

UNIVERSIDADE ESTADUAL DE CAMPINAS



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**EFEITOS PRECOCES DO ESTRÓGENO SOBRE
A PRÓSTATA VENTRAL DE RATOS**

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Orientador: Prof.Dr. Hernandes F. Carvalho

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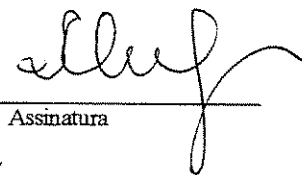
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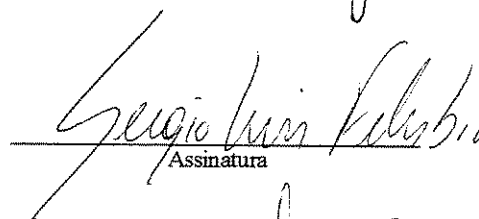
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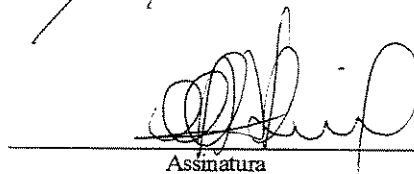
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“Se, por um instante, Deus se esquecesse de que sou uma marionete de trapo e me presenteasse com um pedaço de vida, possivelmente não diria tudo o que penso, mas, certamente, pensaria tudo o que digo. Daria valor às coisas, não pelo que valem, mas pelo que significam. Dormiria pouco, sonharia mais, pois sei que a cada minuto que fechamos os olhos, perdemos sessenta segundos de luz. Andaria quando os demais parassem, acordaria quando os outros dormem. Escutaria quando os outros falassem e gozaria um bom gelado de chocolate. Se Deus me presenteasse com um pedaço de vida, vestiria simplesmente, me jogaria de bruços no solo, deixando descoberto não apenas meu corpo, como minha alma”.

Gabriel García Marquez

Dedicado a mis padres, Víctor Julio e Lilia Ines, porque desde siempre celebraron mis triunfos y dividieron mis derrotas, por el valor que me dieron para enfrentar cada día, por ser simplemente, personas excepcionales.

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Por mostrarme que las cosas simples son las que se guardan en el corazón y permanecen allí sin ser olvidadas en el tiempo.

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RESUMO

A glândula prostática encontra-se exclusivamente em mamíferos, produzindo importantes componentes do sêmen como frutose, íons, e proteínas. Devido ao variado número de patologias que afetam esta glândula, sendo algumas destas ligadas a um alto índice de mortalidade, muitos estudos têm centrado o seu interesse na compreensão da sua fisiologia. O crescimento desta glândula está sob controle endócrino finamente regulado, sendo este susceptível a alterações hormonais. Assim como a testosterona, o estrógeno (E_2) tem marcados efeitos na fisiologia prostática, sendo, às vezes, contraditórios; sabe-se que uma exposição a baixas doses durante a gestação em camundongo incrementa o peso da próstata e a expressão do receptor de andrógeno (AR) na idade adulta, enquanto a exposição a elevadas doses produzem efeitos opostos causando retardo no crescimento e uma pobre expressão do AR. O objetivo do presente estudo foi avaliar as alterações prostáticas sob diferentes condições hormonais. Para este fim, seis diferentes tratamentos foram feitos: ratos intactos (controle), ratos intactos tratados com E_2 , ratos intactos tratados com Tamoxifeno, ratos castrados tratados com E_2 , ratos castrados tratados com Tamoxifeno e ratos castrados sem nenhum tratamento. O volume relativo e o volume absoluto da próstata ventral, foram determinados dentro de um período de sete dias. Além disto, foi determinada expressão do receptor de andrógeno (AR) e do receptor de estrógeno beta ($ER\beta$) por imunocitoquímica no tecido prostático, assim como, os índices de apoptose para cada tratamento através da reação de Tunel e reação de Feulgen. A proliferação celular foi identificada pela contagem de células positivas para PCNA. Os resultados demonstraram que a castração e o tratamento com E_2 levam a uma marcada perda de peso, com uma redução no compartimento epitelial associado com um relativo constante volume luminal e um aumento no estroma junto com um aumento da célula muscular. O Tamoxifeno mostrou pequenos efeitos sobre os compartimentos teciduais. O aumento no volume da célula muscular lisa obtido pelo E_2 sobre os ratos não castrados e tamoxifeno sobre os ratos castrados, correspondem a hiperplasia e hipertrofia, respectivamente. A castração, tratamento com E_2 e a combinação dos dois reduz a marcação para AR e $ER\beta$. O índice de apoptose demonstra diferenças cinéticas na perda celular, entre os ratos castrados e aqueles

tratados com E_2 . A castração leva a um pico de apoptose no dia 3, enquanto que o E_2 leva a um platô, o qual começa no dia 2 e é mantido até o dia 5. A administração de E_2 a ratos castrados resulta num incremento no pico observado no dia 3. Por outro lado, aparentemente o tamoxifeno retarda a morte celular epitelial, resultando em um baixo índice apoptótico até o dia 5, mas este, aumenta, atingindo seu máximo valor no dia 7. A marcação para PCNA demonstrou que a proliferação celular desaparece com todos os tratamentos. Estes resultados demonstram que a castração e o tratamento com E_2 levam a uma marcada redução do peso prostático e função. Não obstante, os dois tratamentos causam diferentes cinéticas dos compartimentos teciduais.

ABSTRACT

The prostate gland is exclusively found in mammals and produces many components of semen such as fructose, ions, and proteins. A number of pathologies that affect this gland, associated to a high mortality, is supporting many studies to understand the prostatic physiology. The prostate growth is under fine endocrine control, and it is susceptible to hormone alterations. Estrogen also has marked effects in prostate physiology, though the results are contradictory, exposure to low doses of estrogen during gestation in mouse was reported to increase adult prostate weight and androgen receptor (AR) levels. In contrast, several studies where neonatal rats have been exposed to higher doses of estrogen resulted in a permanent suppression of prostate growth and a decrease in AR expression. The aim of the present work was to study the prostatic alterations under different hormonal conditions. Six different treatments were made: intact rats (control), non-castrated rats treated with E_2 , non-castrated rats treated with Tamoxifen, castrated rats treated with E_2 , castrated rats treated with Tamoxifen and castrated without treatment. These rats were analyzed on days 1, 2, 3, 5 and 7. The relative and absolute volume variation within a 7 days time line was determined. Furthermore, we have checked the expression of androgen receptor (AR) and estrogen receptor beta ($ER\beta$) by immunocytochemistry and determined the apoptotic indices for each treatment, after TUNEL labelling and Feulgen reaction and investigated cell proliferation, by the identification of PCNA-positive cells. The results demonstrated that castration and exogenous E_2 leads to a marked weight loss, with reduced epithelial compartment associated with a relatively constant luminal volume and an increased stroma in which the smooth muscle cells exhibited a progressively higher contribution. Tamoxifen alone showed very little effect on the tissue compartments. The increase in the smooth muscle volume obtained by E_2 on non-castrated rats and tamoxifen on castrated rats correspond to hyperplasia and hypertrophy, respectively. Castration, E_2 and a combination of both reduced the labelling for AR and $ER\beta$. The apoptotic indices demonstrated a differential kinetics in epithelial cell loss, when E_2 and castration are compared to each other. Castration leads to a peak of apoptosis at day 3, while E_2 leads to a plateau, which begins at day 2 and is sustained up to day 5. E_2 administration to castrated rats resulted in

an increased peak, observed at day 3. On the other hand, tamoxifen seems to delay the epithelial cell death, resulting in low apoptotic indices up to day 5, but reaching the highest value at day 7. PCNA-staining results demonstrated that cell proliferation is abolished with all treatments. The results demonstrated that E₂ and castration lead to marked reduction of prostatic weight and function. However, the two treatments resulted in differential kinetics of the tissue compartments.

INTRODUÇÃO

A estrutura da próstata ventral de ratos

A próstata ventral é composta por um conjunto de estruturas túbulo-alveolares, nas quais as células epiteliais encontram-se envolvidas por um estroma (Aümuller et al., 1979). Na próstata ventral de rato, cada lobo prostático consiste de oito conjuntos de ductos que se originam a partir da uretra como uma simples estrutura tubular a partir dos quais emergem ramos e sub-ramos. Esse conjunto de ductos é dividido em três regiões morfológica e funcionalmente distintas, denominadas de distal, intermediária e proximal, em relação à uretra (Lee, 1990; Shabsigh et al., 1999). Na região distal, são encontradas células epiteliais colunares altas com núcleo apical, indicando atividade proliferativa, enquanto as células musculares lisas apresentam-se como uma camada esparsa e descontínua, associada a uma abundante camada de tecido fibroso. Na região intermediária, as células epiteliais também são colunares altas, porém quiescentes, apresentando características de células secretoras. Nesta região, a camada de células musculares lisas é fina e contínua. Na região proximal, as células epiteliais são cúbicas e baixas, muitas das quais sofrem apoptose. 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 (Nemeth e Lee, 1996).

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

As células musculares lisas (CML) representam 22% da área total da próstata humana (Shapiro et al., 1992), predominando ao redor dos ductos epiteliais e estando 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 (Antonioli et al., 2002, manuscrito em preparação). As CML têm um papel preponderante nos mecanismos de estimulação parácrina, especialmente sobre o epitélio (Farnsworth, 1999) e, provavelmente, também sobre as demais células estromais.

Como em qualquer glândula, a atividade das células epiteliais é fortemente influenciada pelos componentes estromais. Assim, as diferenças fenotípicas das células epiteliais encontradas ao longo dos ductos prostáticos parecem estar relacionadas à distribuição diferenciada dos tecidos fibroso e muscular liso (Lee 1990; Prins 1992; Nemeth e Lee, 1996).

As interações epitélio-estroma desempenham um papel fundamental na regulação da atividade funcional e na manutenção da próstata (Lee, 1996). Durante a embriogênese, sob ação de andrógenos, células invaginam da uretra na base da bexiga para formar o mesênquima do seio urogenital (Timms et al., 1994). Este tecido induz a formação dos ductos e suas ramificações, a proliferação epitelial, a expressão de receptores de andrógenos e secreção de proteínas específicas da próstata (Cunha et al., 1987; Cunha et al., 1992). Na idade adulta, a manutenção da fisiologia da próstata continua dependente de andrógenos.

