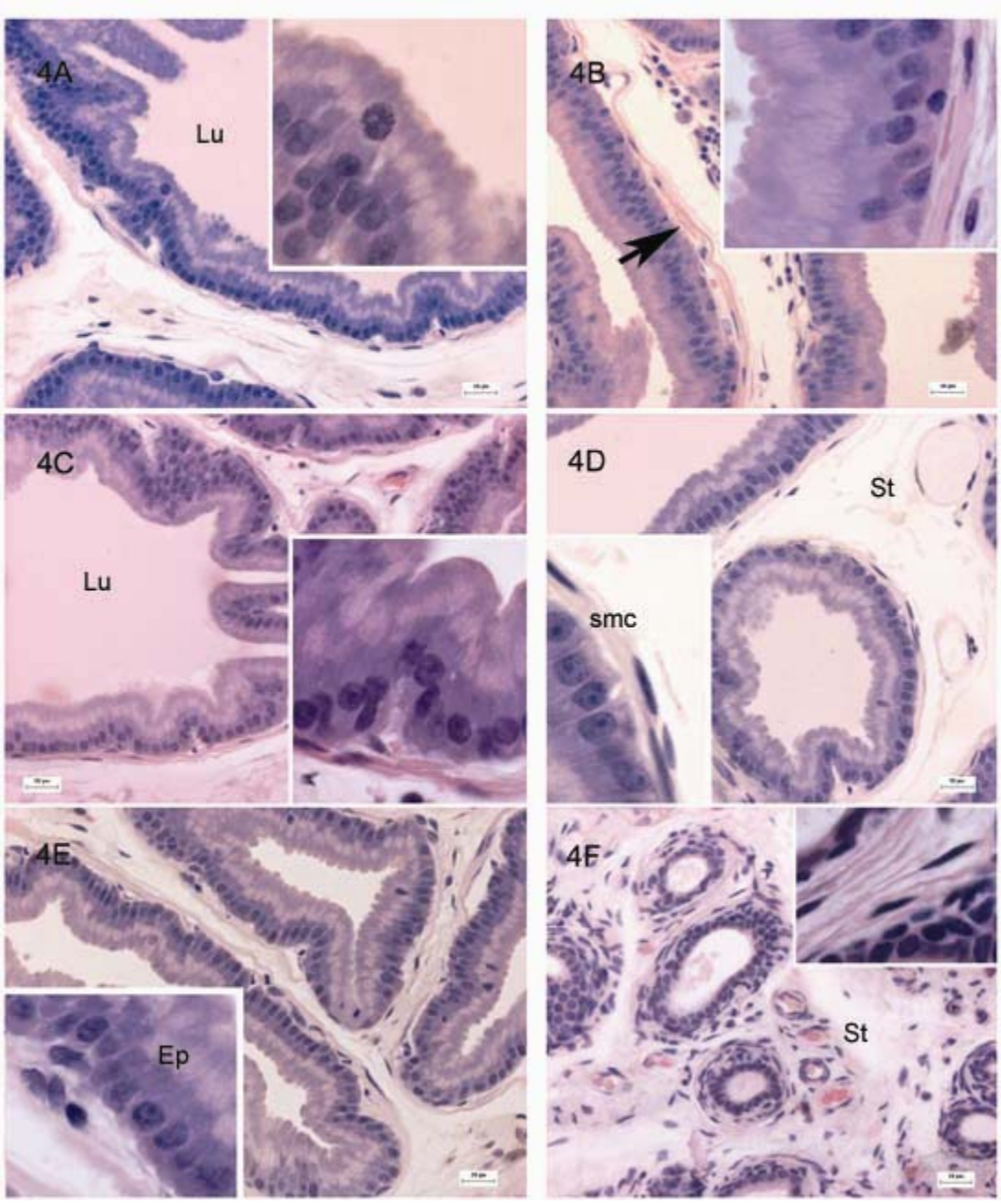


Figure 5

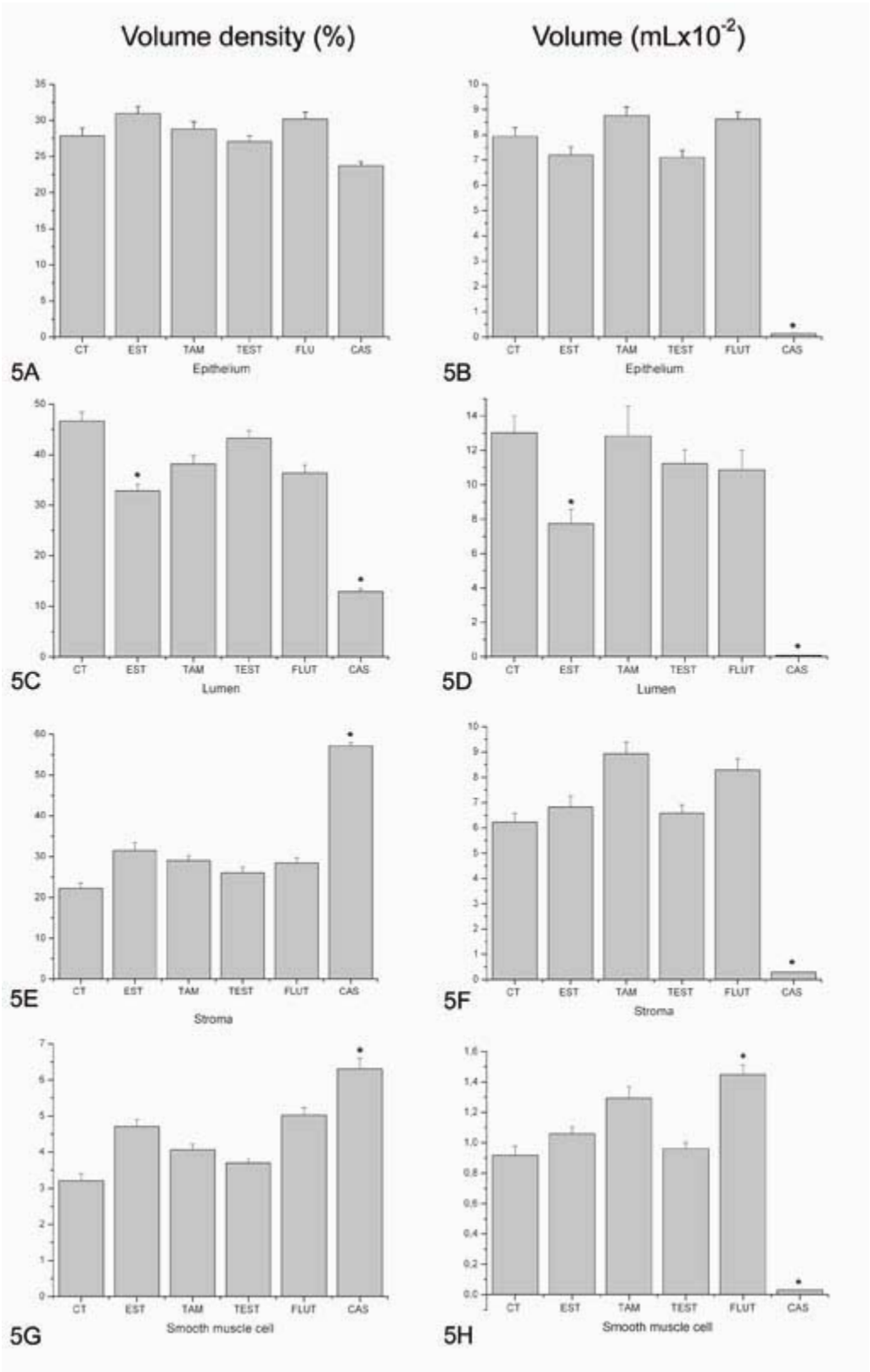

Volume density (%)
Volume (mLx10-2)
5A
Epithelium
5B
Epithelium
5C
Lumen
5D
Lumen
5E
Stroma
5F
Stroma
5G
Smooth muscle cell
5H
Smooth muscle cell
CT
EST
TAM
TEST
FLU
FLUT
CAS

