

ANA PAULA DA SILVA PEREZ

**"EFEITOS DA EXPOSIÇÃO À BAIXA DOSE DE
ETINILESTRADIOL DURANTE AS FASES PRÉ-NATAL
E PUBERAL SOBRE A PRÓSTATA MASCULINA E
FEMININA DE GERBILOS SENIS"**

**"EFFECTS OF EXPOSURE TO LOW DOSE OF
ETHINYLESTRADIOL DURING THE PRENATAL AND
PUBERAL PHASES ON MALE AND FEMALE
PROSTATE OF SENILE GERBILS"**

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UNIVERSIDADE ESTADUAL DE CAMPINAS
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ANA PAULA DA SILVA PEREZ

**"Efeitos da exposição à baixa dose de etinilestradiol durante
as fases pré-natal e puberal sobre a próstata masculina e
feminina de gerbilos senis"**

Orientador: Dr. Sebastião Roberto Taboga

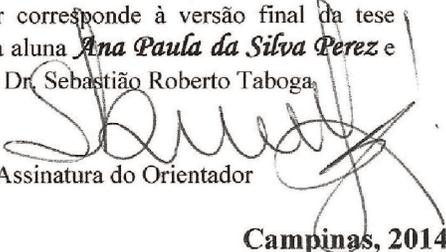
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**"Effects of exposure to low dose of ethinylestradiol during
the prenatal and puberal phases on male and female
prostate of senile gerbils"**

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

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Este exemplar corresponde à versão final da tese defendida pela aluna *Ana Paula da Silva Perez* e orientada pelo Dr. Sebastião Roberto Taboga.


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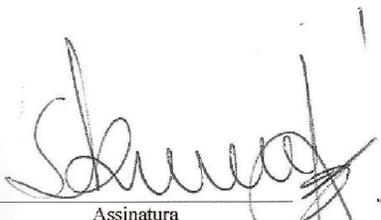
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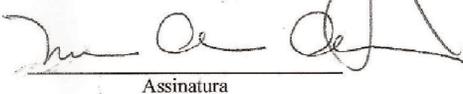
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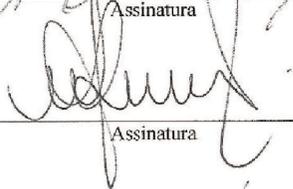
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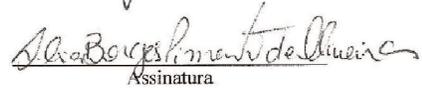
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Resumo

Os disruptores endócrinos (DE) são agentes exógenos que interferem no funcionamento do sistema endócrino. O 17 α -etinilestradiol (EE), um importante componente dos contraceptivos orais é um exemplo de DE. Alguns estudos com roedores machos e fêmeas relataram que a exposição aos DE durante o período pré-natal foi capaz de eliciar proliferações patológicas no sistema reprodutor, incluindo a próstata do animal adulto. Entretanto, pouco se sabe sobre a real ação do estrógeno nas próstatas de machos e fêmeas, principalmente quando se leva em consideração o comportamento de ambas às próstatas durante o desequilíbrio dos níveis de hormônios esteroides que ocorre na puberdade e durante o envelhecimento dessas glândulas. Assim o presente estudo, teve como objetivos avaliar por análises morfológicas, sorológicas e imunohistoquímica quais foram os efeitos da exposição à baixa dose de EE durante os períodos pré-natal e puberal sobre a próstata ventral masculina e na próstata feminina de gerbilo senil. Deste modo, nós dividimos os grupos experimentais de acordo com o período de exposição ao EE (15 μ g/kg/dia). EE/PRÉ durante o período pré-natal, EE/PUB durante a puberdade e o EE/PRÉ-PUB durante o período pré-natal e puberdade. A exposição à estrógenos sintéticos durante o desenvolvimento afeta o eixo hipotálamo-pituitária gonadal, alterando a produção de hormônios esteroides. Os resultados revelaram que a exposição ao EE durante o desenvolvimento prostático alterou os níveis de hormônios esteroides, diminuindo os níveis de testosterona nos machos senis dos grupos EE/PRÉ e EE-PRÉ/PUB e aumento, nas fêmeas senis do grupo EE/PRÉ. Os níveis de estradiol aumentaram nas fêmeas do grupo EE/PRÉ-PUB. A interação epitélio-estroma também foi afetada pela exposição ao EE durante o desenvolvimento, dado evidenciado pela diminuição da imunorreatividade de α -actina de músculo liso em regiões com presença de lesões invasivas, principalmente na próstata ventral de macho senil. A frequência de células positivas de p63 diminuiu na próstata ventral masculina do grupo EE/PRÉ, provavelmente a camada basal diminuiu em locais onde se observa foco com NIP e, aumentou na próstata feminina do grupo EE/PUB. Entretanto, esses dados mostraram que a próstata ventral de machos senil foi mais sensível aos efeitos da exposição ao EE, comparado à próstata de fêmeas senis. A senescência é caracterizada pela queda dos hormônios sexuais, e nessa fase aumenta as doenças prostáticas em machos e fêmeas. A exposição ao EE durante fases críticas como o pré-natal e a

puberdade acentuaram as alterações na estrutura glandular da próstata e aumentaram o desenvolvimento de lesões prostáticas na senescência. Assim como o período pré-natal, o puberal também foi considerado uma fase crítica na exposição ao EE sobre a próstata de gerbilos machos e fêmeas senis. O consumo de EE durante a gestação ou na puberdade altera significativamente a saúde prostática masculina e feminina durante o envelhecimento, e o gerbilo foi considerado um bom modelo para esse estudo.