Entre as células epiteliais e o estroma encontra-se a membrana basal. Esta estrutura é extremamente importante no controle das atividades celulares e, principalmente, na manutenção da fisiologia das células epiteliais (Hayward et al. 1998). 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 (Labat-Robert et al., 1990). Os principais componentes das membranas basais foram detectados na próstata humana normal (Knox et al., 1994) 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.

Carvalho e Line (1996) descreveram as modificações associadas à membrana basal das células epiteliais e das células musculares lisas ocorridas após a privação de andrógenos, demonstrando que eventos apoptóticos seguem à perda de adesão da célula à membrana basal e que existe um retardo na absorção das membranas basais residuais, as quais se tornam extremamente onduladas e contêm moléculas intactas de laminina, mesmo após 21 dias de castração.

Por outro lado, Ilio e colaboradores (2000) observaram que a laminina 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 está 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 períodos de castração, enquanto a laminina permanece intacta. Após o período de pico de apoptose (3º e 4º dia após a castração), o colágeno tipo IV volta a ser encontrado na membrana basal dos ductos. Isto sugere uma direta associação entre um aumento da apoptose celular e a perda de colágeno tipo IV.

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 (Vilamaior et al., 2000).

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 desempenham um papel estrutural, mantendo a integridade do órgão (Carvalho e Line, 1996; Carvalho et al., 1997a).

Desenvolvimento prostático

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

O brotamento prostático é iniciado pela ação androgênica pré-natal, a morfogênese ductal, canalização e citodiferenciação epitelial também precisam de estimulação androgênica. No rato, a maior parte da ramificação ocorre logo após o nascimento quando os níveis de andrógenos são baixos (Donjacour et al., 1988), porém ocorre um pico nos níveis de testosterona próximo ao dia de nascimento, o que poderia induzir o processo de ramificação.

A diferenciação do epitélio prostático ocorre paralelamente à maturação do estroma. Andrógenos atuam sobre receptores de andrógeno (AR) no mesênquima urogenital (UGM) para induzir a proliferação epitelial, ramificação ductal e citodiferenciação nos subtipos celulares basal e luminal. Por sua vez, o epitélio prostático em desenvolvimento direciona os padrões de diferenciação do músculo liso prostático (Hayward et al., 1998). O desenvolvimento do epitélio e da CML é interdependente, ou seja, um tecido não se desenvolve na ausência do outro (Hayward e Cunha, 2000).

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

A próstata de neonato é sensível aos andrógenos. Assim, a administração de testosterona acelera o crescimento da próstata, sendo possível atingir precocemente o crescimento máximo (Berry e Isaacs, 1984).

Embora a testosterona seja o primeiro andrógeno produzido pelos testículos fetais, a diidrotestosterona (DHT) é a responsável pela morfogênese prostática (Taplin e Ho, 2001). A DHT é produzida no seio urogenital (UGS) pela redução enzimática de testosterona pela 5- α redutase. Esta enzima foi detectada no UGS e na genitália externa de ratos, coelhos e humanos (Wilson et al., 1983). Na puberdade, apresenta-se o início do crescimento prostático que é caracterizado por um aumento do peso seco da próstata e por um pequeno incremento no número das ramificações (Sugimura et al., 1986a). Estes dados indicam que a próstata em desenvolvimento é sensível às baixas concentrações de andrógenos para a ramificação ductal, e que a sua resposta aos níveis de andrógenos altos na puberdade

(aumento de peso seco) é diferente da resposta inicial à ramificação ductal (Hayward e Cunha, 2000).

Regulação Hormonal

O controle neuroendócrino do metabolismo, do crescimento e de certos aspectos da reprodução é mediado por uma combinação de sistemas neurais e endócrinos localizados no hipotálamo e na hipófise (Klonoff e Karam, 1995).

A hipófise é constituída por um lobo anterior (adeno-hipófise), um lobo intermediário e um lobo posterior (neuro-hipófise). A secreção de gonadotrofinas (hormônios luteinizante – LH e folículo-estimulante – FSH) pela adeno-hipófise sofre a influência do hipotálamo (Fig 1), através do hormônio liberador de gonadotrofinas (GnRH) (Klonoff e Karam, 1995).

Os hormônios produzidos pelos testículos estão envolvidos em várias funções fisiológicas, como a manutenção das funções sexuais secundárias e o efeito de retroalimentação sob o hipotálamo e a hipófise, responsáveis pela liberação de gonadotrofinas. O LH tem como alvo a célula de Leydig estimulando a secreção do androgênio chamado testosterona, enquanto que o FSH atinge os seus receptores nas células de Sertoli, regulando a espermatogênese. (O' Donnell et al., 2001). Os andrógenos são necessários para o crescimento normal e atividades funcionais da próstata. No homem, os principais androgênios circulantes são a diidrotestosterona (DHT) e a testosterona (T), sendo esta última produzida na sua maior parte (aproximadamente 95%) pelos testículos. As glândulas adrenais contribuem com menos de 5% da produção dos esteróides sexuais (Coffey, 1992). Nos dois tecidos existem pelo menos duas vias sintéticas a partir da pregnenolona; a δ -5, que resulta na produção de androstenediona e testosterona, é a via predominante; não obstante a δ -4, uma via sintética menor, leva à síntese de deidroepiandrosterona (DHEA) e androstenediol.

Uma vez sintetizada, a maior parte da DHEA é inativada via sulfatação, enquanto

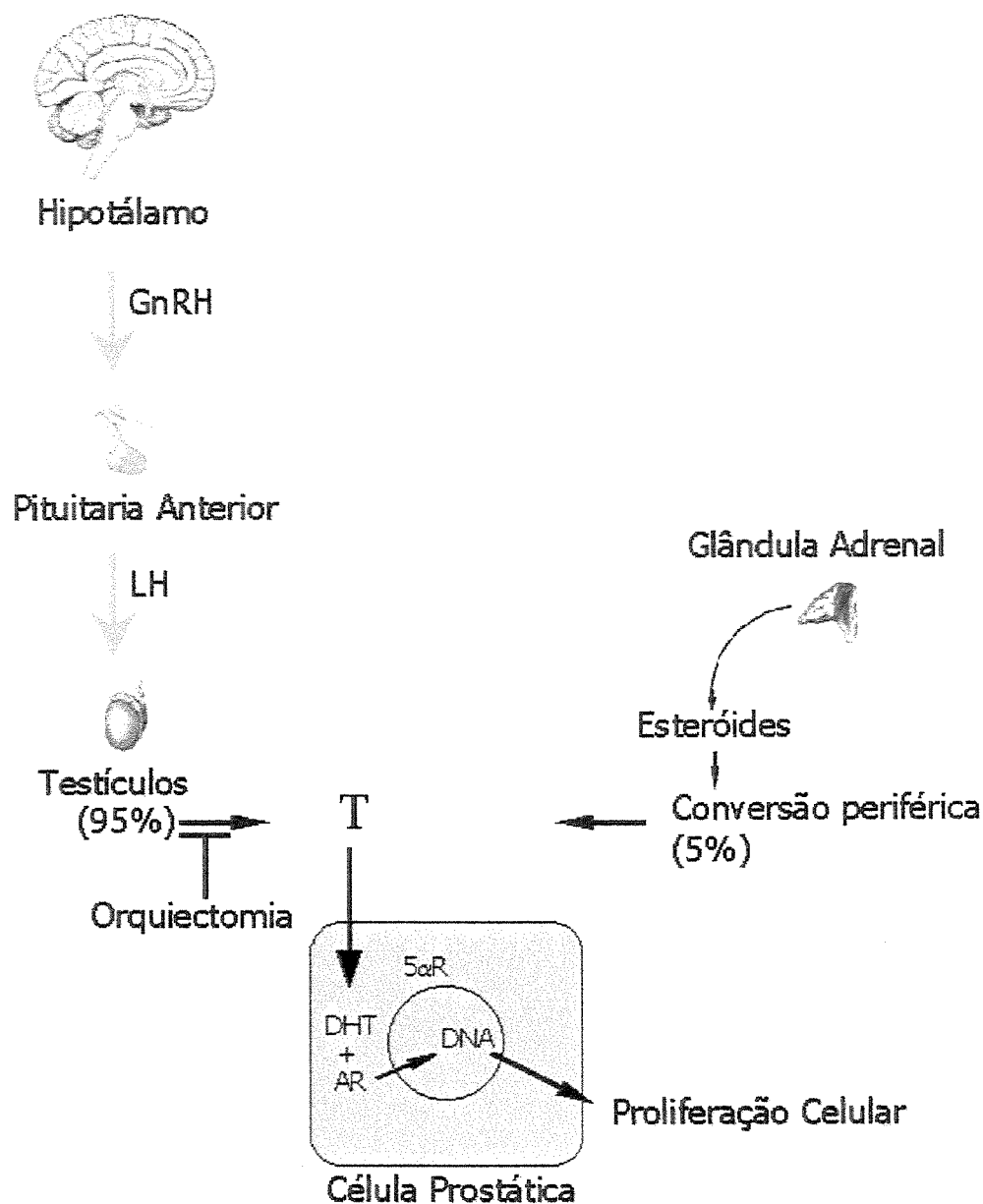


Figura 1. Eixo-hipotálamo-hipófise-gônadas-próstata. A estimulação da pituitária anterior é produzida pelo hipotálamo através dos hormônios liberadores de gonadotrofinas (GnRH), que leva à produção do hormônio luteinizante (LH). A produção de testosterona é feita principalmente nos testículos, pela estimulação do LH sobre a célula de Leydig. Nas células prostáticas a testosterona é convertida em diidrotestosterona (DHT) através da ação da 5 α -redutase. Esta finalmente liga-se ao receptor de andrógeno (AR) com maior afinidade do que a testosterona.

uma pequena fração é convertida em androstenediona e, a seguir em T, nos tecidos periféricos e na próstata (Cheng et al., 1993). A esteroidogênese testicular nas células de Leydig é regulada primariamente pelo LH, enquanto a produção androgênica pela adrenal está sob o controle do ACTH. Em homens saudáveis, os andrógenos adrenais contribuem ligeiramente na função prostática normal, embora em estados tumorais prostáticos a contribuição da adrenal possa promover o crescimento prostático, principalmente após orquiectomia (Geller et al., 1984).