Figure 6

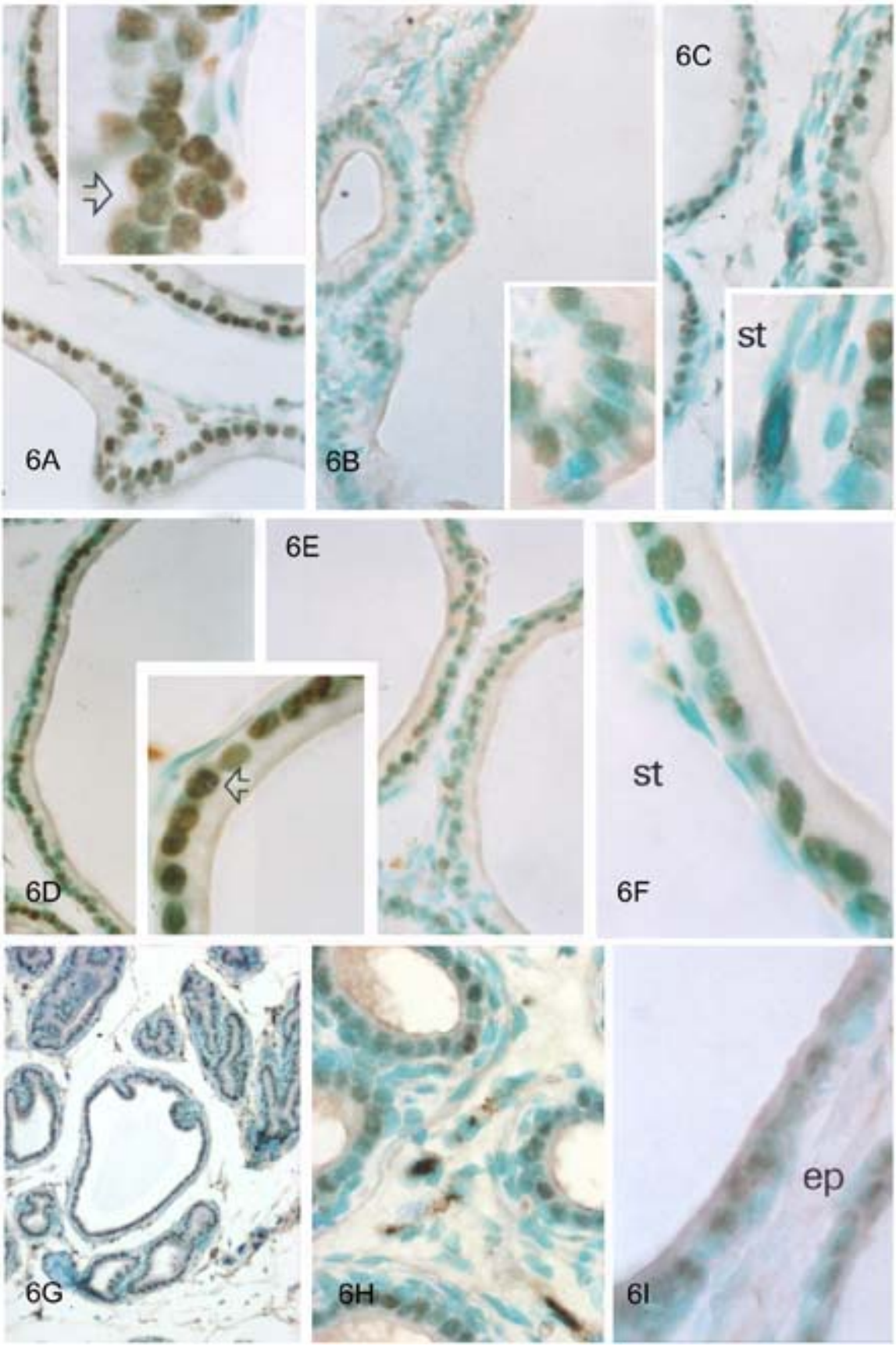


Figure 7

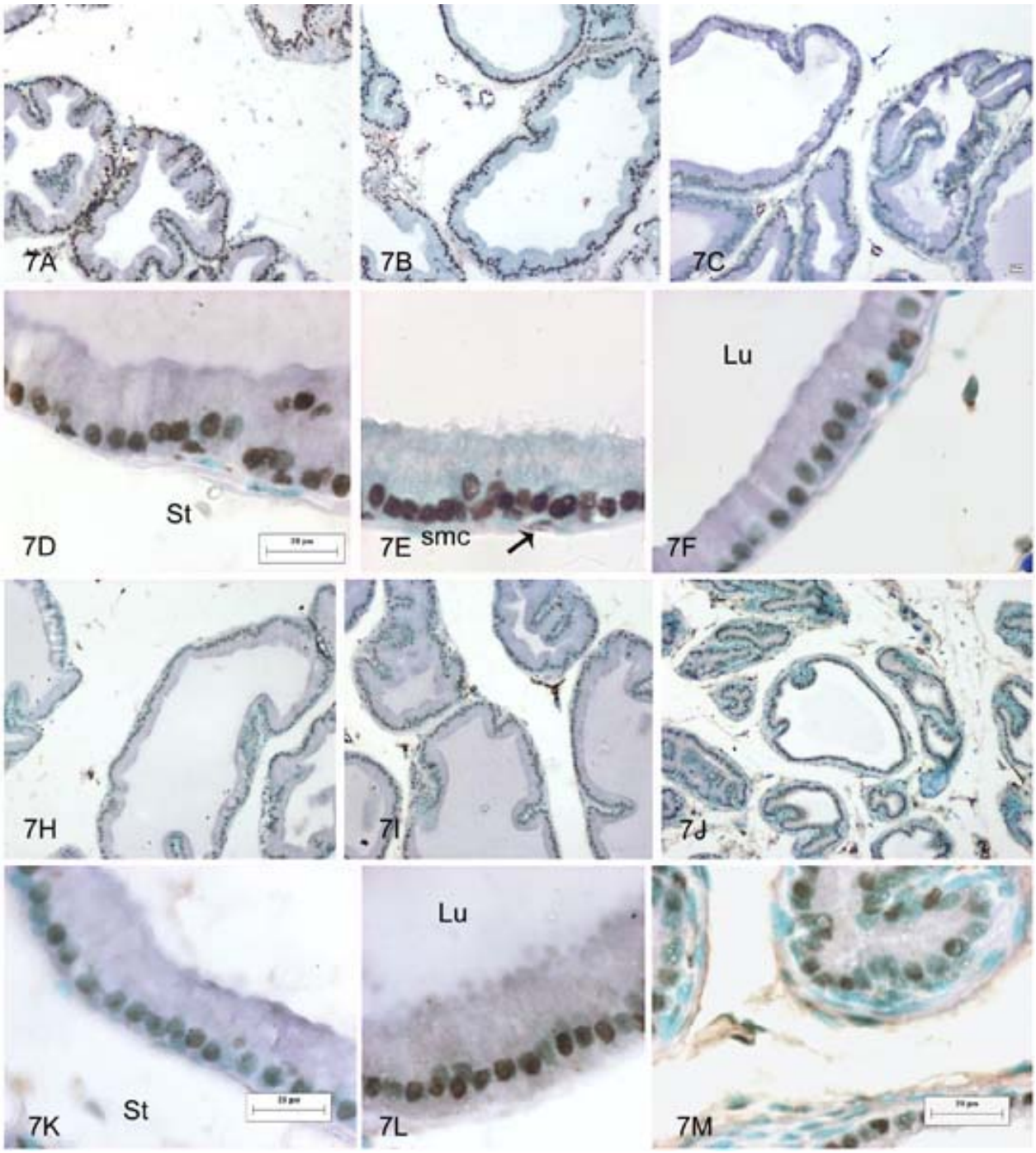
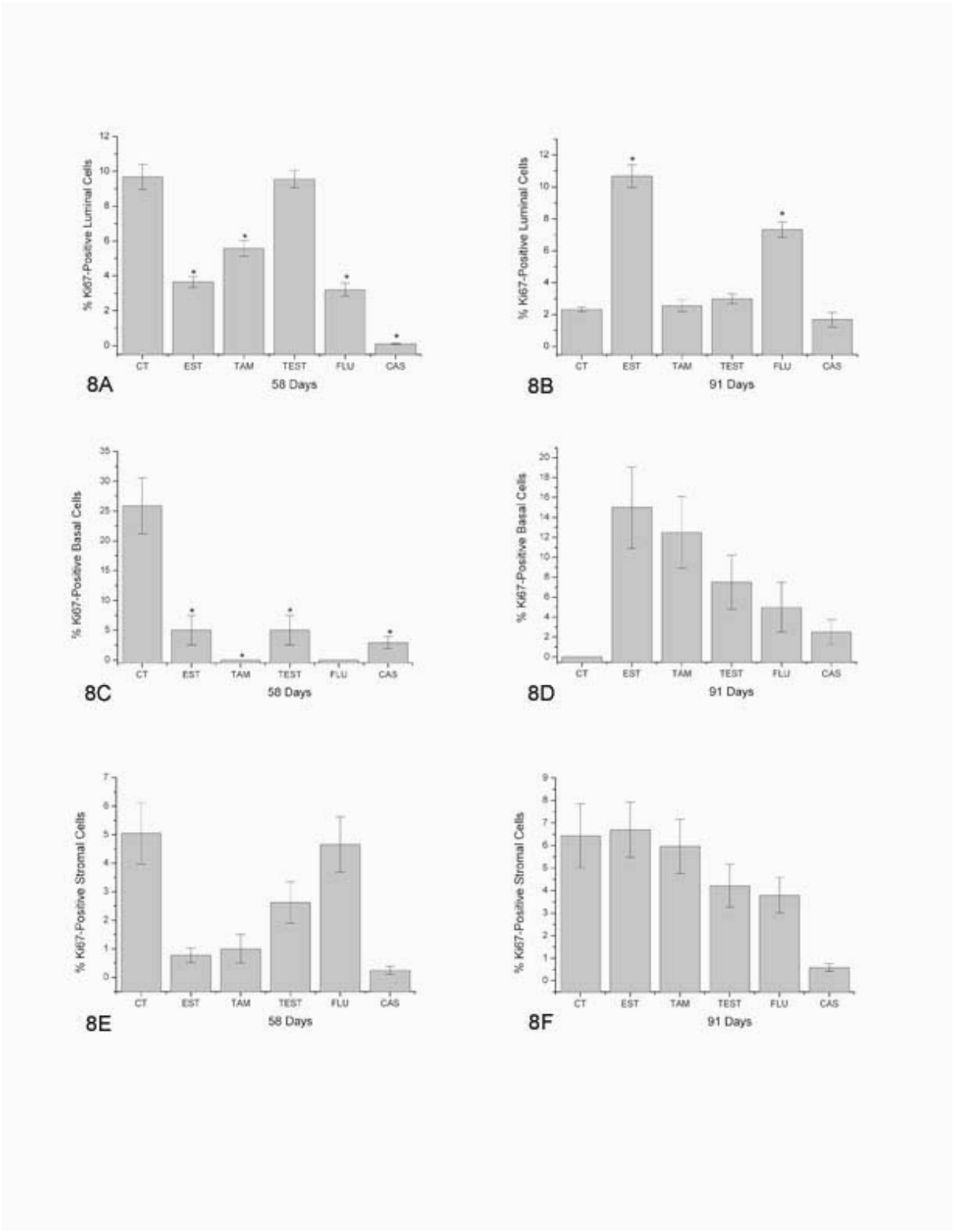