Abstract

Endocrine disruptor (ED) are exogenous agents that interfere in the endocrine system function. 17α -ethinylestradiol, an important component of oral contraceptives is an example of ED. Studies with male and female rodents have reported that exposure to ED during the prenatal period is able to elicit aberrant pathological proliferations in the reproductive system, inclusive in the prostate of the adult animal. However, the amount known about the actual action of estrogen on male and female prostate, mainly as is the behavior of both prostates during imbalance between androgen and estrogen that occurs in pubertal period and during senescence of these glands. Thus the present study aimed to evaluate by morphological, serological, histopathological and immunohistochemical methods, as the exposure to low dose of ethinylestradiol during the prenatal and pubertal periods acts on ventral male prostate and female prostate of senile gerbil. Thus, we divided the experimental groups according to the period of exposure to EE (15 μ g/kg/day). EE/PRE during the prenatal period, EE/PUB during puberty and EE/PRE-PUB during the prenatal period and puberty. Exposure to synthetic estrogens during development affects the hypothalamic-pituitary-gonadal axis this alters the production of steroid hormones. The results showed that exposure to EE during developing prostate changed steroid hormones levels, decreasing testosterone levels in senile male of EE/PRE and EE-PRE/PUB groups and increased in senile female of EE/PRE group. The estradiol levels enhanced in females of EE/PRE-PUB group. In addition, EE exposure during the prenatal and pubertal periods altered the immunoreactivity of AR, ER α and ER β , as the function of these receptors are critical for prostate development, changes these signaling pathways contributed to increase of development of lesion and inflammation prostate in males and females during senescence. The stromal-epithelium interaction was also affected by exposure to EE during developing, was observed by decreased immunoreactivity of smooth muscle α -actin in regions where noted invasive lesions, mainly in the ventral male prostate of senile gerbil. Frequencies positive cells of p63 decreased in the ventral male prostate of EE/PRE group, the basal layer decreases in locals with NIP focus, and these frequencies increased the female prostate EE/PUB group. However, these data showed that the ventral male prostate of senile gerbil was more sensible to effects of exposure to EE compared to female prostate. Senescence is characterized by reducing of sexual hormones, in this phase increases the prostatic diseases in males and females. Exposure to EE during critical

periods as prenatal and puberty accentuated the changes in prostate glandular structure and increased the developing prostatic lesions in senescence. As prenatal period, pubertal was also considered a critical phase during the exposure to EE on male and female prostate of senile gerbil. The use of EE during gestational or puberty phases significantly alters male and female prostate health during aging, and the gerbil was considered a good model for this study.

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Pai,

*“A longa distância apenas serve para unir o nosso amor.
A saudade serve para me dar
a absoluta certeza de que ficaremos para sempre unidos...”*

*E nesse momento de saudade,
quando penso em você,
quando tudo está machucando o meu coração
e acho que não tenho mais forças para continuar;
eis que surge tua doce presença,
com o esplendor de um anjo;
e me envolvendo como uma suave brisa aconchegante...*

Tudo isso acontece porque amo e penso em você...”

William Shakespeare

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“Não há solidão mais triste do que a do homem sem amizades. A falta de amigos faz com que o mundo pareça um deserto”.

Francis Bacon

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“Não há no mundo exagero mais belo que a gratidão.”
Jean de la Bruyere

Introdução

Morfogênese prostática e hormônios esteróides

A glândula prostática desenvolve-se a partir do seio urogenital (SUG), sendo este um tubo endodérmico derivado do intestino e que termina na cloaca (Cunha et al., 2004). O SUG é constituído por uma camada epitelial (SUE) derivada da endoderme circundada por uma camada mesenquimal (SUM) derivada da mesoderme (Marker et al., 2003; Cunha et al., 2004). Esta estrutura é encontrada entre a base da bexiga e a uretra e surge em machos e fêmeas após 13 dias de concepção nos camundongos e ratos, e após 7 semanas de gestação em humanos. Em machos e fêmeas o SUG é morfológicamente indistinguível até o 17^o-18^o dia de gestação em ratos e camundongos, e até a 10^a-12^a semana em humanos. Depois desse período começa a morfogênese prostática que é iniciada e dependente da ação de andrógenos (Marker et al., 2003).

Em machos, o evento inicial da morfogênese prostática é o crescimento de brotos epiteliais sólidos do epitélio do seio urogenital em direção ao mesênquima circundante do seio urogenital (Hayward et al., 1996). Em roedores, a maioria dos ductos prostáticos não é ramificada ao nascimento. Entretanto, durante o período neonatal, na maioria dos roedores, conforme os cordões prostáticos crescem, eles invadem o SUM e começam a bifurcar-se em ramos laterais, originando três lobos prostáticos distintos: o lobo ventral, o lobo dorsolateral e a glândula coaguladora ou lobo anterior (Marker et al., 2003). Simultaneamente ao processo de morfogênese de ramificação ductal, ocorre a canalização ductal (formação do lúmen) e a citodiferenciação epitelial e estromal (Wang et al., 2001).

A morfogênese prostática é dependente de hormônios esteróides fatores parácrinos. Os andrógenos são necessários para iniciar o desenvolvimento prostático, para continuar o seu crescimento embrionário e neonatal e, posteriormente, para iniciar a atividade secretória prostática na puberdade (Isaacs et al., 1994). No entanto, a ação androgênica não é exercida diretamente sobre as células epiteliais. Sob a influência de andrógenos, as células mesenquimais produzem e secretam fatores parácrinos específicos que ditam o crescimento e diferenciação da glândula prostática (Cunha et al., 2004) (Fig. 1).

Interações epitélio-mesenquimais desempenham um papel chave no direcionamento do crescimento e do desenvolvimento da próstata, sendo que a sinalização parácrina do mesênquima para o epitélio é essencial para a embriogênese prostática. Desse modo, a ação de andrógenos sobre as células mesenquimais resulta em fatores parácrinos específicos que atuam sobre as células epiteliais induzindo a sua proliferação (Thomson et al., 2002). Por outro lado, também existe uma sinalização parácrina do epitélio para o mesênquima. Esta sinalização regula a diferenciação do mesênquima que circunda os brotos em formação em um estroma composto por células musculares lisas e fibroblastos (Hayward et al., 1996). Assim, durante a morfogênese prostática, os receptores de andrógenos (AR) são necessários no mesênquima e não no epitélio, sendo que sua expressão precede o surgimento dos brotos prostáticos. Nas células epiteliais, a função do AR se restringe à regulação de proteínas secretórias e, talvez, à diferenciação celular (Donjacour e Cunha, 1993) (Fig. 1).