Estima-se que apenas 2-3% da T encontra-se disponível em sua forma livre, sendo que o restante encontra-se ligada a proteínas séricas como a SHBG (*Sex hormone-binding globulin*), albumina e globulina de ligação com corticoesteróide (Vermeulen, 1973). Entre as três proteínas, a SHBG possui a maior afinidade pela testosterona. A conversão de T no mais potente andrógeno intracelular, 5 α -diidrotestosterona (DHT) pela ação da 5 α -redutase, ocorre no tecido testicular assim como no prostático.

Dois tipos de 5 α -redutases foram identificados. A 5 α -redutase tipo I encontra-se na maioria dos tecidos, enquanto a 5 α -redutase tipo II, que é codificada pelo gene SRD5A2 localizado no cromossomo 2p23 (Labrie et al., 1992), predomina nos tecidos genitais, incluindo a próstata. Quando a finasterida, inibidor específico da 5 α -redutase tipo II, foi administrada a homens para o tratamento de hiperplasia prostática benigna (BPH) os níveis séricos de DHT diminuíram cerca 70 %; enquanto os níveis prostáticos diminuíram entre 85 e 90% (Bartsch et al., 2000). A quantidade remanescente de DHT na próstata foi o resultado da ação da isoenzima tipo I.

A DHT intracelular é metabolizada em uma reação reversível para 3 α , 17 β -androstenediol (3 α -diol), ou para 3 β , 17 β -androstenediol (3 β Adiol). A enzima responsável por esta via de inativação é a 3 β -hidroxiesteróide desidrogenase tipo II. O 3 β Adiol é finalmente convertido irreversivelmente em esteróides inativos hidrossolúveis, através da ação da 3 β Adiol hidroxilase (CYP7B1) (Fig. 2) (Martin et al., 2001; Weihua et al., 2002).

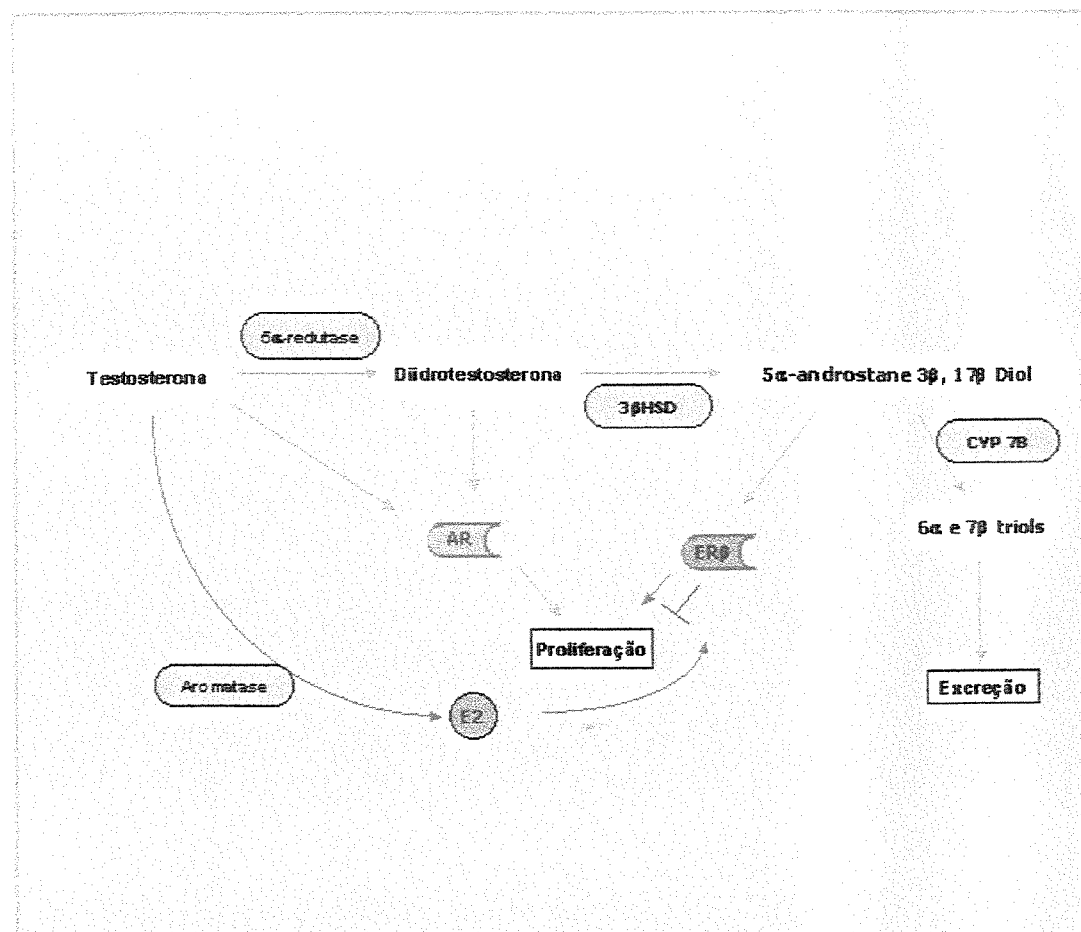


Figura 2. Relações entre andrógenos e estrógenos na regulação do crescimento prostático e seus inibidores. A testosterona tem papel fundamental na fisiologia prostática. No órgão, ela é convertida em diidrotestosterona (DHT) pela enzima 5 α -redutase. Tanto a testosterona quanto a DHT ligam-se aos receptores 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 5 α -androstane 3 β , 17 β diol, pela enzima 3 β -hidroxiesteroide desidrogenase Tipo II (3 β HSD). O 5 α -androstane 3 β , 17 β diol liga-se ao receptor de estrógenos, competindo com o estrógeno circulante e impedindo sua atividade proliferativa. O excesso de 5 α -androstane 3 β , 17 β diol é processado pela enzima 3 β Adiol hidroxilase (CYP7B1) a 6 α e 7 β triols, que são eliminados. Nos machos, o estrógeno circulante origina-se da ação da enzima aromatase sobre a testosterona. AR = Receptor de andrógeno; ER β = Receptor de estrógeno subtipo β ; E2 = Estrogênio; $\longrightarrow$ = Ação do E2 sob seu receptor; $\longrightarrow$ = Metabolismo da testosterona; $\longrightarrow$ = Vias inibidas

Estrógeno e Próstata

A biossíntese de estrógeno é catalisada por um membro da superfamília p450, chamado aromatase p450 (p450 arom, produto de gene CYP19) (O'Donnel et al., 2001). Esta proteína é responsável pela ligação ao substrato andrôgenico e por catalisar uma série de reações, que levam à formação de um anel fenólico no anel A da molécula (característico dos estrógenos). Esta reação emprega três moléculas de oxigênio e três de NADPH para cada molécula de substrato metabolizada (Thompson e Siiteri, 1974)

Em humanos alguns tecidos têm a capacidade de expressar a aromatase e promover então a síntese de estrógenos. Estão incluídos ovários, testículos, placenta, fígado de feto, tecido adiposo, condrócitos e osteoblastos, numerosos sítios do cérebro incluindo varias áreas do hipotálamo, sistema límbico e córtex cerebral (O'Donnel et al, 2001) e na próstata sendo implicada com a etiologia da hiperplasia benigna prostática (Tsugaya et al., 1996)

Mecanismos de ação dos estrógenos

Os receptores de estrógeno foram inicialmente caracterizados nos anos 70 (Jensen e DeSombre, 1973), e pertencem a uma família de receptores nucleares, entre os quais se encontram receptores para outros hormônios esteróides, vitamina D e retinóides entre outros (O'Donnel et al., 2001).

Até relativamente poucos anos, acreditava-se que só existia uma forma de receptor de estrógeno ($ER\alpha$) não obstante uma segunda forma foi encontrada ($ER\beta$) a qual foi identificada em camundongo, rato e humano (Kuiper et al., 1996, Tremblay et al., 1997, Mosselman et al., 1996, Enmark et al., 1997).

Os receptores de hormônios esteróides de um modo geral são compostos de três domínios funcionais independentes mas inter-relacionados (Figura 3): o domínio amino-terminal ou domínio A/B, o domínio de ligação ao DNA, ou domínio C, e o domínio de interação com ligantes, ou domínio D/E/F (Nilsson et al., 2001).

O domínio amino-terminal está envolvido na interação proteína-proteína (McIerney et al., 1993, Onate et al., 1998), sendo a região menos conservada da molécula, com apenas 17% de homologia entre os ERs (O'Donnel et al., 2001)

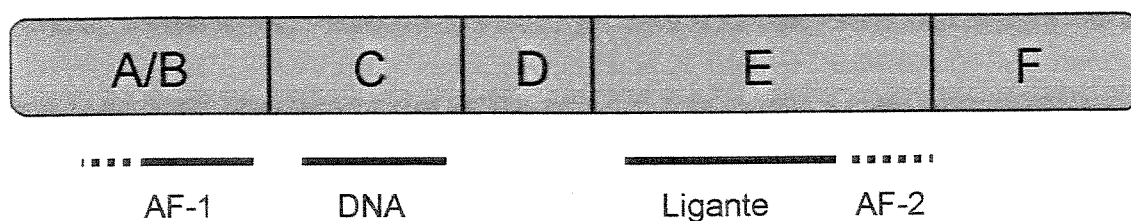


Figura 3. Representação do domínio estrutural dos receptores nucleares. O domínio A/B na região N-terminal contém o sítio AF-1 onde outros fatores de transcrição interagem. Os domínios C e D contêm duas estruturas em forma de dedos de zinco (*ZINC Fingers*) que se ligam ao DNA, e o domínio C/F contém uma região ligante, assim como o domínio AF-2.

O domínio de ligação ao DNA atua na dimerização do receptor e na ligação do receptor às seqüências específicas de DNA (Nilsson et al., 2001), sendo esta a região mais altamente conservada (O'Donnel et al., 2001).

A região carboxi-terminal (E/F) ou *ligand binding domain* atua na translocação nuclear, na dimerização do receptor e na ativação da expressão de genes alvos (Nilsson et al., 2001).

Os ERs contêm duas funções ativadoras distintas: Uma no domínio A/B chamada *Activation Function I* (AF1), e o segundo, *Activation Function II* (AF2), hormônio dependente, localizada no domínio E. AF1 e AF2 são requeridos de forma acoplada para uma completa atividade dos ERs na maioria das células (Katzenellenbogen et al., 2000).