Figure 8


% Ki67-Positive Luminal Cells
CT
EST
TAM
TEST
FLU
CAS
8A
58 Days
% Ki67-Positive Luminal Cells
8B
91 Days
% Ki67-Positive Basal Cells
8C
58 Days
% Ki67-Positive Basal Cells
8D
91 Days
% Ki67-Positive Stromal Cells
8E
58 Days
% Ki67-Positive Stromal Cells
8F
91 Days

The aromatase inhibitor letrozole down regulates androgen receptor in rat ventral prostate

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Short title**: Letrozole down regulates androgen receptor in rat ventral prostate.**

Key-words: prostate, androgen receptor, letrozole, stereology, cell proliferation

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Abstract

The prostate gland is regulated by steroid hormones and complex interactions based on a subtle balance between androgen and estrogen (E_2) regulate prostatic development and physiology. Interestingly, the changes in steroid hormone levels at old ages affect the hormonal milieu and contribute to the evolution of the pathological changes of the gland. We have analyzed the effects of letrozole, an aromatase inhibitor, on the structure and androgen receptor expression by the ventral prostate of control and castrated adult rats. The results demonstrated alterations in prostate physiology after letrozole treatment. Serum levels of testosterone, prostate weight and proliferative index in luminal and basal cells were increased. Estrogen serum levels were not altered dramatically, in contrast to slight increase in gonadotrophin hormones seen in the castrated animals. Castration did not alter the proliferation index of basal cells. Reorganization of tissue compartments was seen with significative increase in letrozole treated animals. A decrease in androgen receptor expression was seen 21-days after the beginning of treatment with letrozole. These results were confirmed by immunohistochemistry and Western blotting. These results reveal new aspects in the relationship between androgen receptor and steroid metabolism in the prostate gland, demonstrating that alteration in hormone levels during a short time period induces significative alterations in prostate homeostasis.

INTRODUCTION

Prostate development is primarily influenced by androgens; testosterone (T) and its active metabolites elicit regulatory responses of urogenital sinus (UGS) from epithelial bud induction up to the total differentiation and functional maturity. However, prostate is also an estrogen-targeted organ and the expression of the estrogens receptors, α and β, in prostate gland (1,2) together with the presence of enzymes involved in estrogen metabolism suggest important role of this hormone in differentiation and metabolism of prostate gland (3).

The biosynthesis of estrogens occurs via metabolism of an androgenic substrate and the enzyme involved in the irreversible transformation of androgen in estrogens is a complex known as aromatase, which is present in the endoplasmic reticulum of numerous tissues (4), including the prostate gland, suggesting a direct intraprostatic effect of estrogen (5). The aromatase complex is composed of two proteins; the NADPH-cytochrome p450 reductase and a cytochrome p450 aromatase, which contains the heme and the steroid binding pocket (6). In humans, the p450arom is the product of a single *Cyp*19 gene, belonging to the cytochrome p450 family (7).

The aromatase-modulated transgenic mice, such as the Aromatase-overexpressing mouse (8) and the aromatase-knockout mouse (4) allowed the determination of the effects of estrogen/androgen unbalance in male reproductive functions. From these effects, it is remarkable the prostate atrophy in AROM+ mice and prostate enlargement, elevated peripheral and intraprostatic androgen levels and increased androgen receptor expression in ArKO mice (9).

Other important alterations are age-dependent changes in the hormonal balance, marked by a decrease in serum testosterone to estradiol ratio, when coupled with alterations in the concentrations of steroid receptors, may contribute to the evolution of pathological changes observed in benign prostatic hyperplasia (BPH) and carcinoma of prostate gland among older men (10).

As shown in other reports, androgen receptor is expressed along the entire prostatic ducts in all three lobes from adult rats where it elicits the different responses after T and DHT exposure (11, 12). Many studies are focused in the altered pattern of expression after exogenous compounds. For example, the normal developmental of the rat prostate lobes is affect by exposure of the neonatal animals to estrogens resulted in an altered pattern (13). The purpose of this study was to investigate the effect of letrozole, an aromatase inhibitor, on androgen receptor expression in the ventral prostate structure and androgen receptor expression of the non-castrated and castrated rats, aiming at identifing processes that affect AR levels and AR function, and that might thereby influence proliferation and viability of androgen dependent cells.

MATERIALS AND METHODS

Treatments

A total of 40 male Wistar rats (12 weeks old) were housed in standard plastic cages, maintained under controlled conditions (lights on 0700-1900h, temperature 20-24 ^{o}C) and allowed to water and rat chow *ad libitum*. The rats were divided into ten different groups: intact rats (Control), non-castrated rats treated with letrozole during seven days (LET 7), castrated letrozole-treated rats (CASLET) and castrated (CAS) with no further treatment. Analyses were performed 7, 14 and 21 days after treatments. Treated rats received 1 mg/kg/day of aromatase inhibitor, letrozole, dissolved in corn oil by oral gavage (14). This dose has been previously demonstrated to produce a significant decrease in the concentration of estrogen in the serum of female rats (15). Control rats received the same volume of the vehicle. Animals were killed by cervical dislocation. The prostates were removed, and ventral prostate (VP) were dissected. For immunohistochemical studies, VPs were fixed in 4% paraformaldehyde and embedding in paraffin; for Western blotting, VPs were frozen in liquid nitrogen. The experiments were carried

out according to the Guide for Care and Use of Laboratory Animals and were approved by the Committee for Ethics in Animal Experimentation of UNICAMP (Protocol number 592-1).

Chemicals and antibodies

Letrozole was purchased from Novartis (Basileia, Swiss), corn oil and protease inhibitor cocktail, were obtained from Sigma Chemical Co. (St Louis, MO, USA). Monoclonal mouse anti-Ki67 antibody was purchased from Dako (Carpentaria, CA, USA). Anti-AR rabbit polyclonal anti-AR (N-20) primary antibody and luminal reagent were from Santa Cruz Biothecnology (Santa Cruz, CA. USA) and The EasyPath ABComplex/HRP kit from Novocastra Laboratories (Newcastle upon Tyne, UK).

Hormone measurement

Plasma levels of testosterone (T), estradiol (E_2), luteinizing hormone (LH) and follicle stimulating hormone (FSH) were estimated by RIA. Blood samples were obtained by cardiac puncture immediately before death. The plasma was separated by centrifugation and stored at -20 C for subsequent hormone assays. T and E_2, concentrations were measured in serum samples using Coat-a-count kits (Diagnostic Products, LA, CA, USA). Serum samples were assayed in duplicate.

Plasma LH and FSH were measured by double-antibody radioimmunoassay using specific kits provided from the National Hormone and Peptide Program (NIH / NIDDK, USA). All samples in the same experiment were measured in the same assay. The lowest detectable amount of $LHRP_3$ standard was 0.05 ng/ml and the intra-assay coefficient of variation was 4%. The lowest detectable amount of FSHRP2 standard was 0.2 ng/ml and the intra-assay coefficient of variation was 3.2 %.

Histology

The VP was immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr. Samples were then washed, partially dehydrated and embedded in Leica historesin. Two micrometer sections were obtained and stained with hematoxylin and eosin (16).