Dentre os fatores parácrinos que atuam sobre a morfogênese prostática, pode-se citar os fatores de transcrição da família homeobox NKx3.1, Hoxa-13, Hoxb-13 e Hoxd-13, os fatores da família FGF (fatores de crescimento de fibroblasto) (FGF-7 e -10) (Huang et al., 2004), a glicoproteína Sonic hedgehog (Shh), o receptor para Shh, *patched* (*ptc*), os fatores de transcrição da família Gli e BMP-4 (Pu et al., 2004).

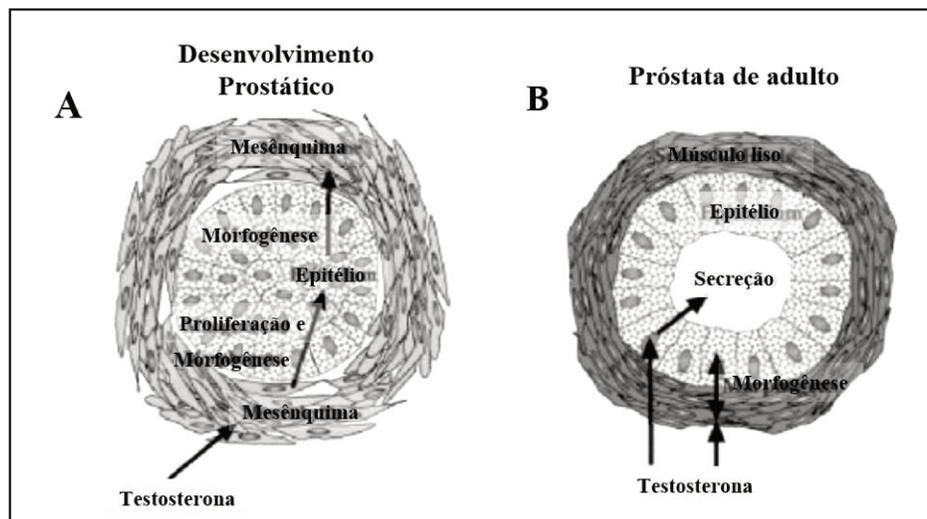


Figura 1. Representação da interação entre o epitélio/estroma da próstata durante o desenvolvimento e a fase adulta. **A.** Durante o desenvolvimento prostático, baixos níveis de andrógenos atuam nos receptores de andrógenos (AR) mesenquimais, estimulando a proliferação e diferenciação do epitélio prostático. Simultaneamente, o epitélio induz a diferenciação do mesênquima em músculo liso. **B.** Altos níveis de andrógenos circulantes atuam nos AR localizados no músculo liso, mantendo a morfologia e a função secretora da glândula prostática na fase adulta. (Adaptado de Cunha et al., 2002)

Em fetos femininos de ratos e humanos, a ausência de testosterona induz o SUG a originar a porção inferior da vagina e a uretra (Shapiro et al., 2004). O SUM passa a circundar o epitélio uretral, sendo subdividido em três zonas: o mesênquima periuretral, a zona mesenquimal que sofre diferenciação em músculo liso e a zona que contém o mesênquima condensado ventral (VMP). Esta última estrutura apresenta localização análoga à próstata ventral masculina e representa o mesênquima do SUG sem a invasão do SUE (Thomson et al., 2002; Staack et al., 2003).

A diferenciação de parte do mesênquima do SUG em músculo liso é crucial para a morfogênese prostática em machos e fêmeas, pois é este evento que regula a sinalização parácrina entre o epitélio e o mesênquima (Staack et al., 2003; Thomson et al., 2002).

Durante a diferenciação do mesênquima, os andrógenos regulam a espessura e a continuidade da camada de músculo liso formada, de modo que a ausência de andrógenos em embriões femininos ocasiona a formação de uma camada muscular espessa e contínua. Esta camada separa o VMP do epitélio uretral, impedindo que os brotos epiteliais prostáticos que estão emergindo da uretra entrem em contato direto com o VMP. Desse modo, a espessa camada de músculo liso impede a interação do VMP com os brotos prostáticos em formação, bloqueando a comunicação parácrina entre o epitélio e o mesênquima (Thomson et al., 2002). Em machos, na presença de andrógenos, a formação do músculo liso é inibida ou atrasada, e os brotos prostáticos emergem da uretra e podem penetrar no VMP. Assim, a interação parácrina epitélio-mesênquima é estabelecida, ocasionando a ramificação e expansão da próstata ventral.

Formação da próstata em fêmeas

A próstata é a maior glândula acessória do sistema reprodutor masculino (Ross e Pawlina, 2012). É uma glândula exócrina tubuloalveolar revestida por tecido epitelial e circundada por um

estroma fibromuscular (Lee, 1997; Ross e Pawlina, 2012). As células epiteliais secretam um fluido claro ligeiramente alcalino (pH 7,29), no qual contribui para a formação do líquido seminal (Ross e Pawlina, 2012). Na maioria dos roedores machos, a próstata se divide em três pares de lobos: ventral, lateral e dorsal, de acordo com a localização ao redor da uretra prostática, e um par de glândulas coaguladoras ou próstata anterior, localizadas na face côncava das vesículas seminais (Aumuller e Seitz, 1990; Rochel et al., 2007). Segundo Campos e colaboradores (2006), em gerbilos machos adultos (Campos et al., 2006), a próstata apresenta alvéolos revestidos por epitélio prismático simples e altamente secretório imersos em um estroma com tecido conjuntivo vascularizado, poucas fibras colágenas e elásticas e células musculares lisas bem compactadas dispostas ao redor dos alvéolos, características muito semelhantes às encontradas na próstata masculina humana.

Entretanto vários estudos mostram que a próstata não é uma glândula exclusiva do sistema reprodutor masculino, sendo encontrada em fêmeas de diversos mamíferos, incluindo humanos (Zaviačič, 1999; Zaviačič et al., 2000a) e roedores (Mahoney e Witschi, 1947; Gross e Didio, 1987; Flamini et al., 2002; Custódio et al., 2004; Santos et al., 2006).