Ação dos estrógenos sobre os seus receptores

Estudos em humanos mostram que a administração de estradiol (E_2) pode promover a supressão da secreção de gonadotrofinas (Handelsman et al., 2000).

A demonstração da presença $ER\alpha$ e $ER\beta$ no hipotálamo e pituitária de roedores junto com a imunolocalização da aromatase no cérebro indicam que o E_2 tem um importante papel nestes tecidos. (Carretero et al., 1999)

Gill-Sharma e colaboradores (2001) mostraram que, em ratos adultos, doses aumentadas de E₂ (0,1-1 µg/kg/dia) durante um tratamento prolongado, produziam uma significativa redução na concentração circulante de FSH e LH, o que posteriormente leva a uma redução na concentração de testosterona sérica e testicular. Em outros trabalhos, foi possível notar um efeito estimulador das baixas doses de E₂ sob o FSH (Atanassova et al., 2000). Isto indica que o E₂ pode participar como promotor ou indutor do FSH.

O maior componente da ação da retroalimentação negativa dos andrógenos sobre a secreção de gonadotrofinas é mediada via aromatização e conseqüente produção de estrógeno (Finkelstein et al., 1991).

Sabe-se que a exposição a estrógeno exógeno tem pronunciados efeitos sobre o trato urogenital masculino em desenvolvimento e na fase adulta (Nilsson et al., 2001). Não obstante não está claro quais destes efeitos são relacionados à ação direta do estrógeno via ERs nos tecidos reprodutivos e quais são resultado dos efeitos indiretos da ação do estrógeno sobre o eixo hipotálamo-hipófise-gônadal (eixo HHG).

Prins e Birch (1997) e Chang e colaboradores (1999) localizaram o ER α na próstata de rato, através de procedimentos imunohistoquímicos, encontrando-o em células estromais periductais (fibroblastos e células musculares lisas), mas não em células epiteliais.

Prins et al. (1998) revelaram, através de técnicas de hibridação *in situ*, que a expressão de ER β na próstata ventral de rato é baixa após o nascimento e aumenta inicialmente quando as células epiteliais são diferenciadas em células luminais epiteliais, ocorrendo sua expressão máxima 90 dias após o nascimento. Já no adulto, observa-se uma apreciável expressão do ER β . Esta expressão forma um gradiente, sendo baixa nas porções glandulares proximais e aumentando nas porções mais distais, em conformidade com a existência de uma heterogeneidade histológica, funcional e estrutural do epitélio prostático ao longo da rede de ductos, desde sua origem na uretra, até as ramificações ductais (Sugimura et al. 1986b, Sugimura et al. 1986c, Lee et al., 1990, Rouleau 1999, Sensibar 1991). A importância do gradiente do ER β é ainda desconhecida, mas poderia estar determinando a heterogeneidade do epitélio no sistema ductal prostático (Chang e Prins, 1999).

Recentemente descobriu-se que os estrogênios são necessários para manter a função normal dos testículos e da próstata. Já a importância dos andrógenos em fêmeas é indubitável uma vez que são eles os precursores de estrogênios, sendo substratos da aromatase, enzima que produz o estrógeno (Pettersson e Gustafsson, 2001).

Estudos experimentais e epidemiológicos têm demonstrado que o estrógeno está envolvido tanto na indução como na prevenção do câncer prostático, mas seu papel exato não está ainda definido (Weihua et al, 2001). Uma excessiva exposição ao estrógeno durante etapas críticas do desenvolvimento leva à neoplasia prostática (Messina et al, 1994 e Bosland *et al*, 1995). Walsh e Wilson (1976), corroboraram isto, usando um modelo canino e demonstraram que o estrógeno é necessário para a produção de uma hiperplasia glandular, sendo que em rato, o estrógeno atuando com andrógenos induz displasia e formação de adenocarcinoma na próstata (Lau et al., 1998). Em aparente contradição, uma dieta com altos conteúdos de fitoestrogênios (especialmente produtos de soja) é associada com um baixo risco de câncer prostático (Barnes et al, 1995, Goodman et al, 1997 e Landstrom et al, 1998). Algumas destas observações contraditórias podem ser explicadas pelo fato que existem dois diferentes receptores de estrogênio, o ER α e o ER β , Estes receptores possuem papéis únicos e, às vezes, opostos. Além destes receptores também é possível encontrar algumas isoformas e dímeros ($\alpha\alpha/\beta\beta$) ou heterodímeros ($\alpha\beta$), o que possivelmente proporciona diferentes formas de ação dos estrogênios e a ativação de diferentes genes (Chang e Prins, 1999).

Objetivo

Sabendo dos efeitos contraditórios do estrógeno sobre a próstata este trabalho teve por objetivo analisar os efeitos precoces do tratamento com E₂ sobre a próstata ventral de ratos, procurando-se identificar parâmetros que possam definir a sua forma de ação. Para isto utilizou-se da castração e da aplicação de E₂, assim como do tamoxifeno, antagonista parcial do E₂ e de análises estereológicas, imunolocalização de AR e ER β , além de contagens de células apoptóticas e proliferativas.

ARTIGO

O Presente trabalho deu origem ao artigo **Early effects of estrogen and tamoxifen on the rat ventral prostate** o qual foi submetido à revista **Biology of Reproduction**.

Early effects of estrogen and tamoxifen on the rat ventral prostate

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Abstract

Complex interactions between androgen and estrogen (E_2) regulate prostatic development and physiology. We have analyzed the early effects of exogenous E_2 and tamoxifen (a partial E_2 antagonist) on the ventral prostate of control and castrated rats. The relative and absolute volume variation within a 7-day time line was determined. The expression of the androgen receptor (AR) and estrogen receptor beta ($ER\beta$) was checked by immunocytochemistry and apoptotic indices for each treatment obtained after Tunnel labeling and the Feulgen reaction, and cell proliferation investigated by the identification of PCNA-positive cells. Castration and exogenous E_2 leads to a marked weight loss, with reduced epithelium constant luminal volume and increased stroma, which showed a progressively greater contribution to the smooth muscle cells (SMC). Tamoxifen alone showed very little effect on the tissue compartments. The increases in the SMC volume obtained by E_2 on non-castrated rats and tamoxifen on castrated rats correspond to hyperplasia and hypertrophy, respectively. The apoptotic indices demonstrated a differential kinetics in epithelial cell loss, when E_2 and castration are compared to each other. Castration leads to a peak of apoptosis at day 3, while E_2 leads to a plateau. E_2 administration to castrated rats resulted in an increase in the peak observed at day 3. Tamoxifen seems to delay epithelial cell death. E_2 and castration leads to prostatic involution through a different mechanism. Apparently, E_2 has an immediate effect on the prostate, inducing the arrest of epithelial cell proliferation and apoptosis, then followed by a secondary effect, similar to castration, by reducing the circulating T.

Introduction

Androgens are required for the growth and physiology of the prostate. The circulating androgens are testosterone (T) and dihydrotestosterone (DHT). The former is produced mainly by the testis (~95%) and the adrenal gland (~5%) [1]. The conversion of T to the more potent androgen DHT, by the enzyme 5α -reductase, takes place in the testis and also in diverse tissues, including the prostate. Both T and DHT bind to the same androgen receptor (AR). Two isoforms of 5α -reductase have been described. 5α -reductase type 1 is

found in most tissues, while 5 α -reductase type 2, which is coded by the gene SRD5A2 located in chromosome 2p23 [2], predominates in the reproductive tissues.

The intracellular DHT is rapidly metabolized in a reversible reaction to 3 α , 17 β -androstenediol (3 α -diol) or to 3 β , 17 β -androstenediol (3 β Adiol). The enzyme responsible for this inactivation is 3 β -hydroxysteroid dehydrogenase type II. 3 β Adiol is finally and irreversibly converted into inactive water-soluble steroids by the action of the enzyme 3 β Adiol hydroxylase (CYP7B1) [3, 4].

On the other hand, it is well known that the circulating androgens are converted to estrogen in several tissues by the action of the enzyme aromatase [5]. Interestingly, the enzyme aromatase has been identified in the human prostate, suggesting that the prostate is a site of aromatization and a possible source of estrogens [6]

In spite of being an androgen-dependent tissue, the physiology and pathology of the prostate is also under the influence of estrogen. Their action is mediated by the estrogen receptors (ER α and ER β) in the prostatic stroma and epithelium, respectively [7].

E₂ has a series of effects on the prostate. It promotes involution of the epithelium and growth of the stroma in the adult animal, probably by reducing the levels of circulating T [7,8], and, in castrated dogs, E₂ (8 mg/dog/week) showed a proliferative effect in both the stroma and the epithelium [9]. When administered to neonates, it causes permanent changes in the prostate. The gland becomes irresponsive to androgen, attains a reduced size in adulthood, and develops hyperplasia and/or dysplasia more frequently [10, 11]

Estrogen stimulation results in apoptotic cell death in the uterine epithelium of neonatal mice, but not in the stroma. With high exposure to E₂ (25 μ g/mouse/day), the apoptotic index of the uterine epithelium increased markedly, however daily injections of E₂ (7.2 μ g/g body wt) completely inhibited the increase in the apoptotic index [12].

A series of evidences suggest that epithelial growth in male and female reproductive organs takes place through paracrine mechanisms mediated by stromal receptors. Cunha and others [13] showed that androgen-induced epithelial proliferation in the prostate gland is mediated indirectly through mesenchymal/stromal androgen receptors. Cook and others [14] used a model of tissue separation/recombination and showed that the mitogenic effects

of E₂ on the uterine epithelium of the estrogen receptor beta knockout mouse (βERKO), are mediated indirectly via stromal ER. Besides, neither E₂ nor testosterone (T) has a mitogenic effect on normal epithelial cells in cultures [15, 16]. These findings reinforce the idea that epithelial growth regulation in male and female reproductive organs proceeds through common paracrine mechanisms mediated by stromal hormone receptors.

Tamoxifen, acts as an antiestrogen agent by competitive binding to the estrogen receptor. The biological effects of tamoxifen are complex, depending on the concentration, the sex of the animal and the target organ [17]. Oral administration of tamoxifen appeared to exert both estrogenic and antiestrogenic effects on gonadotropins [18, 19, 20]. Perinatal exposure to tamoxifen is associated with altered prostate weights and increased occurrence of prostate inflammation in adulthood. [21]

As noted from the data presented above, the hormonal control of prostatic development and function is complex and based on a dense network of interactions between steroid hormones. The pathways in this network are still poorly known and their analysis is hampered by the predominant effect of testosterone.