Stereological Analysis

For stereological purposes, five microscopical fields from the hematoxylin and eosin stained sections from three animals for each group were photographed with a Zeiss Axioskop microscope (Jena, Germany) and the Weibel multipurpose graticule system (120 point, 60 test lines) (17) was utilized in a systematic field sample of the ventral prostate. The following parameters were evaluated on the basis of area measurement-volume densities (percentage of tissue volume occupied by defined tissue compartment) of epithelium, stroma, lumen and smooth muscle cell. For the estimation of volume fractions, the number of counting grid points falling in each tissue compartment was counted. The volume of the VP served as the reference volume. For approximation it was assumed that the specific gravity of the prostate tissue was 1.0 (17, 18).

Immunohistochemical localization of Androgen receptor

Paraffin-embedded sections (6 μm) of VP were dewaxed and rehydrated in an ethanol graded series. Antigens were retrieved by boiling in 10 mM citrate buffer (pH 6.0) 3 times of 5 min. The cooled sections were incubated in 1% H_2O_2 for 15 min to quench endogenous peroxidase. To block nonspecific binding, sections were incubated in BSA 3% for 1h at room temperature. Sections were then incubated with anti-AR (1:100) or anti-Ki67 (1:100) in 1% BSA at 4°C. Negative controls were incubated with 1% BSA without primary antibody. The ABC kit was used to visualize the signal, according to manufacturer's manual. The sections were incubated in appropriated secondary antibody solution for 30 min followed by washing for 10 min with PBS and incubation in Avidin and Biotinylated HRP solution for 30 min. After washing

in PBS, sections were developed with 3, 3´diaminobenzidine tetrahydrochloride substrate, lightly counterstained with methyl green, dehydrated through ethanol series and xylene, and mounted.

Preparation of tissue extracts for Western blot

Frozen tissue samples and pellets of LNCaP human prostate cancer cell were homogenized in tissue lysis buffer (0.05 M Tris, pH 6.8, 10% sucrose, 2% SDS and 4% (v/v) β–mercaptoethanol and 10 μL/mL protease inhibitors cocktail). The tissue homogenates and cell extracts were clarified by centrifugation at 14000 X g for 20 min at 4 C. An aliquot of each sample was used for the determination of protein content using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The clarified supernatants were mixed (1:1) with 2 X sample buffer [100 mM Tris-HCl (pH 6.8), containing 10% 2-mercaptoethanol, 4% SDS, and 20% glycerol], transferred to bath and heated at 95 ºC for 5 min, rapidly frozen on ice, and stored at -70 C until use.

Samples with equivalent 75 μg of protein were electrophoresed in 10% SDS polyacrylamide gels. After separation, proteins were electrophoretically transferred at 400 mA for 45 min at low temperature to Hybond nitrocellulose membrane (Amershan, Pharmacia Biotech, Arlington Heights, IL. USA). For AR Western blotting, membranes were blocked with 5% skim milk powder in TBS for 60 min, rinsed in TBS and incubated at 4ºC overnight with rabbit polyclonal anti-AR (N-20) primary antibody diluted 1:500 in TBS containing 1% skim milk. Membranes were then incubated for 60 min with anti-rabbit horseradish peroxidase conjugated secondary antibody diluted 1:2500 in TBS containing 1% skim milk. Between incubations, sections were washed 3x10 min in TBS containing 0·2% Tween-20. Antibody binding sites were visualized on Hyperfilm (Kodak) by exposure for 2 min using the enhanced chemiluminescence detection system luminol according to the manufacturer's instructions. Molecular weight markers were run on each gel to confirm the molecular size of the androgen receptor.

Counting of proliferating cells

To quantify and compare proliferation rates between VP of control and treatments groups, dividing cells were assessed by counting Ki67-positive and Ki67-negative (methyl green-stained) nuclei at 400 x magnification. A total of three immuno-stained sections per treatment were used. Separated counts were made for the epithelial and stromal compartments of the gland. Approximately 2,000 cells were counted for each treatment. The number of positive cells was expressed as the percentage of Ki67-positive cells.

Statistical Analysis

Body weights, tissue weights, hormone serum levels (T, E_2, LH and FSH), stereological data, cell proliferation and cell death index, were analyzed for statistical significance by analysis of variance (ANOVA). Comparisons among individual treatments conditions within each time period were further examined by Tukey's test for multiple comparisons using MINITAB® Release 14.20. Results were considered to be statistically significant when $p<0.05$.

RESULTS

Body and organ weights

Body, prostate, and relative testis were not affected by letrozole treatment (Table 1). After seven days, castration led the prostate to a 70% regression. After 14 and 21 days of castration the regression was 80%. The letrozole administration in castrated rats did not alter this effect.

Serum hormones concentrations

Testosterone levels were altered after treatment with letrozole during the 14 day of the experiment (Table II). At seven and 21 days of treatment the intact rats did not present significative alterations when compared with the control. In castrated animals (treated or not with letrozole) T levels were below to detection level. LH and FSH levels in control group were 3.8 ± 0.7 and 8.7 ± 1.6, respectively. Letrozole treatment did not affect these values. However there

was a tendency in castrated animals for increased gonadotrophin levels. Serum estrogen levels were stable in all treatments.

Histological analyses

Examination of ducts in rat ventral prostate of adult rats (91-day-old) revealed a simple columnar epithelium, lined with tall columnar secretory epithelial cells (Fig. 1A), the characteristic infolding of the distal region is also present (Fig. 1B).

After 7 (Fig. 1C), 14 (Fig. 1D) and 21 (Fig. 1E) days of beginning of treatment the proximal ductal region did not show differences when compared to the control. The proximal region of letrozole-treated animals in 7th day presented small vesicles in the layers of stromal cells (Fig. 1C), and proximal regions of the 21st day presents a multilayered sheet of smooth muscle cells. However in absolute terms, a decrease of the smooth muscle cell was detected in the 7th and 21st but not in 14th day of treatment. The non-muscular stroma compartment showed the reverse behavior situation (Figs. 2G and H). Intermediate ductal regions (Figs. 1F-H) did not show significative alterations between treatments. Distal ductal regions presented pronounced mitotic activity (Figs. 1I-K), particularly in the 7th day after treatment absolute values of epithelium and lumen were increased and, after 21st days, there was a stimulatory effect only in the luminal compartment (Fig 2B and D).

Volumetric density of smooth muscle cell compartment was diminished after 21 days of letrozole-treatment while the other tissue compartments remain unaltered (Fig 2).

After seven days of castration, the prostate remodeled dramatically losing its characteristic morphology. The epithelial regression is evident and stromal cells became disorganized (Fig. 2). In castrated rats (Fig. 1L) and castrated-letrozole treated rats (Fig. 1M) different patterns of smooth muscle cells were seen. After 14 days of castration the frequency of rudimentary ducts increase in the prostatic area and the stromal cells are more evident (Fig. 1N). Letrozole-treated rats conserved this pattern (Fig. 1O). After 21 days of castration the acini were

totally atrophic and their frequency was increased. (Figs. 1P and Q). No alteration was seen in the smooth muscle cell compartment.

Stereological analyses showed a dramatic reduction in absolute volumes in all compartments (Fig. 2). There were an increase in stromal compartment and a reduction in the epithelial and luminal compartments. When compared no significative difference in absolute volume of the different compartments, but a decrease in volume density of smooth muscle cell in letrozole-treated animals (Fig 2).

Immunolocalization of androgen receptor in ventral prostate lobes and Western blot

In control rats the total epithelial cells were AR-positive (Fig. 3A) and staining was also present in some stromal cells (Fig. 3B). The distal ductal region showed AR- positive cells with different intensity staining (Fig. 3C). In letrozole-treated groups, in the 7^{th} (Figs. 3D, G and J) and 14^{th} day (Figs. 3E, H, and K) the AR-staining in proximal, intermediate and distal regions of the prostate was not modified. The main alteration in letrozole treated animals was found in 21-day group that showed a decrease in a staining in the three regions of ventral prostate. In the proximal region there was a decrease in staining in epithelial and stromal cells (Fig. 3F). In the intermediate and distal regions AR-staining was decreased (Fig. 3I) in the epithelial cells (Fig. 3L and Fig. 4). Castration had a strong effect on AR-staining, which was completely abolished. Letrozole application did not alter this pattern (Fig. 3B).

A single immunore-active protein of approximately 110 kDa was detected in all rat prostate tissue extracts, and well as extract from LNCaP cells (positive control). When androgen receptor levels were compared, we found that the level was approximately 30% lower after 21 days of letrozole treatment (Fig 4). Castrated rats treated or not with letrozole present very low levels of immunoreactive AR protein (data not shown).