A formação de brotos prostáticos é um processo constitutivo de machos e fêmeas, porém a ramificação e expansão desses brotos são reguladas por andrógenos. Em fêmeas, o isolamento entre o VMP e o epitélio uretral, que é provocado pela camada de músculo liso, impede a formação de uma glândula prostática desenvolvida e lobulada. Desse modo, o reduzido tecido prostático observado em fêmeas de várias espécies é originário do SUG que não sofreu estímulo androgênico. No entanto, embora a próstata feminina adulta seja menor que a masculina (cerca de 15% a 25% do tamanho da próstata ventral masculina), ela apresenta um epitélio secretor diferenciado e funcional (Zaviačič et al., 2000a; Santos et al., 2003; Custódio et al., 2004). Como a próstata de fêmeas cresce e desenvolve-se em um ambiente com baixos níveis de andrógenos (apenas 5% do total de precursores androgênicos produzidos no organismo masculino), acredita-se que outros fatores, além desses hormônios, possam atuar no desenvolvimento e manutenção da função dessa glândula em adultos (Timms et al., 1999).

Em algumas espécies, a frequência de desenvolvimento de uma glândula prostática funcional em fêmeas é muito elevada. Na espécie humana, cerca de 90% das mulheres desenvolvem um tecido prostático maduro e ativo nos processos de secreção (Zaviačič et al.,

2000b) (Fig. 2). Em roedores, a ocorrência de próstata em fêmeas é variável de espécie para espécie (Mahoney e Witschi, 1947). Em ratos Wistar a frequência de próstata feminina pode variar de 1% a 98% de acordo com a cepa utilizada (Manhoney, 1940). Em gerbilos, o desenvolvimento de próstata é muito freqüente, se assemelhando ao que ocorre na espécie humana (Santos et al., 2003; Santos e Taboga, 2006).

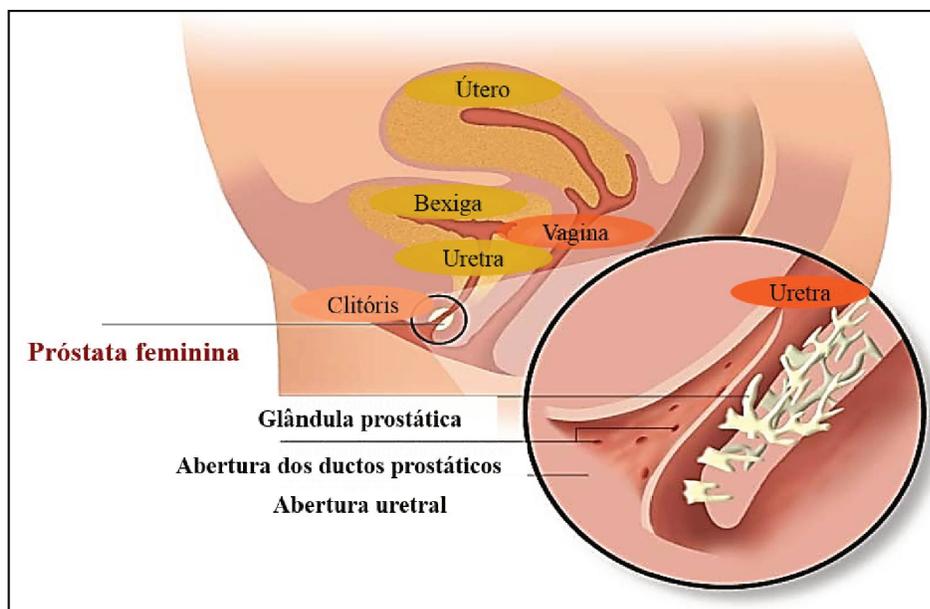


Figura 2. Representação anatômica da próstata feminina de humanos, mostrando a localização parauretral da glândula e a presença de ductos prostáticos (Adaptado de Moalem, 2009).

Diversos trabalhos têm relatado a ocorrência de uma glândula prostática desenvolvida em várias espécies de fêmeas de roedores, como *Praomys natalensis* (Gross e Didio, 1987), *Rattus norvegicus* (Shehata, 1980), *Lagostomus maximus maximus* (Flamini et al., 2002), *Meriones libycus* (Shehata, 1974; 1975) e *Meriones unguiculatus* (Santos et al., 2003; Custódio et al., 2004). Estes trabalhos demonstraram a existência de grande semelhança entre a próstata feminina de roedores e humanos (Zaviačić, 1999). Além disso, as características morfológicas apresentadas por estas pesquisas indicam que a próstata feminina dessas espécies é equivalente à próstata ventral masculina, e que suas células epiteliais exibem um fenótipo de células funcionalmente ativas.

A próstata de fêmeas adultas de gerbilo da Mongólia (*Meriones unguiculatus*), modelo experimental utilizado em nosso grupo de pesquisa (Fig. 3), apresenta grande similaridade morfofuncional com a próstata feminina humana, apresentando localização parauretral (Fig. 4) (Santos et al., 2003; Santos e Taboga, 2006). Ela apresenta cerca de 15% a 25% do tamanho da próstata ventral de gerbilos machos, sendo constituída por um pequeno conjunto de ductos e alvéolos circundados por um estroma desenvolvido (Santos et al., 2003). A porção alveolar é revestida por células secretoras altamente sintéticas, que produzem abundante secreção rica em fosfatase ácida (Custódio et al., 2004) e que expressam o antígeno específico da próstata (PSA) (Santos et al., 2006).



Figura 3. Gerbilo da Mongólia (*Meriones unguiculatus*)

A ação de estrógenos na morfofisiologia prostática

O desenvolvimento prostático é dependente da ação hormonal, sendo mediada primariamente por andrógenos que ordenam o crescimento e a diferenciação dessa glândula. Entretanto, o desenvolvimento prostático é sensível a outras proteínas e hormônios esteroides, incluindo os estrógenos (Prins et al., 2006; McPherson et al, 2008).