Androgen deprivation elicited by physiological or chemical castration induces apoptosis in prostatic epithelium. In the ventral prostate (VP) of the rat, epithelial apoptosis reaches its peak three days after androgen withdrawal [22]. Seven days after surgical castration the rat prostate is rapidly involuting and both basal and secretory cellular subtypes persist. However the number of glandular cells is found to be significantly reduced (66% decrease) [23]. This castration-induced atrophy of the VP is associated with a significant increase in p27, which is selectively up-regulated in the non-proliferating secretory epithelial compartment. After testosterone propionate treatment, both proliferating basal and secretory epithelial cells no longer express p27. [24]

In an attempt to understand the early effects of steroid hormones on the different prostatic tissue compartments, we have employed castration, exogenous E₂ administration and tamoxifen, a partial antagonist of E₂, associated with stereology, apoptotic index determination and PCNA-immunocytochemistry to verify the behavior of the rat ventral prostate under different hormonal conditions within a 7 day experimental time line.

Material and Methods

Animals

Adult male 90-day-old Wistar rats were used. They were maintained in a controlled environment with free access to food and water. Experiments were performed according to the *Guide for Care and Use of Laboratory Animals*, and were approved by the Ethical Committee of the State University of Campinas

Experimental protocol

A sole dose of each compound was injected subcutaneously in a volume of 0.4 mL of corn oil. Control rats received only corn oil. These treatments included 17 β -estradiol (E₂) (Sigma Chemical Co.; St. Louis, MO), and the partial estrogen antagonist tamoxifen (Tam) (Sigma Chemical Co.) at concentrations of 0.0367 mM and 0.0176 mM, respectively. The rats were divided into six different groups: intact rats (control), non-castrated rats treated with E₂, non-castrated rats treated with Tam, castrated rats treated with E₂, castrated rats treated with Tam and castrated with no further treatment. Analyses were performed on days 1, 2, 3, 5 and 7.

Necropsy and prostate examinations

Rats were weighed before have been killed. At 91, 92, 93, 95 and 97 d of age, the different groups of rats were killed by cervical dislocation within 15 s after being removed from their cages. Testis, seminal vesicle, ventral prostate and coagulating gland (the ventral lobe and coagulating gland was carefully separated from the other lobes and from the seminal vesicle, respectively) were removed and weighed. The organs were excised and processed immediately or stored in liquid nitrogen. The ventral prostate was dissected out and immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr. The samples were then washed, dehydrated, cleared in xylene, and embedded in historesin for stereological analyses and in Paraplast Plus embedding medium for immunocytochemistry.

Stereological analyses

Two-micrometer sections were obtained and stained with hematoxylin and eosin. Six microscope fields from at least two animals from each group were photographed and the stereology carried out using Weibel's system and a 168-point grid [25]. According to DeKler & Coffey [26], 1 mg of fresh rat ventral prostate tissue had a volume of approximately 1mm^3 . Consequently, the weight of the ventral lobe (mg) could, in practice, be used as equivalent to volume (mm^3). The relative volumes of the epithelium, lumen, smooth muscle and non-muscular stroma were determined. The total stroma was the sum of the smooth muscle and non-muscular stroma. For the calculation of the total volume of each of these compartments, we considered the mean prostatic weight measured for each group.

Immunocytochemistry

Five-micrometer sections were collected on silanized glass slides, dewaxed with xylene and rehydrated in a descending ethanol dilution series. Endogenous peroxidase activity was blocked by treating tissue sections with 3% hydrogen peroxide in water for 30 min. Blocking of non-specific protein-protein interactions was achieved by incubating sections for 1 h with 3% bovine serum albumin (BSA; (Sigma Chemical Co.) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T).

The immuno-localization of the proliferating cell nuclear antigen (PCNA), used a mouse monoclonal antibody (PC-10), specific for the PCNA p36 protein. Immunolocalization of the estrogen receptor beta ($\text{ER}\beta$) used a goat polyclonal antibody (Y-19), raised against a peptide within the N-terminal domain of the mouse $\text{ER}\beta$. Immunolocalization of the androgen receptor (AR) used a rabbit polyclonal antibody (N-20), raised against a peptide within the N-terminal domain of human AR. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies were diluted in a solution of 1% BSA in TBS-T, according to the manufacturer's directions, and applied to sections for 1 h at room temperature. After three 5 min washes with TBS-T, the sections were incubated with adequate peroxidase-conjugated antibodies, diluted 1:100 in 1% BSA in TBS-T, for 1 h. The sections were washed again and the peroxidase activity

revealed using the 3,3'-diaminobenzidine reaction. They were counterstained with methyl green, air dried and mounted in Entellan (Merck, Darmstadt, Germany).

Observations were carried out using a Zeiss Axioskop microscope and photographs were taken using a Kodak 100 Proimage film.

Apoptosis detection and apoptotic index determination

DNA fragmentation was detected using The Apoptosis Detection System - Fluorescein (Promega, Madison, WI, USA) and all the conditions were performed according to the manufacturer's instructions. The apoptotic nuclei were identified using a Zeiss Axioscop Microscope and the micrographs obtained using a Kodak 400 Kodacolor film.

Historesin sections were subjected to Feulgen's reaction. They were hydrolyzed with 4N HCl for 1h15 and then reacted with Schiff's reagent for 40 min. After extensive washing, the sections were dehydrated and mounted in Canada's balsam. Six microscope fields from at least two animals were analyzed and the apoptotic index determined by dividing the number of apoptotic nuclei by the total number of nuclei found in the microscope field, using a X40 objective. Apoptotic nuclei were identified by the characteristic picnosis and/or fragmentation [27]. The number of nuclei counted was between 1290 and 2657 for the different experimental situations.

Statistical Analyses

The 1-factorial analysis of variance (ANOVA), followed by a Dunnett's test for multiple comparisons was employed for the comparison of the measurements obtained after each treatment and of those of the controls. Value of less than 0.05 was considered to be statistically significant.

Results

Table I exhibits the variation in body, testis and accessory gland weights following the different treatments for 7 days. E₂ caused a significant reduction in testis weight. E₂ and castration caused similar reductions in the weight of the accessory glands. The SV was less responsive to E₂. The weight loss caused by tamoxifen was not significant. On the other

hand, tamoxifen reduced the weight loss in VP ($p<0.05$) and CG ($p<0.1$), as compared to castration alone.

The histology of the rat ventral prostate revealed a simple, cylindrical epithelium with basal nuclei (Fig. 1A). The stroma was scarce, with few smooth muscle cells (SMC) and fibroblasts. E_2 treatment for 7 days also resulted in shorter epithelial cells. The SMC formed a dense bundle below the epithelium (Fig. 1B). Tamoxifen showed no apparent effect on the epithelial morphology. However, a thicker layer of SMC was observed (Fig. 1C). Seven days of androgen deprivation by surgical castration resulted in a loss of the apical cytoplasm, resulting in shorter epithelial cells. The stroma, in turn, became more prominent, with a dense array of SMC, fibroblasts and other cell types (Fig. 1D). Exogenous E_2 administration to castrated rats resulted in a histological organization intermediate between those seen in castrated rats and E_2 -treated rats, especially with respect to the SMC (Fig. 1E). Tamoxifen administration to castrated rats resulted in no apparent effect to the epithelium, when compared to castration alone, but resulted in a thick layer of SMC (Fig. 1F). In contrast to the thickening of the SMC layer observed after E_2 treatment, that promoted by tamoxifen resulted from an apparent hypertrophy of the SMC (Fig. 1G).

Stereology was employed to follow the kinetics of different tissue compartments under the different hormonal situations. E_2 resulted in a marked reduction of the relative volume of the epithelial compartment 2 days after the beginning of the treatment. This loss was maintained up to day 5 and was then partially restored at day 7 (Fig. 2A).

It was observed that tamoxifen had very little effect on the relative volume of the epithelial compartment (Fig. 2A). The luminal compartment showed little variation within the 7 days of treatment (Fig. 2B). All hormonal conditions resulted in an increment of the relative volume of the stroma (Fig. 2C). The relative volume of the stromal compartment increased progressively after day 2, with a pronounced contribution of the SMC in all hormonal conditions, as compared to that of the control (Figs. 2C-E).

The absolute volume showed more dramatic changes in the prostatic tissue compartments. Except for tamoxifen, all the hormonal conditions differing from the control resulted in a decrease in the absolute volume of the epithelium (Fig. 2F). E_2 caused an initial reduction (day 1), an increase at day 2 and then a progressive reduction up to day

seven in the luminal compartment. A small reduction was noted in the luminal compartment, irrespective of the treatment (Fig. 2G). Tamoxifen caused little increase in the absolute volume of the stromal compartment, most remarkably on the SMC, which was sustained for the 7 days of the experiment (Figs. 2H-J). When applied to the castrated animals, tamoxifen resulted in the maintenance of the absolute volume of the stroma, in contrast to the reduction observed by androgen deprivation, E₂ administration (Figs. 2H-J).

Immunocytochemistry has localized the expression of AR in the prostatic epithelial cell nuclei and in part of the stromal cells in control rats (Figs. 3A and 3B). E₂ treatment eliminates the immunostaining, except for a slight reaction in a dot like structure found in the epithelial cell nuclei (Fig. 3C), while tamoxifen had almost no effect (Fig. 3D). AR immuno-reactivity was greatly reduced in castrated rats. Not every nucleus was labeled and the dot like pattern of staining was observed (Fig. 3E). This pattern was not changed by the administration of E₂ to castrated rats (Fig. 3F) or tamoxifen (Fig. 3G).

Immunocytochemistry for ER β resulted in a weak dot like labeling in the epithelial cell nuclei (Fig. 3H). E₂ administration resulted in a slightly stronger staining (Fig. 3I), while tamoxifen did not change the control pattern (Fig. 3J). Castration completely eliminates the staining of ER β (Fig. 3K). E₂ administration to castrated rats resulted in the maintenance of the control pattern (Fig. 3L). Tamoxifen administration to castrated rats did not modify the lack of staining observed for castration alone (Fig. 3M).