Ki67 positive cell counts

To assess the proliferative status of the basal and luminal compartments, the expression of the Ki67 antigen was measured. After seven days of treatment an increase in the proliferation index in basal cells of letrozole-treated animals was observed (Table III). Fourteen and 21-days after treatment, the proliferative index was similar to that of control animals. After seven days of castration the proliferative index changed from 2.46% in luminal cells to 0.40%. However these values were not significative. At the 14^{th} and 21^{st} day not proliferative activity was detected in the epithelial compartment. Ki67 staining was detected after seven days of castration in basal cells. Treatment with letrozole in castrated rats did not alter this pattern. The prostate of castrated and castrated-letrozole treated animals were Ki67 negative.

DISCUSSION

Multiple examples of physiological interplay between androgens and estrogens led us to hypothesize that there may be a direct interaction between the androgen receptor and estrogen receptors. In breast cancer for example, tumors containing both receptors (AR and ER) presented higher levels of endogenous steroid hormones than tumours showing either receptor alone (19).

In prostatic tissue, androgen receptor (AR) synthesis is regulated by androgens. Therefore, it might serve as an effective molecular marker of androgen action in this tissue. In this study we showed that a deregulation of hormonal environment alters the normal expression of AR and interfere with the kinetics of cell proliferation.

Previous reports showed a relationship between AR and aromatase inhibitors. A breast cancer cell (MCF-7) was sensitive to aromatase inhibitors and blockade of the AR inhibited the antiproliferative effect of letrozole. These results suggest that aromatase inhibitors might exert their antiproliferative effect not only by reducing the intracellular production of estrogens but also by unmasking the inhibitory effect of androgens acting via the AR (20). In the present study

letrozole did not cause an inhibiting effect on the proliferating cells. On the contrary, a slight increase of proliferating cells was present which led us to hypothesize that an inhibition of the conversion of T to E_2 by letrozole resulted in a significant increase in serum T levels as well as in alterations in the pattern of AR location, thus demonstrating that its expression is dependent on the hormonal environment and probably control cell proliferation. This inhibition of estrogen synthesis by letrozole was effective and the hormonal quantifications showed larger levels of testosterone serum levels after treatment. The general tendency of an increased epithelial and luminal compartments and a decrease of the stromal compartment indicate a reorganization of the gland after disruption of the normal hormonal environment, besides an increase in proliferation index of the basal cells.

Variation in normal AR synthesis among the different treatment groups was noted, with the highest levels seen in the VP of control rats and the expression of the AR-staining shows a quick effect in AR-labeling in animals treated after 14 and 21 days.

The possible pathways activated after letrozole treatment was the increase of testosterone levels, after aromatase inhibition that stimulates the cell proliferation and produces the growth in the tissue compartments of the gland. Cell proliferation is also stimulated by the loss of the physiological ligands of estrogen receptors, as demonstrated by Weihua and co-workers (21) Androgen receptor down regulation likely results from the disruption of the hormonal milieu.

We conclude that letrozole down-regulates AR in the rat ventral prostate and alters the serum levels of androgens. This model represents a valuable model for analyzing the direct and indirect effects of unbalanced estrogen and androgen action.

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Table I. *Body, prostate, relative prostate, and testis weight in grams, of adult rats after treatment with different drugs (values are given as means ± SEM n=4).*

Treatment	Day	Body Weight (g)	Prostate (mg)	Relative prostatic weight	Testis (g)
Control		336.75 ± 16.66	305.08 ± 30.67	0.090 ± 0.00	3.73 ± 0.07
Letrozole	7	327.50 ± 13.67	340.68 ± 21.61*	0.104 ± 0.00	3.59 ± 0.13
	14	314.75 ± 7.97	293.20 ± 32.83	0.093 ± 0.00	3.36 ± 0.14
	21	329.75 ± 25.41	330.73 ± 21.24	0.101 ± 0.00	3.36 ± 0.11
Castrated	7	317.25 ± 6.40	87.05 ± 18.51*	0.027 ± 0.00*	_
	14	330 ± 21.09	33.58 ± 5.52*	0.010 ± 0.00*	_
	21	326.50 ± 19.77	34.53 ± 8.19*	0.010 ± 0.00*	_
Castrated plus Letrozole	7	340.50 ± 25.51	74.73 ± 15.56*	0.022 ± 0.00*	_
	14	312 ±12.33	38.23 ± 13.69*	0.012 ± 0.00*	_
	21	331 ± 20.30	32.43 ± 8.83*	0.009 ± 0.00*	_

* = $P < 0.05$, as compared to the control

Table II. *Serum Testosterone, Estradiol, Luteinizing hormone and Follicle stimulating hormone levels of adult rats after treatment with different drugs (values are given as means ± SEM n=4).*

Treatment	Day	Testosterone (ng/dL)	Estradiol (pg/mL)	LH (ng/mL)	FSH (ng/mL)
Control		308.8 ± 29.35	16.64 ± 1.73	3.8 ± 0.68	8.65 ± 1.64
Letrozole	7	364.78 ± 15.80	23.45 ± 1.12	5.15 ± 0.71	10.9 ± 0.89
	14	625 ± 72.50	19.84 ± 2.10	5.73 ± 0.99	16.63 ± 2.09
	21	359 ± 55	24.65 ± 0.31	7.3 ± 0.58	9.53 ± 0,66
Castrated	7	_	20.69 ± 1.73	6.5 ± 0.72	24.18 ± 2.41
	14	_	23.93 ± 0.68	10 ± 0.43*	22.50 ± 0.31
	21	_	21.69 ± 4.08	12 ± 0.60*	53.50 ± 2.10*
Castrated Plus Letrozole	7	_	10.74 ± 0.22	10.8 ± 0.20*	38.8 ± 7.60*
	14	_	12.04 ± 1.18	6.6 ± 0.56	46.30 ± 2.79*
	21	_	17.64 ± 0.02	10.25 ± 0.92*	40.90 ± 9.85*

* = P< 0.05, as compared to the control

Figure Legends

Figure 1. The morphology of control, letrozole-treated, castrated and castrated letrozole-treated rats was examined by H&E staining after seven days of treatment with letrozole, and then collected after 7, 14 and 21 days of the beginning of treatment. A and B, control animals showing the active columnar epithelium and the characteristic infolding of the distal region (arrow).

Proximal, intermediate and distal region of letrozole-treated animals after 7, (C, F and I), 14 (D, G and I) and 21 (E, H and K) days of the experiment. The proximal regions of the treatments show different characteristics in the stromal compartment (arrow). Intermediate regions conserved the same morphological organization and distal regions present intense proliferate activity (arrows)

Castrated animals after seven days (L) and treated with letrozole (M) present an epithelial regression. It was also notable different patters in the stromal cells pattern (inset). After 14 days of castration the atrophied acini, in detail apoptotic figure (N) the treatment with letrozole present disorganization in an epithelial and stromal comparments (O). 21 days after castration the acini are reduced and the stroma is a predominant compartment (P) treatment with letrozole did not alter this pattern (Q). Apparently the letrozole treatment in castrated rats results in a dense cellular stromal phenotype. Lu = lumen; smc = smooth muscle cell, St = stroma.

Figure 2. Changes in volumetric density and absolute volume in control, letrozole-treated, castrated and castrated letrozole-treated rats after seven days of treatment with letrozole or vehicle, and then collected after 7, 14 and 21 days of the beginning of treatment. Volumetric density and absolute volume of epithelial (A and

B), Luminal (C and D), Non-muscular (E and F) and smooth muscle cells (G and H) compartments respectively. The insets in the graphs correspond to the stereological analyses of the same compartment in the castrated and castrated-letrozole-treated animals.

Figure 3. Histological sections of the rat ventral prostate immunocytochemically stained for AR. In the control 91 day-old rats (3A-C) most of the epithelial cells within the acini were AR (+) (arrows). It was detected a presence of the stromal cells AR-positive (arrow).