Vários estudos com roedores alegam que os estrógenos desempenham um papel fisiológico no desenvolvimento prostático, particularmente na programação das células estromais prostáticas (Risbridger et al., 2001; Jarred et al., 2002). Em humanos o desenvolvimento da glândula ocorre por completo durante a vida fetal. Porém em roedores a próstata sofre uma extensão morfogênica seguida da diferenciação celular, após os primeiros 15 dias de vida. Desse

modo, os roedores tem sido um eficiente modelo para avaliar o papel dos estrógenos durante o desenvolvimento prostático (Hayashi et al., 1991; Prins et al., 2007).

Durante a gestação fetos machos são expostos no útero ao estrógeno endógeno maternal. Esses níveis de hormônio são capazes de iniciar proliferações patológicas aberrantes (Sugimura et al., 1986).

Em roedores observou-se que a exposição ao estrógeno sintético durante o período pré-natal promoveu distúrbios no crescimento prostático e o aumento às predisposições a lesões cancerosas na vida adulta (Timms et al., 2005; Perez et al., 2012). Um processo denominado “estrogenização do desenvolvimento” ou “imprint estrogênico” (Fig. 4) (Prins et al., 2008).

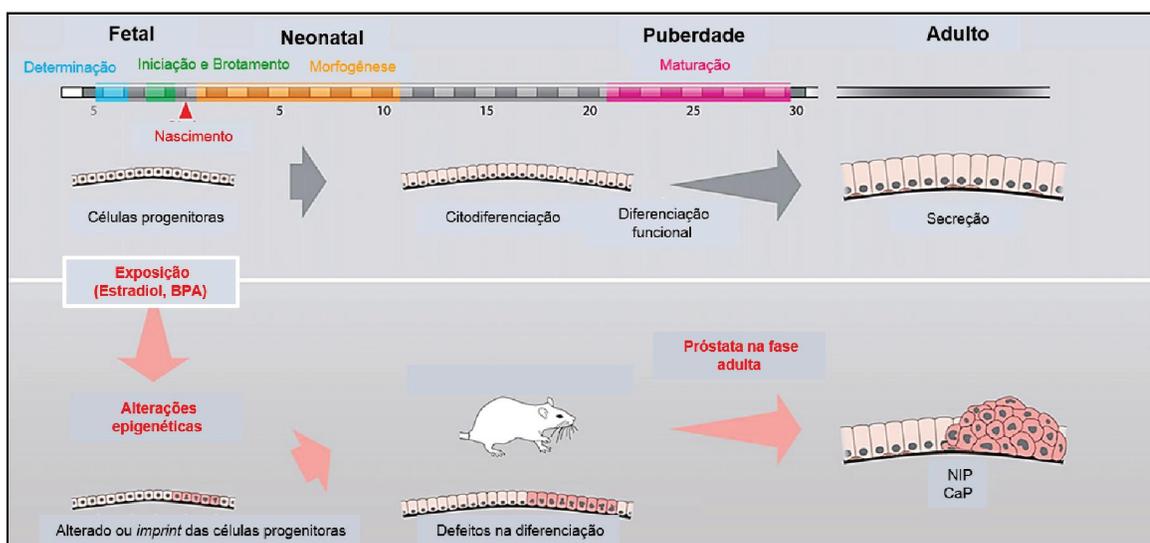


Figura 4. Esquema representa o modelo de mecanismo epigenético do “imprint estrogênico” da glândula prostática de ratos expostos ao estradiol ou ao bisfenol-A (BPA) durante o período pré-natal. A exposição aos estrógenos sintéticos durante o desenvolvimento prostático predispõe ao câncer de próstata na fase adulta (neoplasia intraepitelial prostática (NIP) e câncer de próstata (CaP) (Adaptado de Prins et al., 2007).

Doses altas de estrógenos durante a fase pré-natal e puberal causam distúrbios permanentes na próstata, que incluem redução do crescimento, defeitos na diferenciação das células epiteliais, alteração da função secretora e reduzida resposta a andrógenos na vida adulta (Risbridger et al., 2005). Inversamente, doses baixas de estrógenos têm sido associadas ao

aumento do tamanho e atividade secretória da glândula prostática no indivíduo adulto, e ao surgimento de doenças prostáticas em indivíduos senis (Omoto et al., 2005; Timms et al., 2005).

Durante o desenvolvimento prostático normal, o AR é o receptor dominante tanto no epitélio quanto no estroma (Cunha et al., 2004), enquanto que o ER α (receptor de estrógeno alfa) é expresso a baixos níveis nas células estromais próximas aos ductos prostáticos em expansão (McPherson e Ellem, 2008). Com a exposição a altas doses de estrógenos sintéticos, os níveis de ER α aumentam, fazendo com que a morfogênese prostática torne-se um processo dirigido por estrógenos. Esse evento causa interrupções na expressão coordenada de genes críticos para o desenvolvimento prostático, entre eles, Hox13, NKx3.1, Shh, FGF-10 e BMP-4, ocasionando uma supressão no brotamento e ramificação prostática (Huang et al., 2004). Por outro lado, estudos com camundongos demonstram que, a baixas doses, os estrógenos não só interagem com o ER α , mas também atuam como agonistas do AR, aumentando sua expressão e atividade, fato que ocasiona o maior desenvolvimento prostático (Gupta, 2000; Omoto et al., 2005).

Enquanto a ação dos receptores AR e ER α são fundamentais durante as fases iniciais do desenvolvimento prostático (Cunha et al., 2004; McPherson et al., 2008), a ação do ER β sobre a próstata é mais significativa na fase adulta, onde realiza o papel antiproliferativo (Weihua et al., 2002), ao contrário do ER α que tem ação proliferativa (McPherson et al., 2008)

Vários estudos envolvendo a exposição à estrógenos sintéticos durante o período pré-natal e neonatal de roedores foram desenvolvidos com a finalidade de avaliar o impacto que esses hormônios podem causar sobre os órgãos reprodutivos de machos e fêmeas de roedores (Markey et al., 2005; Prins et al., 2007; Perez et al., 2011). O foco de interesse desses experimentos advém do fato que nas últimas décadas milhões de mulheres têm exposto seus filhos a estrógenos sintéticos durante a gestação (Thayer et al., 2001; Schrager e Potter, 2004). Dentre esses compostos sintéticos que apresentam atividade estrogênica pode-se citar o bisfenol-A (BPA), o dietilestilbestrol (DES) e o 17 α -etinilestradiol (EE), os quais são denominados disruptores endócrinos (DE) (Casals-Casas e Desvergne, 2011, Frye et al., 2012).