Table II summarizes the immunocytochemistry results for AR and ER β

Tunnel labeling has demonstrated the occurrence of apoptosis in the rat ventral prostate. The occurrence of apoptosis was almost absent in the control rats (Fig. 4A), but marked in E₂ treated rats (Fig. 4B) and in castrated rats (Figs. 4C). The determination of the apoptotic indices demonstrated differential kinetics of cell death for each of the treatments (Fig. 5). Castration resulted in a peak of apoptosis at day 3. The E₂ treatment resulted in apoptotic cell death. This reached a plateau on day 2 and was maintained up to day 5. The apoptotic index observed at day 3 was similar to that obtained by castration. Tamoxifen alone had very little effect, showing no significant difference to control at day 3, but slightly greater at day seven ($p < 0.05$). E₂ administration to castrated rats resulted in an increase in apoptotic cell death, significantly greater than castration alone ($p < 0.05$), but the profile was

similar to that obtained for castration alone. Interestingly, tamoxifen administration to castrated rats diminished the peak observed on day 3 and caused an apparent delay in epithelial cell death, the apoptotic index reaching its maximum on day 7.

Immunocytochemistry to PCNA allowed for the observation that cell division ceases within the first two days of any treatment (Fig. 6). E₂ and tamoxifen was more effective in reducing the number of cell divisions in 24h than castration, and less efficient when administered to castrated rats.

Discussion

Estrogens are involved with prostate development and functioning. They have a general anti-androgen effect by negatively regulating the hypothalamo-pituitary-testis axis and thus reduce testosterone secretion by the testes [28]. However, the identification of both ER α and ER β in prostatic cells indicates that E₂ may have a direct effect on the prostate.

When administered neonatally, E₂ results in several defects in prostatic development, including an increased susceptibility to prostatitis [29] and to the occurrence of prostatic metaplasia [30]. Long-term treatment of adult rats with E₂ causes prostatic involution [31] and ultimately leads to epithelial squamous metaplasia [32].

Furthermore, there seems to be a dose dependent variation in the effects of E₂. Low doses have a stimulatory effect on the growth of epithelium and stroma, while high doses have an inhibitory effect [31,33]

In this work we tried to determine the early effects of E₂ treatment on adult rats and compare them with its action on castrated rats, as well as to test the effect of the partial estrogen blocker tamoxifen.

E₂ caused a general reduction in the weight of the testes, demonstrating its ability to reduce gonadotropin secretion. Furthermore, it also caused a reduction in the VP and CG that was comparable to the effect of castration, but not in the SV. When administered to castrated rats it did not affect the weight reduction of the VP and CG and reduced the weight loss of the SV caused by androgen deprivation.

E₂ caused a reduction in the absolute volume of the epithelium of the VP that was similar to the effect of castration and did not affect the reduction caused by castration after

7 days. However, the reduction in epithelial volume was progressive after 24hs, in a clear correlation with the increase in the number of apoptotic cells seen as early as 24hs after E_2 injection. This contrasted with the reduction in the epithelial volume caused by castration, that was more evident on days 5 and 7, in a clear association with the peak of apoptosis observed on day 3, and demonstrated that the kinetics of epithelial cell loss is different for each treatment. It is still not clear why the increase in apoptosis observed by the treatment of castrated rats with E_2 , resulted in no greater reduction in the epithelial volume and VP weight after 7days than that caused by either castration and or E_2 treatment alone. One possibility is that a further increase in the apoptotic index may take place on the fourth day after castration (not evaluated in the present work). However, a similar peak of apoptosis on the third day has been reported by other researchers [34, 35]. It may also be demonstrated that cells are larger in the castrated rats treated with E_2 , but it is not easily observed by a simple visual analysis of the micrographs.

Zhao [36] reported that the most obvious alteration in the ventral prostate was a reduction in the luminal volume, in contrast to the present results showing little variation in this compartment. However, the dosage employed by those authors was smaller and may have caused different responses of the gland.

E_2 also caused an increase in the relative volume of the stroma, with a major contribution by the smooth muscle cells. This increase was similar to that caused by castration. Furthermore, E_2 did not affect the alterations promoted by castration.

Tamoxifen (Tam) is a partial antagonist of estrogen. It was shown to inhibit the proliferative response of the epithelium to treatment with E_2 alone or in combination with 3 alpha-androstanediol, but showed a partially restrictive effect on the stroma [37].

In our experiments, Tam caused very little, if any, effect on the testis and prostatic weight. Tam did not affect the absolute volume of the epithelium, but caused a slight reduction in the absolute volume of the lumen, probably by impairing the secretory activity. Accordingly, Tam did not increase the apoptotic index of the epithelial cells. On the other hand, Tam showed an inhibitory effect on the weight loss caused by castration. This loss was due to a smaller reduction in the prostatic stroma. Actually, Tam administration to castrated rats apparently caused a hypertrophy of the smooth muscle cells. The effect of E_2

on the Central Nervous System hampers the definition of its direct effects on the prostate. In an *in vitro* system, it was demonstrated that E₂ diminished prostatic growth, though not affecting cell proliferation [38].

The E₂ dose employed is apparently enough to inhibit gonadotropin release. However, it was clearly shown that E₂ has an immediate effect on the prostatic epithelium, causing cell proliferation arrest and inducing apoptosis. It seems to us that after 24 or 48 hours, the reducing levels of testosterone would have a more pronounced effect on the prostate. Then, 7 days after estrogen treatment, the scenario would be very similar to that obtained by castration, with respect to the final prostatic weight and absolute volume of the different tissue compartments.

After 7 days of treatment, castration and E₂ had similar effects on reducing the level of AR in the epithelium. However, while castration eliminates the staining for AR in the stroma, E₂ only reduced its expression. Similar results were found for the ER β , which was diminished in the epithelium by E₂ treatment, and completely eliminated by castration. These differences in steroid hormone receptors expression may represent the basis for the later effects of castration and E₂ treatment on the prostate.

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Figure legends

Figure 1. H&E stained sections of the rat ventral prostate after seven days under different experimental conditions. **Fig. 1A.** The control prostate shows its cylindrical epithelial cells with a basal nucleus and a prominent nucleolus. The stroma is scarce, with few elongated SMC (*) beneath the epithelium. **Fig. 1B.** E₂ treatment resulted in a reduction of the apical cytoplasm and an increase of the stroma. SMC occupies a greater area of the stroma and seems hyperplastic. **Fig. 1C.** Tamoxifen treatment showed little effect on the epithelial cells, though they seemed slightly shorter. The stroma shows a thickened layer of SMC. **Fig. 1D.** Castration resulted in a shortening of the epithelial cells and a thickening of the stroma, with a greater contribution by SMC (*), blood vessels and other cell types. **Fig. 1E.** E₂ administration to castrated rats did not change the basic aspects observed in castrated animals. **Fig. 1F.** Tamoxifen administration to castrated rats also seemed to preserve the major aspects observed after castration. However, the SMC seemed hypertrophic (**Fig. 1G**). Bar = 25 µm (A-F), 10 µm (G)

Figure 2. Stereological analysis of the rat ventral prostate compartments. Figs. 2A-E) correspond to the relative volume (%) of each compartment. Figs. 2F-J) are the absolute volume (mL) of each compartment. Ct = control; CtE₂ = control rats treated with E₂; CtTam = control rats treated with Tam; Cast = castrated rats; CastE₂ = castrated rats treated with E₂; CastTam = castrated rats treated with Tam. 1-7 represent the days after the

beginning of the treatment. The gray bar corresponds to the variation around the mean obtained in each case for the control rats.

Figure 3. Immunocytochemical identification of AR (Figs. 3A-G) and ER β (Figs. 3H-M) in the rat ventral prostate after seven days under different experimental conditions. **Figs. 3A and B.** The AR was located in the nuclei of both epithelial and stromal cells (arrow). **Figs. 3C.** E₂ treatment eliminated most of the staining, which remained as a dot like structure inside the epithelial cell nuclei. The staining in the stroma was eliminated. **Figs. 3D.** Tam did not affect AR staining. **Figs. 3E.** Castration resulted in an interrupted pattern with part of the cells showing weak reactivity. This pattern was neither changed by E₂ (**Fig. 3F**) nor by Tam (**Fig. 3G**). **Figs. 3H.** ER β staining in control rats appeared as a nuclear dot like structure. **Figs. 3I.** Treatment with E₂ reduced the ER β immunoreactivity, while Tam (**Figs. 3J**) showed no effect. **Figs. 3K.** Castration eliminated ER β staining. **Figs. 3L.** E₂ administration to castrated rats resulted in the maintenance of ER β staining in the epithelial cells, while Tam (**Figs. 3M**) did not change the aspect of the castrated animals. Bar = 25 μ m (A, C-H, J-M), 10 μ m (B)

Figure 4. Detection of DNA fragmentation in the rat ventral prostate. Control rats (**Fig. 4A**) showed no reaction, while the E₂ treatment (**Fig. 4B**) and castration (**Fig. 4C**) resulted in DNA fragmentation (arrows). Representative micrographs obtained on day three. Bar = 50 μ m (A-C)

Figure 5. Apoptotic indices obtained for the prostatic epithelial cells under different experimental conditions, after different periods of treatment. Castration resulted in a peak of apoptotic cell death on day 3. E₂ caused the premature death of epithelial cells and showed a sustained effect. Tam showed a minimum effect after 7 days. E₂ administration to castrated rats resulted in an increased incidence of apoptosis, although preserving the peak on the day 3. On the other hand, Tam caused a delay in the apoptotic death of epithelial cells, diminishing the number of dead cells on day 3 and reaching its maximum figure on day 7. Ct = control; CtE₂ = control rats treated with E₂; CtTam = control rats treated with Tam; Cast = castrated rats; CastE₂ = castrated rats treated with E₂; CastTam = castrated rats treated with Tam.

Figure 6. Immunocytochemical localization of PCNA in prostatic epithelial cells under different experimental conditions after different periods of treatment. **Figs. 6A-C** correspond to the identification of proliferating cells in the control on day 1 of the treatment with E₂ and after castration, respectively. **Fig. 6D** shows the number of PCNA-positive epithelial cells in control rats and after different periods under different experimental situations. Ct = control; CtE₂ = control rats treated with E₂; CtTam = control rats treated with Tam; Cast = castrated rats; CastE₂ = castrated rats treated with E₂; CastTam = castrated rats treated with Tam. Bar = 25 µm (A-C)

Tables

Table I – Effect of different hormonal conditions on the body weight, testis and accessory glands in the male rats 7 days postdosing. Figures correspond to the mean $\pm$ standard error expressed in grams (n = 3).