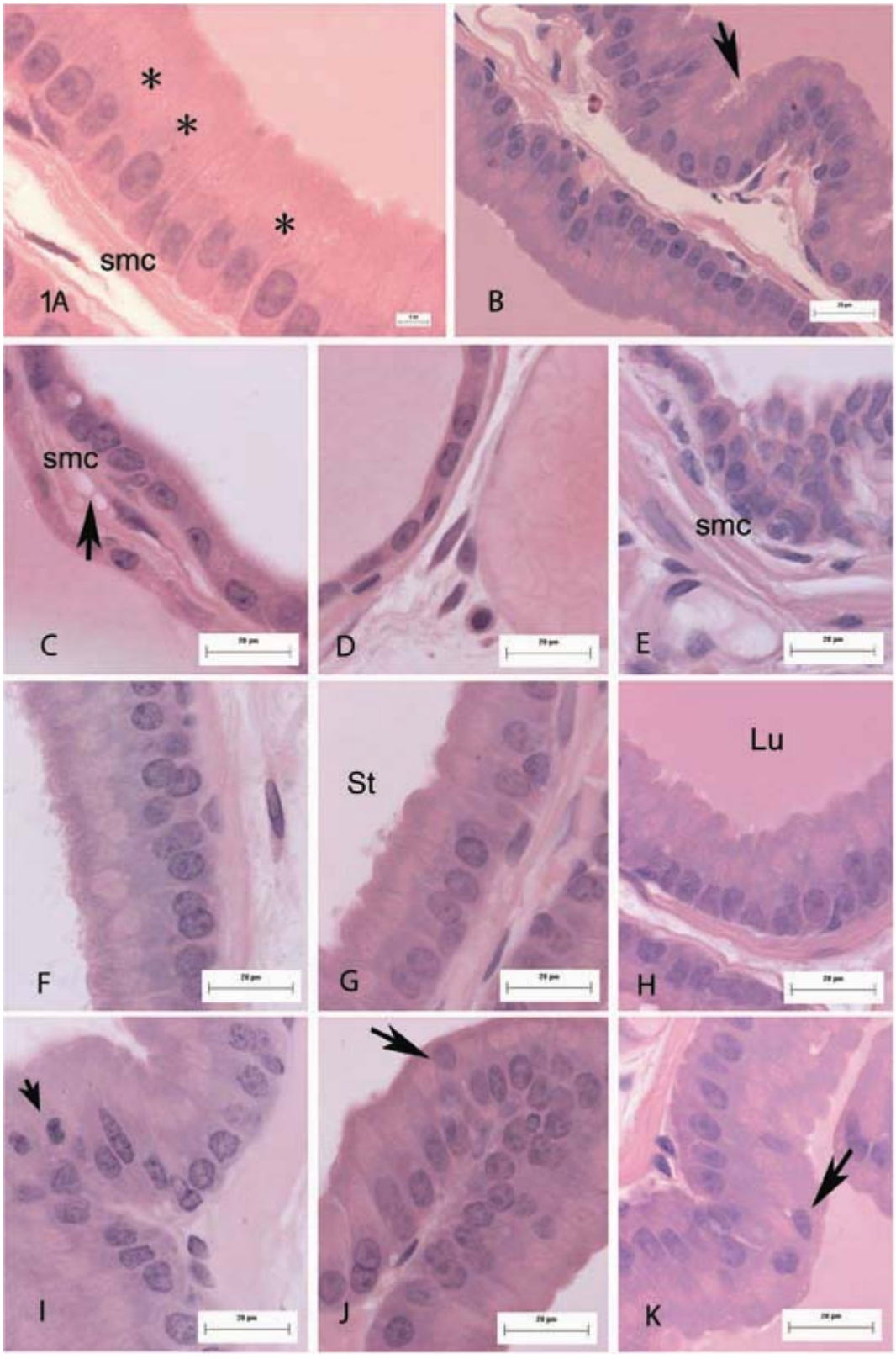
In the animals treated for seven days with letrozole the epithelium showed a strong nuclear staining in the proximal, intermediate and distal region (D, G, and J) respectively. Fourteen days after treatment a intense staining was seen in proximal region (E), while intermediate (H) and distal regions (K) conserve the staining. 21 days (F, I and L), the intense of the nuclear staining was fainter (arrows) staining decrease in the different regions.

Immunocytochemical localization of AR in the prostate of castrate rats. The epithelial cells showed only a weak staining. Neither nucleus nor cytoplasm was stained seven days after castration (M). In castrated letrozole-treated rats after seven days the nucleus and cytoplasm were weakly labeled (P). Fourteen (N) and twenty-one days (O) after castration, the epithelial cells were atrophic and the stroma was more evident. Labeling was abolished. Treatment with letrozole did not alter this pattern (Q and R). (Figs. 2B, 2D, 2F, 2H, 2L (925x)). Lu = lumen; smc = smooth muscle cell, st=Stroma

Figure 4. Representative Western Blot of extracts of rat ventral prostate of different experimental groups. AR expression of letrozole treated intact animals during seven days. The treatment decreases the amount of AR in the prostate of letrozole-treated rats.

Figure 5. Box-plot representations of the median percentage of Ki67 positively stained cell in luminal (A and B) and stromal (C) compartments. The experimental groups are sequentially represented according to the different treatments. The plots correspond to counts of 20 separate fields of view from each experimental group. CT=Control; LET= Letrozole-treated animals; CAST= Castrated animals; CASTLET=Castrated letrozole treated animals