Os DE são compostos exógenos com o potencial de alterar a regulação hormonal e consequentemente afetar a saúde e reprodução de humanos e animais (Diamanti-Kandarakis et al., 2009; Casal-Casas e Desvergne, 2011). Esses compostos podem interferir na produção, no

metabolismo e liberação dos hormônios esteroides (andrógenos e estrógenos) (De coster e Van Larebeke, 2012).

O EE é um disruptor endócrino estrogênico, que faz parte da composição dos contraceptivos orais (Smithells, 1981) (Fig. 5). Nos EUA e na Europa, é estimado que 2% a 5% das 60 milhões de mulheres que tomam contraceptivos orais engravidam acidentalmente e, desconhecendo a gravidez, continuam o uso dos mesmos até o terceiro ou quarto mês de gestação (Smithell, 1981). Esta alta incidência de fertilização ocorre devido a erros na administração da pílula (principalmente por esquecimento) ou, em poucos casos, a variações individuais na sensibilidade aos hormônios contraceptivos. Como a exposição a esse hormônio ocorre no período crítico para o desenvolvimento dos órgãos sexuais, várias anormalidades do desenvolvimento ocorrem neste período, sobretudo nos órgãos que apresentam morfogênese dependente de hormônios esteroides (Thayer et al., 2001; Timms et al., 2005; Perez et al., 2012).

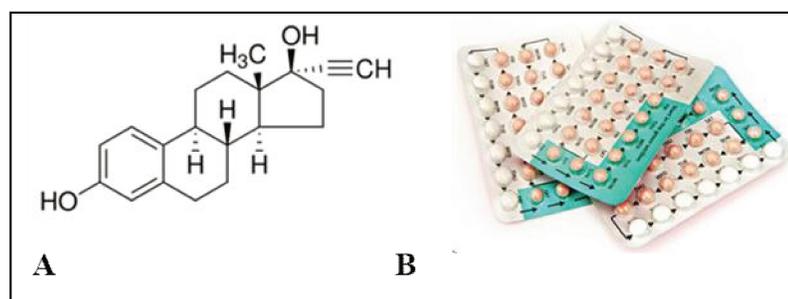


Figura 5. Fórmula estrutural do 17 α -etinilestradiol (EE) (C₂₀H₂₄O₂) (A), um estrógeno sintético componente dos contraceptivos orais (B). Adaptado de www.sigmaaldrich.com

Camundongos machos expostos a baixas doses de EE no período de desenvolvimento pré-natal apresentaram um crescimento prostático anormal na fase neonatal (Timms et al., 2005). Além disso, quando as próstatas desses animais que foram expostos ao EE no útero foram analisadas na vida adulta, pôde-se constatar um grande desenvolvimento glandular e uma alta expressão de AR (Gupta 2000; Thayer et al., 2001). Estes trabalhos demonstram que os efeitos que o EE causa sobre a próstata de camundongos machos recém-nascidos e adultos são equivalentes às alterações glandulares causadas pelo BPA (plástico policarbonato) e pelo DES (medicamento utilizado na década de 70 para evitar aborto).

A maioria dos trabalhos enfatizam os efeitos adversos da exposição precoce aos estrógenos sintéticos na próstata masculina (Garcia-Flórez et al., 2005; Timms et al., 2005; Perez et al., 2012), mas a ação deste DE no período pré-natal também contribui para o surgimento de lesões neoplásicas na próstata feminina de gerbilo durante a vida adulta (Perez et al., 2011). Portanto, é essencial que se avalie os efeitos que a exposição aos estrógenos sintéticos durante períodos críticos do desenvolvimento podem promover na morfogênese prostática, uma vez que tem sido crescentes os relatos de desordens prostáticas na mulher, tais como hiperplasia benigna e adenocarcinoma (Sloboda et al., 1998; Islam et al., 2001; Sharifi-Aghdas e Ghaderian, 2004; Kato et al., 2005).

Os fatores que promovem a atividade da próstata feminina do gerbilo adulto têm sido muito estudados em diversas pesquisas atuais (Custódio et al., 2004; Santos et al., 2006; 2007). Os resultados obtidos com estes estudos literários demonstram que a fisiologia da próstata feminina é regulada por andrógenos e estrógenos, e que desequilíbrios hormonais decorrentes do envelhecimento (Custódio et al., 2010) ou exposição à disruptores endócrinos na fase pré-natal (Perez et al., 2011; Biancardi et al., 2012) podem levar à predisposição de lesões prostáticas.

A proporção andrógeno/estrógeno é regulada em vertebrados por meio da atividade da enzima aromatase. Essa enzima tem como função sintetizar estrógeno a partir de andrógenos, processo conhecido como aromatização (Ellem e Risbridger, 2010). A próstata é um sítio de aromatização e fonte de estrógeno, que através de seus receptores influenciam as células epiteliais e estromais da própria glândula (Tsugaya et al., 1996; Ellem e Risbridger, 2010).

O aspecto temporal influencia o balanço entre a testosterona e o estrógeno, e a expressão dos receptores ER α , ER β e o AR. Isso ocorre devido a diferença da síntese hormonal e a expressão dos receptores nos diferentes períodos do desenvolvimento da próstata dos machos. Na fase neonatal os níveis sorológicos de estrógenos são mais elevados do que os níveis de andrógenos (McPherson et al., 2008). Entretanto, na puberdade, que em roedores se inicia na 6ª semana de vida pós-natal, há uma elevação nos níveis de testosterona (Siegford et al., 2003; Vilamaior et al., 2006). Nesse período ocorre o crescimento e maturação prostática, seguida pelo aumento da proliferação das células epiteliais e início da atividade secretória dessa glândula (Vilamaior et al., 2006).