Group	Body	Testis	VP	SV	CG
Control	337.0 $\pm$ 11.0	3.67 $\pm$ 0.09	0.585 $\pm$ 0.003	0.598 $\pm$ 0.035	0.370 $\pm$ 0.014
Control +E₂	308.3 $\pm$ 2.33 ^{NS}	2.72 $\pm$ 0.05 ^{**}	0.293 $\pm$ 0.014 ^{**}	0.403 $\pm$ 0.026 ^{**}	0.262 $\pm$ 0.010 ^{**}
Tamoxifen	306.3 $\pm$ 22.3 ^{NS}	3.66 $\pm$ 0.09 ^{NS}	0.511 $\pm$ 0.048 ^{NS}	0.527 $\pm$ 0.031 ^{NS}	0.341 $\pm$ 0.023 ^{NS}
Castrated	292.6 $\pm$ 5.24 ^{NS}	-	0.277 $\pm$ 0.007 ^{**}	0.330 $\pm$ 0.013 ^{**}	0.237 $\pm$ 0.001 [*]
Castrated + E₂	291.6 $\pm$ 8.33 ^{NS}	-	0.281 $\pm$ 0.006 ^{**}	0.397 $\pm$ 0.026 ^{**}	0.252 $\pm$ 0.008 [*]
Castrated + Tam	295.0 $\pm$ 6.39 ^{NS}	-	0.306 $\pm$ 0.004 ^{**}	0.396 $\pm$ 0.011 ^{**}	0.296 $\pm$ 0.023 [*]

NS = not significant (p>0.05); * = p<0.01; ** = p<0.05, as compared to the control.

Table II – Arbitrary grading of immunocytochemical staining for AR and ER β in the rat ventral prostate under the different treatments, considering the epithelial and stromal compartments.

		Control	E ₂	Tam	Castration	Castration + E ₂	Castration + Tam
AR	Epithelium	+++	+	+++	+	+	+
	Stroma	+++	+	+++	-	-	-
ER β	Epithelium	++	+	++	-	+	+
	Stroma	+	-	+	-	-	-

(+++) Intense staining; **(++)** Moderate staining; **(+)** Weak staining; **(-)** Negative