Figure 1



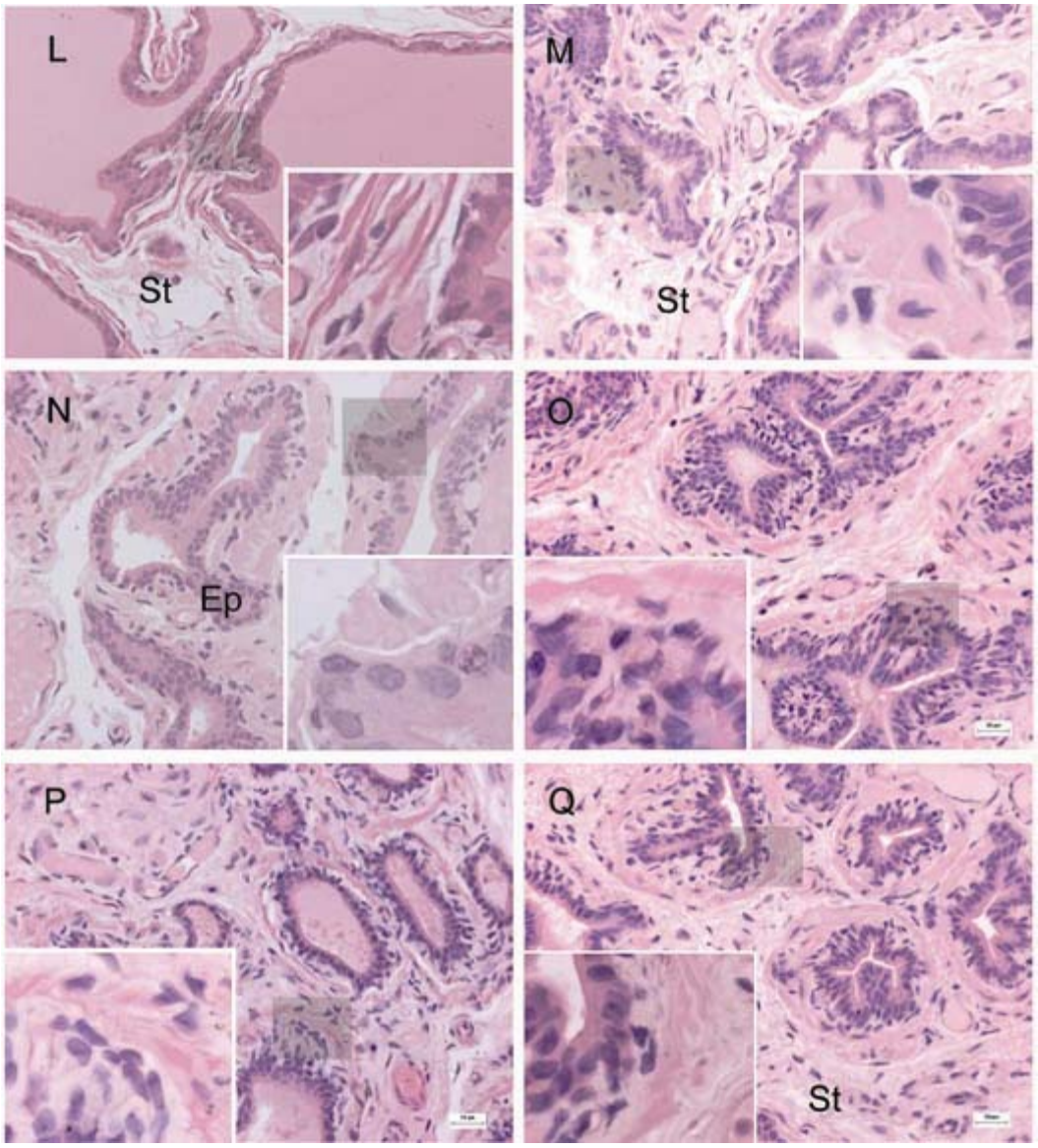

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Figure 2

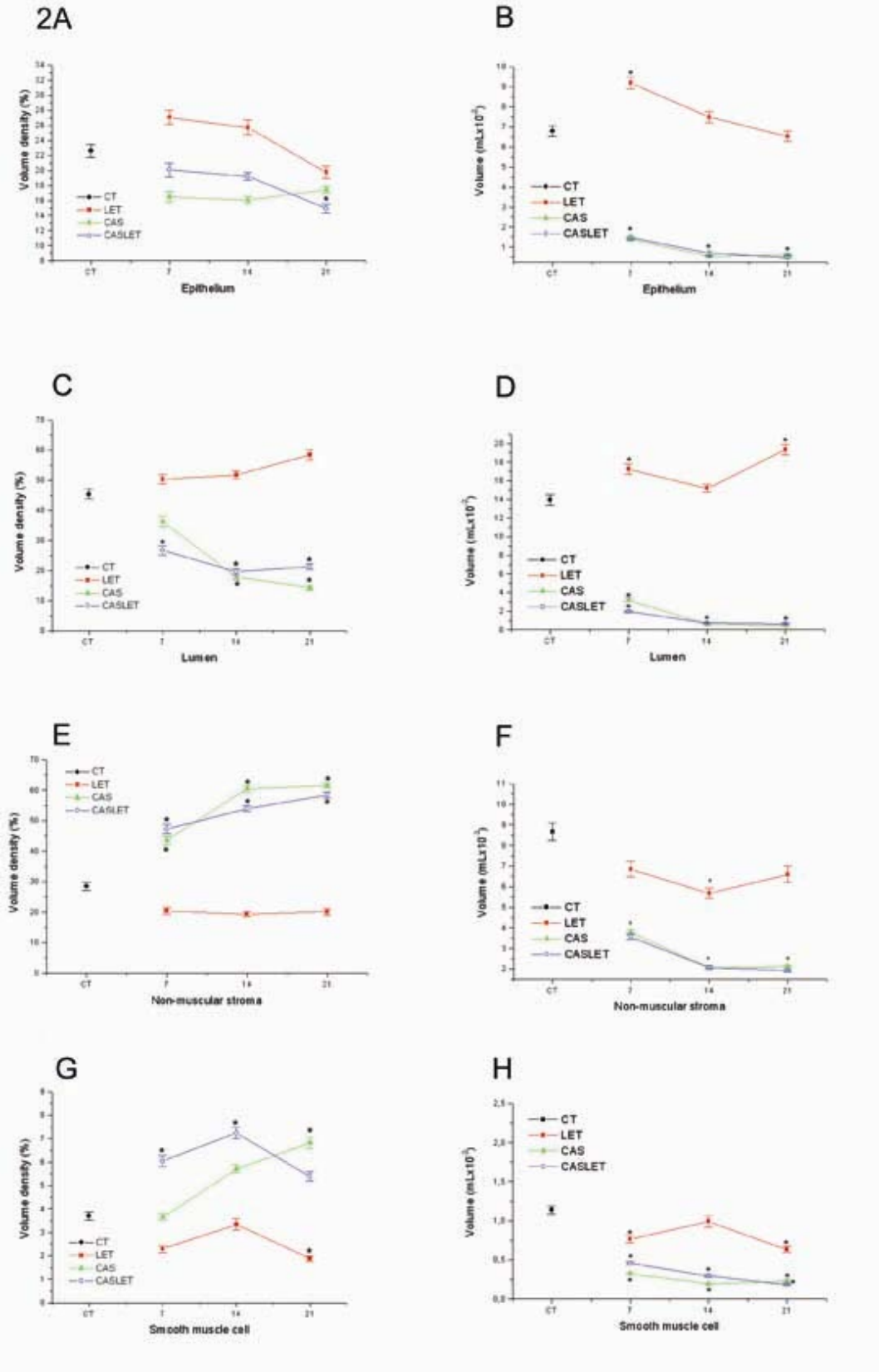
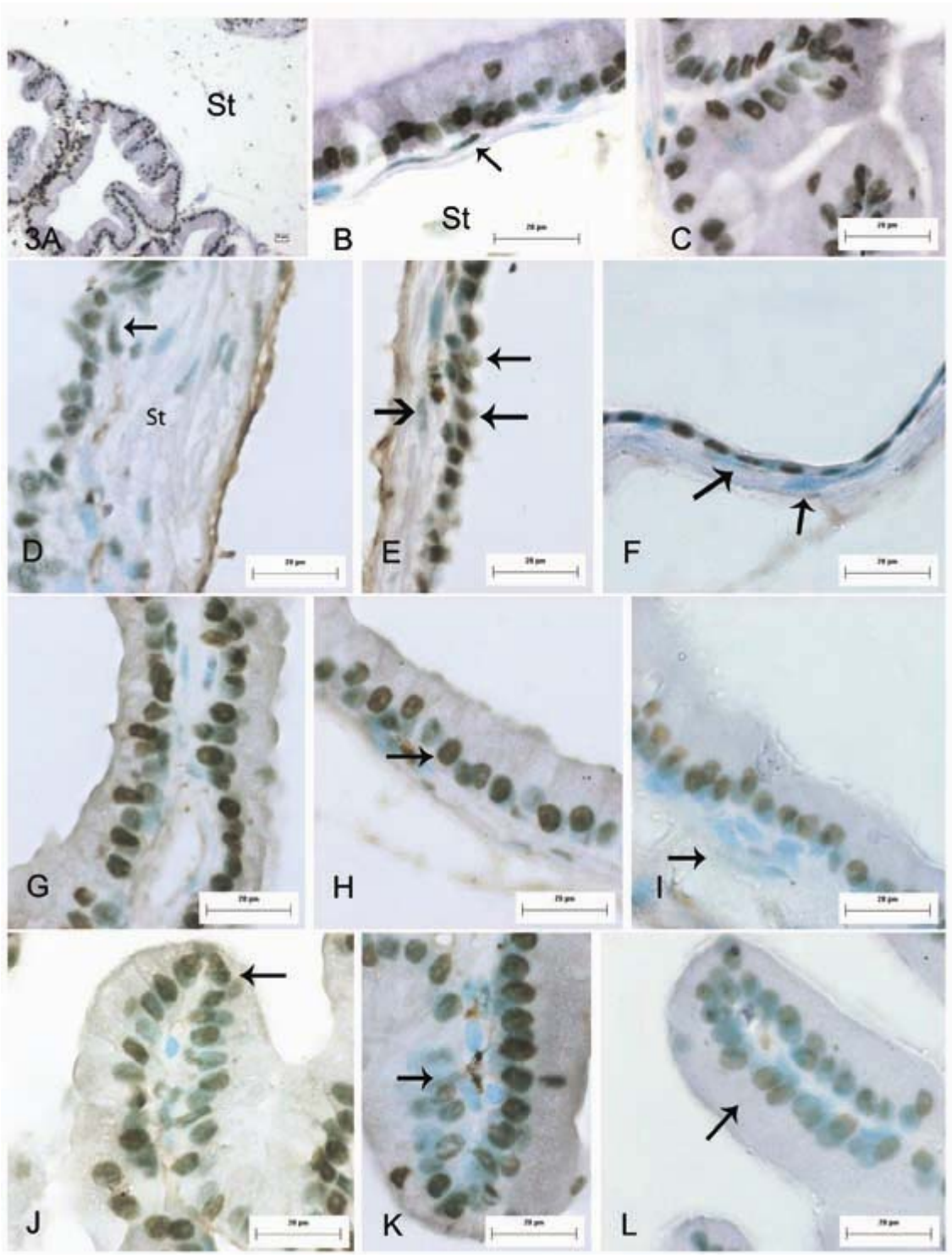

2A
Volume density (%)
CT
LET
CAS
CASLET
Epithelium
B
Volume (mLx10⁻³)
CT
LET
CAS
CASLET
Epithelium
C
Volume density (%)
CT
LET
CAS
CASLET
Lumen
D
Volume (mLx10⁻³)
CT
LET
CAS
CASLET
Lumen
E
Volume density (%)
CT
LET
CAS
CASLET
Non-muscular stroma
F
Volume (mLx10⁻³)
CT
LET
CAS
CASLET
Non-muscular stroma
G
Volume density (%)
CT
LET
CAS
CASLET
Smooth muscle cell
H
Volume (mLx10⁻³)
CT
LET
CAS
CASLET
Smooth muscle cell