Durante a senescência os níveis de testosterona declinam em roedores. Em contraste, os níveis de estrógeno não alteram ou aumentam nesse mesmo período. Esses níveis podem ser mantidos pelo aumento da aromatização de andrógenos adrenais. Conseqüentemente há um significativo aumento na taxa de estrógeno em relação à testosterona (Vermeulen et al., 1972; Culig et al., 1993).

Os maiores níveis de estrógenos tanto na fase adulta quanto na senescência nos machos podem suprimir a síntese de testosterona, privando a próstata de estimulação androgênica. Desse modo a glândula é induzida a atrofia e regressão (McPherson et al., 2008).

Nas fêmeas, os níveis de estrógeno são elevados na idade adulta comparado com a fase jovem e senil, sendo que nesta há um declínio da produção de esteroides nos ovários. Assim, o envelhecimento, segundo estudos com roedores, provoca alterações celulares, hipertrofia e lesões patológicas na próstata de fêmeas, sendo estas alterações também encontradas na próstata de machos. (Greendale et al., 1999; Custódio et al., 2008). Deste modo, é de fundamental importância avaliar os efeitos da ação da exposição ao EE durante períodos críticos do desenvolvimento prostático (pré-natal e puberdade), em virtude do aumento da susceptibilidade ao aparecimento de lesões prostáticas durante o envelhecimento.

Objetivos

O presente trabalho visa:

1. Avaliar, por análises morfológicas (estruturais e ultraestruturais), sorológicas e imunohistoquímica os efeitos da exposição à baixas doses do etinilestradiol durante os períodos pré-natal e puberal sobre a próstata masculina e feminina de gerbilo senil.
2. Verificar se a exposição, pré-natal e puberal, ao etinilestradiol predispõe lesões prostáticas em gerbilos machos e fêmeas durante a senescência.
3. Investigar se a exposição à esse estrógeno sintético é capaz de induzir padrões de alterações diferentes nas glândulas prostáticas masculina e feminina de gerbilo senis.

Artigo 1

**IN THE AGING, THE VENTRAL MALE PROSTATE IS MORE SENSIBLE TO
EFFECTS OF ETHINYLESTRADIOL THAN THE FEMALE PROSTATE WHEN THE
GERBILS WERE EXPOSED TO LOW DOSES OF THIS STEROID DURING
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Submitted to Steroids

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Running Title: Ethinylestradiol effects on the prostate of male and female senile gerbil

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Abstract

The ethinylestradiol (EE) is an endocrine disruptor (ED) which acts as estrogen agonist, being an important compound of oral contraceptive. The aim of this work was to analyse the morphological effects in the ventral male prostate and female prostate of senile gerbils (*Meriones unguiculatus*) that were exposed to low doses of EE (15µg/kg/day) during prenatal period (EE/PRE group). Our results showed that exposure to EE changed the levels of steroid hormone and may alter the pathways of AR, ER α and ER β . These effects have as response alterations in the morphological pattern of the prostatic glands and increase predisposition to emergence of lesions as prostatic intraepithelial neoplasia (PIN) observed in the prostate of both sexes. Despite male and female have been exposed the same doses of EE, the exposure to ED promoted modifications more accentuated in the male prostate than the female gland.

Keywords: ethinylestradiol; prenatal period; Mongolian gerbil; male prostate; female prostate.

Abbreviations: ED, endocrine disruptors; EE, 17 α -ethinylestradiol; PIN, prostatic intraepithelial neoplasia; PCNA, proliferating cell nuclear antigen; AR, androgen receptor; ER α , estrogen receptor alpha; ER β , estrogen receptor beta.

Introduction

Endocrine disruptors (ED) are exogenous chemical compounds capable of disrupting the function of the endocrine system, thus affecting the reproductive systems of animals and humans [1, 2]. Many ED have the potential to mimic, antagonize, or alter the levels of endogenous steroid hormones (androgens and estrogens) [3]. World wide, people are exposed to ED, which are found in products such as bisphenol A (BPA) found in polycarbonate plastic, glycidyl methacrylate for dental use, and ethinylestradiol (EE) used in medicines [3, 4].

Exposure to ED during development alters the male and female reproductive systems, resulting in changes in the neuroendocrine system, reproductive behavior [3], and susceptibility to prostate cancer [5]. The prenatal, neonatal, and pubertal period are sensible to hormonal imbalance because during these phases the levels of steroid hormones are essential for the development of male and female reproductive organs [6, 7]. The hormonal dysregulation caused by exposure to ED during critical stages of development increase the risks of the prostate diseases in adulthood, such as cancer [5, 8, 9].

The prostate is a gland that depends on the action of steroid hormones for its development, and these effects begin in the prenatal period [10]. Exposure to ethinylestradiol, a synthetic estrogen in contraceptive pills, during the prenatal period, increases susceptibility to the emergence of pre-neoplastic lesions in the prostate in male and female gerbils in adulthood [11,12].

Millions of women consume contraceptive pills daily. Some women may not know they are pregnant, and may continue to take the birth control during the first months of pregnancy [13]. According to Thayer and colleagues [14], exposure to low doses of ethinylestradiol (0.002 mg/kg/day; substantially less than the 0.5 mg/kg found in contraceptive pills consumed by women) during pregnancy caused rodents to have decreased daily sperm production during adolescence and increased prostate size in males in the neonatal period. ED exposure during pregnancy, even at low doses, promotes changes in the prostate of rodents in the neonatal phase [14], and such modifications remain into adulthood [11, 12].