Figure 1

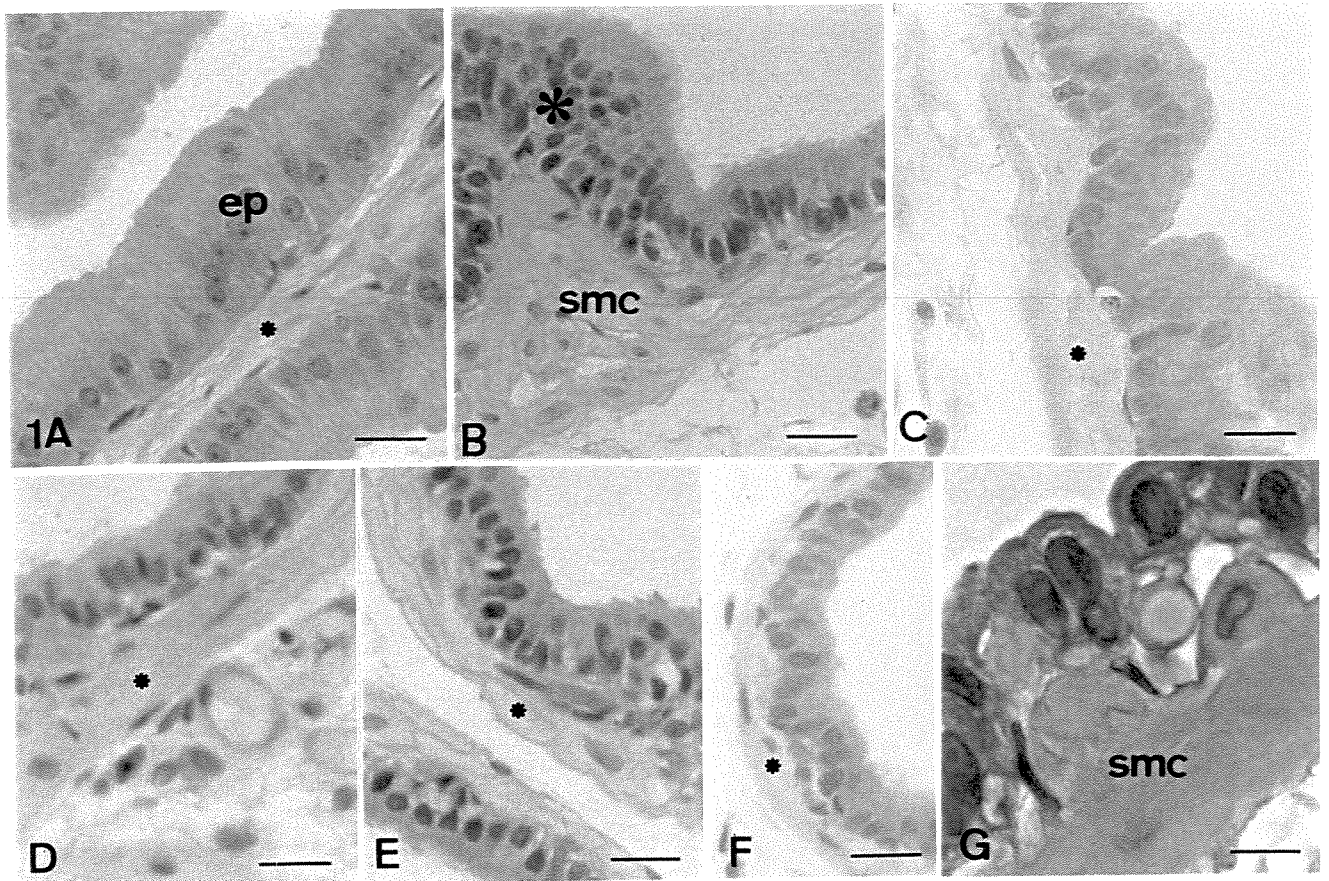


Figure 2

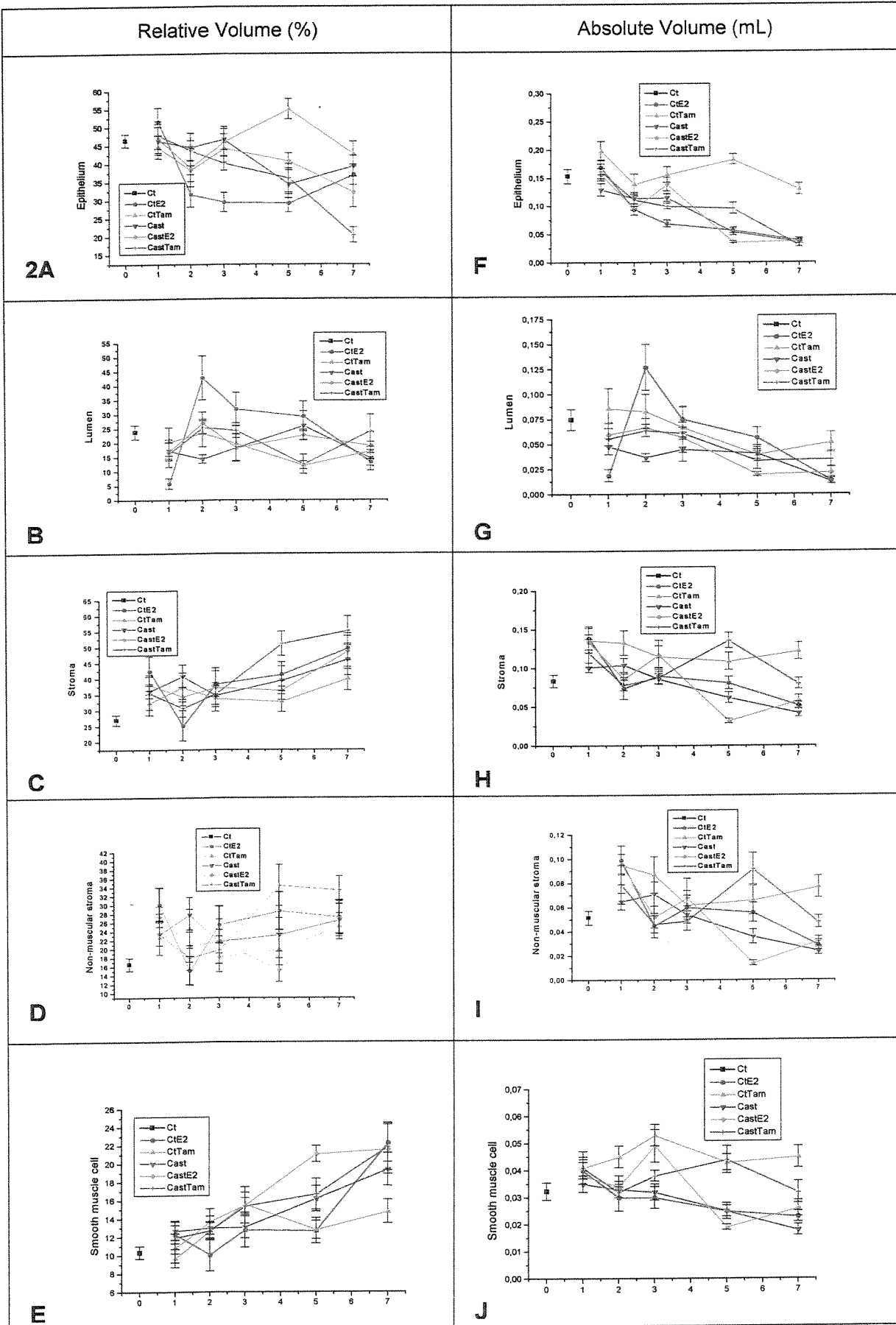
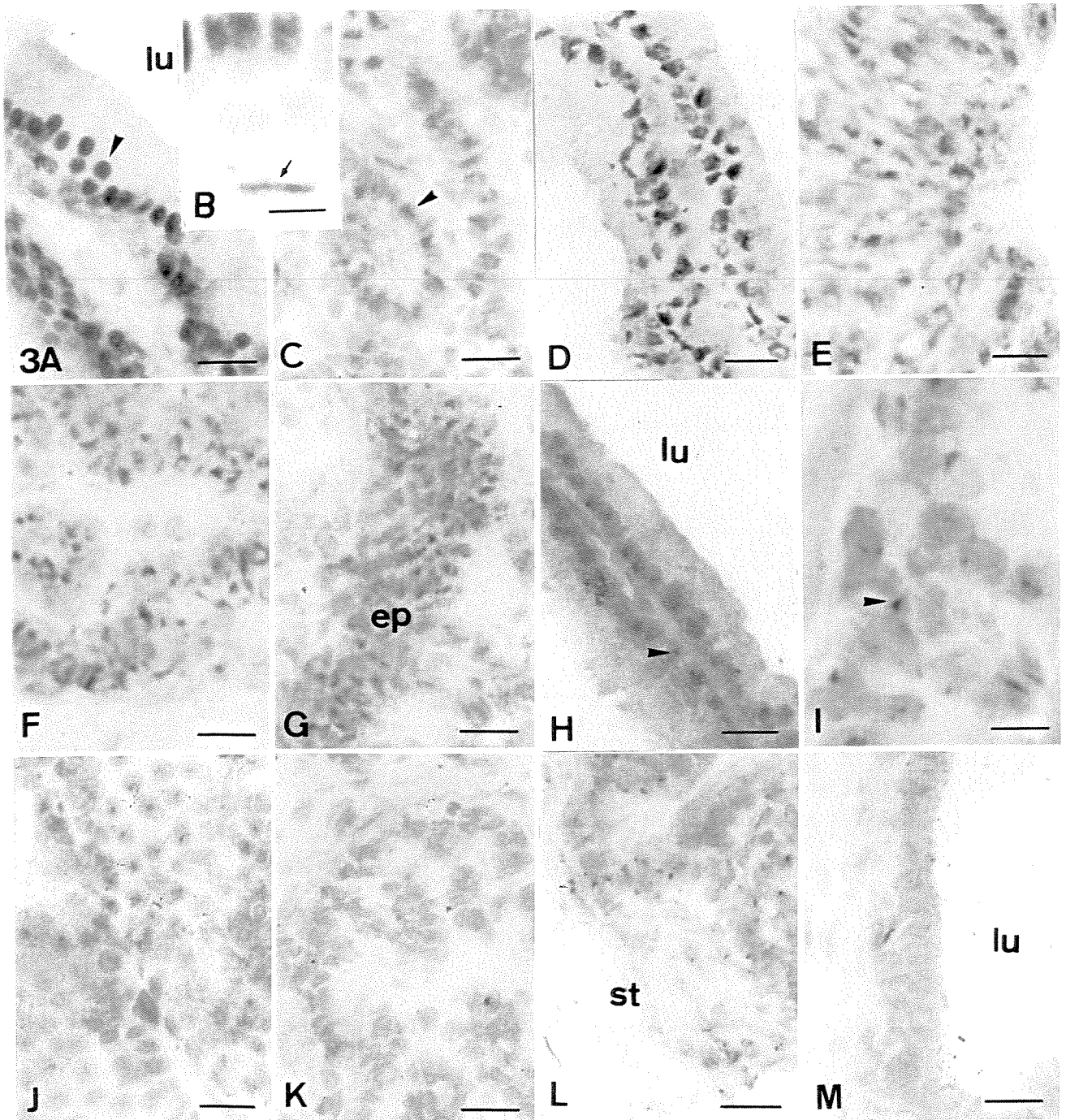
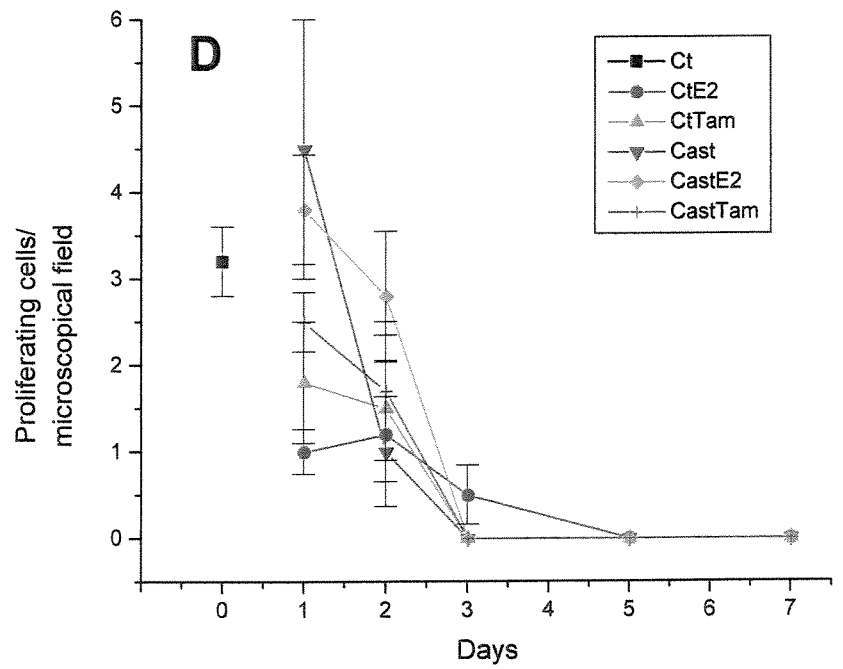
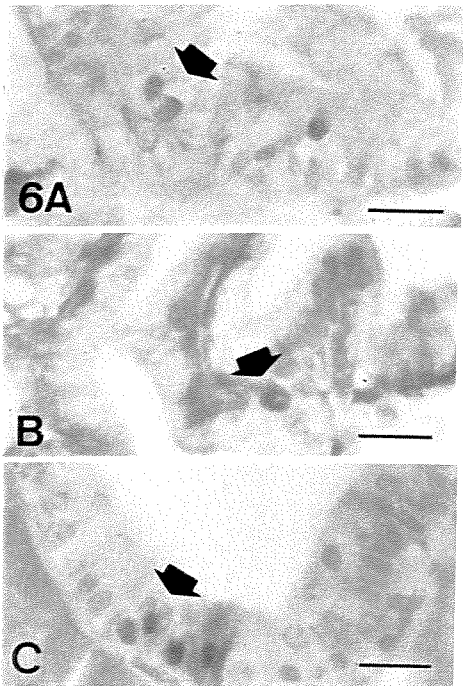
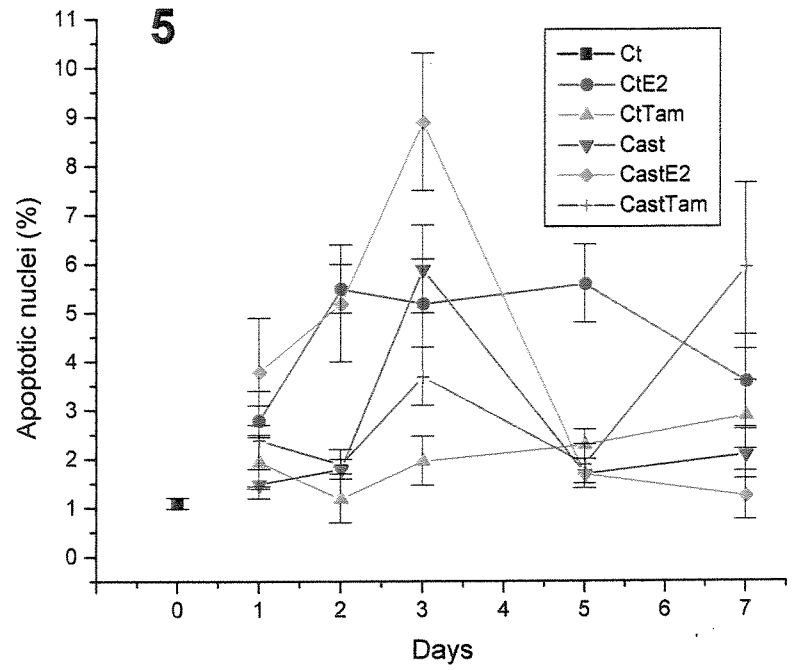
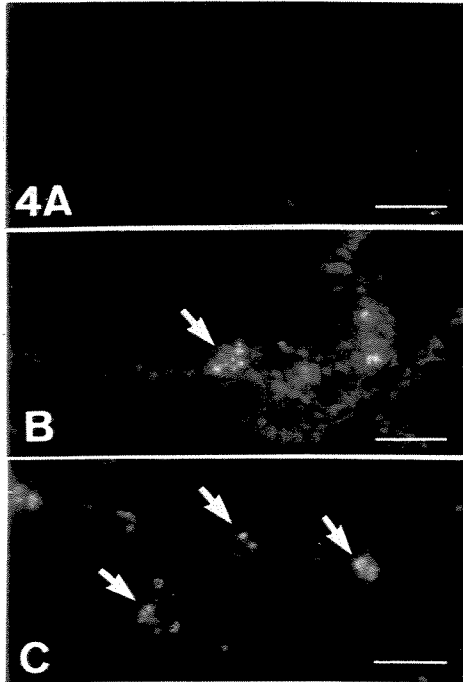


Figure 3



Figures 4, 5 and 6



Conclusões Gerais

- A alteração do equilíbrio hormonal afeta a próstata diretamente, a castração e o tratamento com estrógeno levam a uma perda de peso e ao aumento relativo do compartimento estromal. Embora as duas situações possuam efeitos finais similares as cinéticas teciduais são diferentes.
- O estrógeno na dose utilizada leva à morte precoce das células epiteliais e induz o incremento do índice de apoptose sendo este mantido até o dia 5 após o tratamento. Já a castração apresenta um pico no dia 3 que cai rapidamente nos dias posteriores.
- Um dos efeitos das altas doses de estrógeno é a atrofia prostática em ratos adultos. Isto está estreitamente ligado aos efeitos imediatos de aumento do índice apoptótico e à queda no índice de proliferação celular.
- Na concentração utilizada, 0.0367 mM, o E₂ parece ter um efeito primário direto sobre a próstata e um efeito secundário indireto, possivelmente reduzindo os níveis de T circulante via eixo hipotálamo-hipófise-gônadal
- O tamoxifeno retarda alguns dos efeitos da castração sobre a próstata no rato adulto, sendo a perda de peso relativamente menos dramática possivelmente ligada à diminuição do índice apoptótico.
- A próstata é uma glândula sensível à alteração da sua homeostase hormonal, modificações feitas no equilíbrio destes hormônios leva a uma cascata de reações inicialmente moleculares e em última instância morfológicas.

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