Figure 3



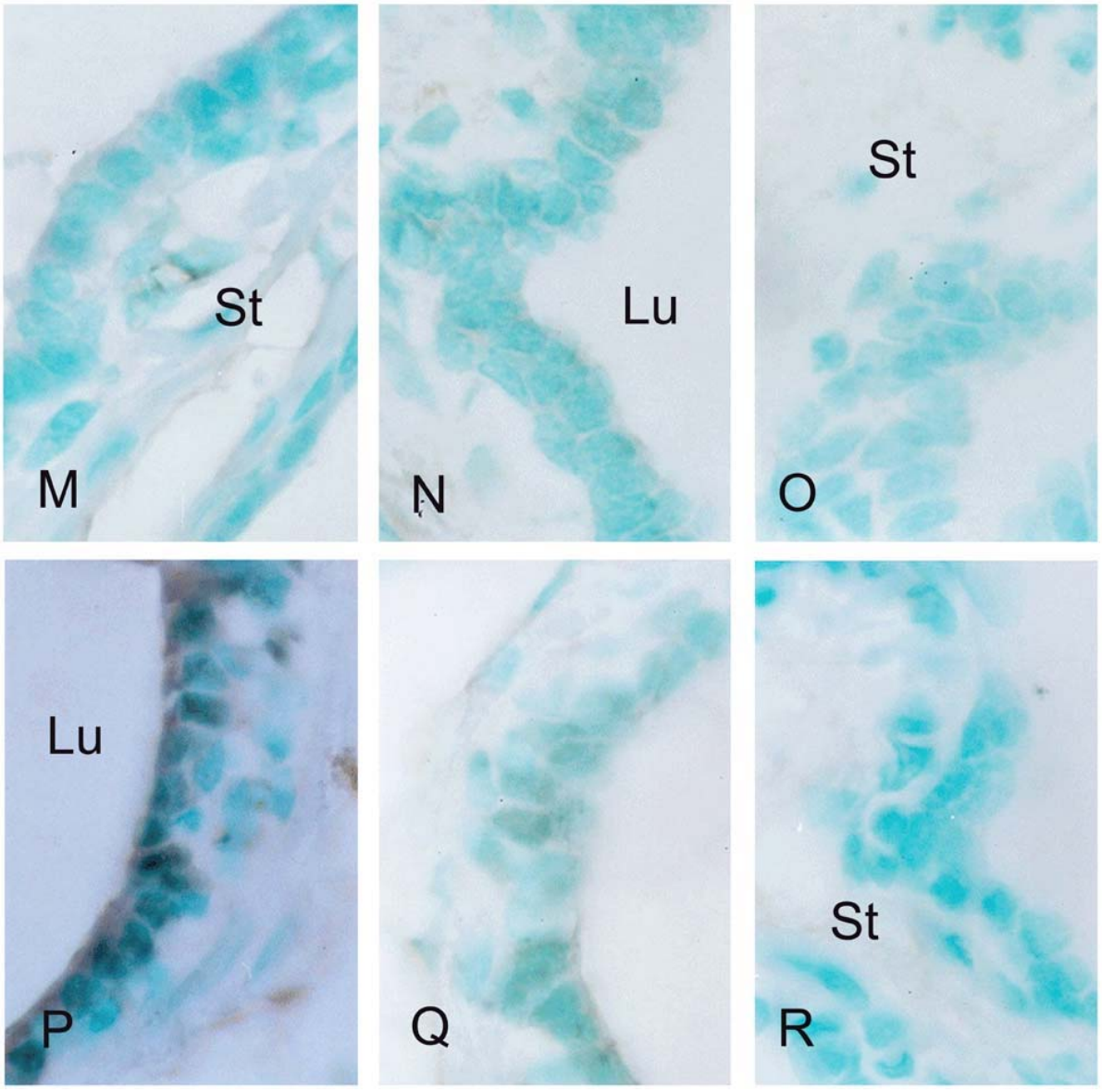

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Figure 4

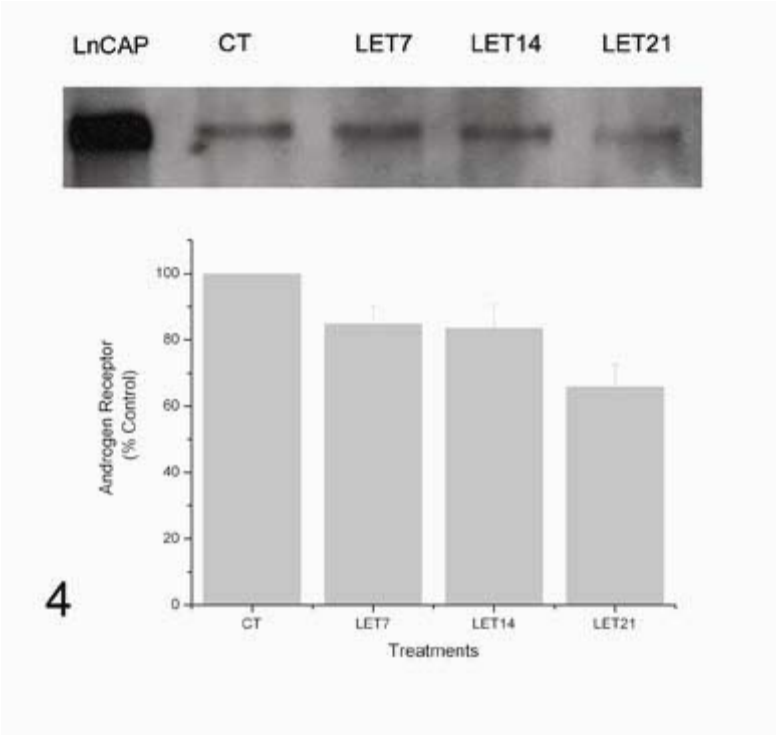
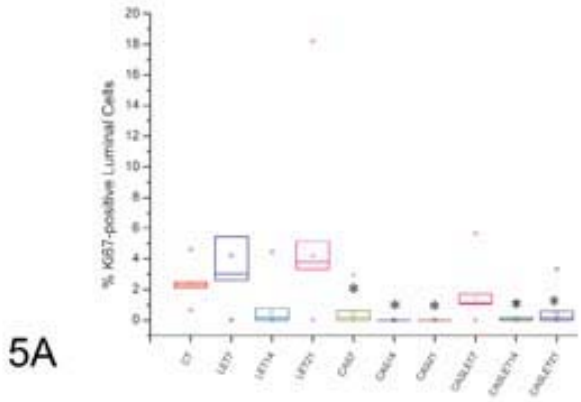
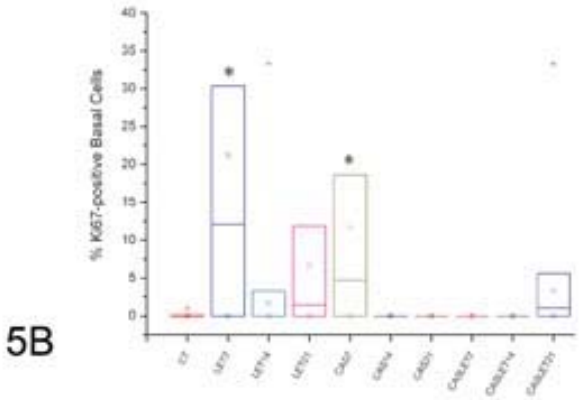
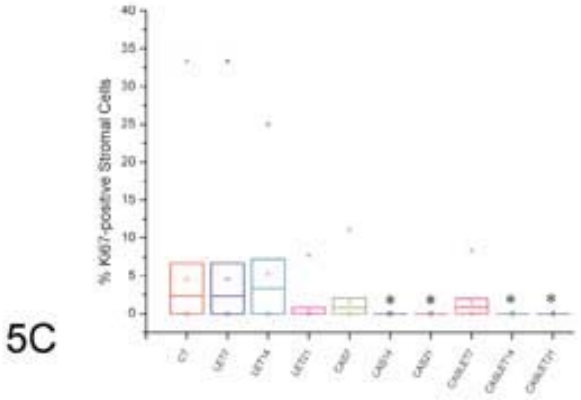

LnCAP
CT
LET7
LET14
LET21
Androgen Receptor (% Control)
100
80
60
40
20
0
CT
LET7
LET14
LET21
Treatments
4

Figure 5


% Ki67-positive Luminal Cells
5A


% Ki67-positive Basal Cells
5B


% Ki67-positive Stromal Cells
5C

Conclusões Gerais

CONCLUSÕES GERAIS

- A aplicação de tamoxifeno e letrozole, durante a fase perinatal compromete o normal desenvolvimento prostático nos animais de 21 dias de idade.
- A exposição perinatal às baixas concentrações de estrógeno estimula a proliferação celular nos compartimentos luminal e estromal dos animais de 42 dias de idade.
- Durante o período pré-púbere a próstata é susceptível ao desequilíbrio hormonal produzido por agentes exógenos e responde com permanentes alterações no crescimento prostático na fase adulta.
- A exposição às baixas concentrações de estrógeno na fase pré-púbere causa inibição do desenvolvimento prostático nos animais de 58 dias de idade.
- O inibidor da enzima aromatase, letrozole, altera as concentrações de testosterona e regula negativamente a expressão do receptor de andrógeno na próstata ventral de ratos Wistar adultos.
- As alterações hormonais afetam os índices normais de proliferação celular, a expressão do receptor de andrógeno e dinâmicas de crescimento prostático, assunto de grande relevância, que em parte explica a alta incidência de algumas patologias prostáticas de etiologia aparentemente desconhecida.

➢

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Anexos

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Comissão de Ética na Experimentação Animal
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CERTIFICADO

Certificamos que o Protocolo nº **592-1**, sobre **"Modulação hormonal do crescimento prostático em diferentes etapas do desenvolvimenti"**, sob a responsabilidade de **Prof. Dr. Hernandes Faustino Carvalho / Manuel Garcia Flórez**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em 09 de setembro de 2003.

CERTIFICATE

We certify that the protocol nº **592-1**, entitled "_____", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on September 5, 2003.

Campinas, 18 de janeiro de 2007.

2ª. VIA

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