Most studies involving ED emphasize the consequences of intrauterine exposure on the prostate of rodents during the neonatal and adult phases [12, 15, 16]. However, few studies have evaluated the effects of this exposure in senile individuals [17]. Senescence has been

characterized as reproductive aging and this, in turn, is associated with the loss of reproductive capacity and deregulation of the hypothalamic-pituitary-gonadal axis. This process may be regulated by genetic and environmental factors [18]. In rodents, there is a decrease in the feedback of gonadal steroid hormones on the hypothalamus and pituitary gland during the senile period [19, 20]. This hormonal deregulation is a major cause for the emergence of prostatic diseases, especially during aging [21].

Therefore, it is of fundamental importance to study the effects of exposure to ED during critical phases of development on the prostate gland of rodents in the senile stage. In view of these aspects, our hypothesis is that prenatal exposure to ethinylestradiol may lead to increased predisposition to develop prostate lesions during the senile phase. Thus, this study aimed to evaluate the morphological effects of prenatal exposure to ethinylestradiol on the ventral male prostate and female prostate of gerbils during aging.

Material and Methods

Animals

The male and females gerbils (*Meriones unguiculatus*) used in this experiment were maintained in a biotherium in the IBILCE/UNESP (São José do Rio Preto-SP) in polyethylene boxes with wood shavings substrate, under controlled light conditions, and at an average temperature 23 °C. They were supplied filtered water in glass bottles and food ad libitum (composition: 23% protein, 12% minerals, 5% fiber, and 4% of total lipids).

Experimental Design

In this experiment, we used eight adult virgin female gerbils (90 to 120 days), and each was maintained with a male of the same age for the formation of different families and separated in two different groups. As shown in Figure 1, the EE/PRE group, the pregnant females received 15 µg/kg/day 17 α -ethinylestradiol (EE- Formula: C₂₀H₂₄O₂) (E4876 Sigma, St. Louis, MO, USA) diluted in 100 µl of Nujol[®] mineral oil (CAS 8020-83-5, Sigma-Aldrich, St. Louis, MO) by gavage. In the control group, the pregnant females received no treatment.

Pregnant females in the EE/PRE groups received ethinylestradiol of the 18th to 22th, considering that the gestational time of the gerbil is 26 days. The treatment was performed during

the prostatic morphogenesis of the gerbils, which starts between the 20th and 21th days of the prenatal period [22].

Males and females of the experimental groups were killed at 12 months for blood collection, by anesthetization in CO₂ followed by decapitation. After death, the male and female prostate complexes were removed and weighed, as along with the testis, the ovary and the adrenal gland. The females were cycled and sacrificed during the proestrus phase [23].

Biometric Analysis

Prior to collection and weighing of the biological materials, both males and females at 12 months of age were submitted to measurement of the anogenital distance (AGD) using a caliper King Tools Digital Caliper (0–300 mm).

Optical Microscopy

Male and female prostates from the experimental groups were fixed in 4% buffered paraformol and metacarn (at a 1:3:6 of acetic acid, chloroform, and methanol, respectively), dehydrated in ethanol, clarified with xylene, and then embedded in Paraplast (Histosec, Merk). The organs were sectioned at 5 μm and stained with hematoxylin and eosin (HE) for morphological, morphometric, and histopathological analysis of the prostate [24]. In addition, we used the Gömöri's reticulin reaction for analysis of reticular fibers.

The histological images were captured using a microscope Zeiss JenaVal-(Jena, Germany) and they were scanned using the Image-Pro Plus software version 4.5 for Windows (Media Cybernetics, Inc., Silver Spring, MA, USA).

Morphometric and Histopathological Analysis

For morphometric analysis, we collected 200 data points for each prostate of the experimental groups and were used to measure the thickness (μm) of the epithelial height of the muscular layer of ventral male prostate and female prostate. The analysis was performed using a photomicroscope Olympus BX60 and the images were captured for photographic documentation and morphometric measurement using the Image-Pro Plus[®] Average Cybernetics software.

Histopathological analyses of the ventral male prostates and female prostates from the control and EE/PRE groups were done through the multiplicity of prostatic intraepithelial

neoplasia (PIN). The identification of these lesions was performed according to the classification of Shappell and colleagues [25]. The multiplicity was evaluated by determining the number of lesions throughout the section using five animals from each group, analyzing 25 sections of the each group in Olympus optical microscope.

Immunohistochemistry

The ventral male prostates and female prostates from the experimental groups were fixed in 4% buffered paraformol or metacarn and submitted to histological processing by immersed in Paraplast (Histosec, Merk), followed by sectioning (5 μ m). The sections were subjected to immunohistochemical detection of antigen proliferating cell nuclear antigen (PCNA; mouse monoclonal IgG_{2a}, P-10: SC 56, Santa Cruz Biotechnology CA, USA), p63 protein (basal cell marker; mouse monoclonal IgG_{2a}, 4A4: sc-8431, Santa Cruz Biotechnology CA, USA), androgen receptor (AR; rabbit polyclonal IgG, N-20: sc-816, Santa Cruz Biotechnology CA, USA), estrogen receptor alpha (ER α ; rabbit polyclonal IgG, H-184: sc-7207, Santa Cruz Biotechnology CA, USA), and estrogen receptor beta (ER β ; rabbit polyclonal IgG, H-150: sc-8974, Santa Cruz Biotechnology CA, USA). Antigenic recuperation was performed in citrate buffer (pH 6.0) at a high temperature (98 °C) for 45 min, followed by three 5 min washes in phosphate buffered saline (PBS). Peroxidase was blocked against using 12% H₂O₂ in methanol for 20 min. Then, the sections were blocked with nonspecific protein (milk powder diluted in PBS) for 30 min and then incubated with antibodies diluted in 1% bovine serum albumin (BSA) in PBS overnight (4 °C; 1:100 PCNA and p63; 1:75 AR; 1:50 ER α and ER β). The sections were incubated with polymer (Novolink, Novocastra) at 37 °C for 45 min, then visualized with diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) and counter stained with Harris hematoxylin. For the reactions for PCNA, p63, and AR, we determined the number of positive nuclei in prostatic epithelial cells in the experimental groups using fields of microscopic images (400X; Olympus BX60) of the prostate from each group (n=3; 2000 cells total). The values of positive nuclei were divided by the total number of cells counted in each field, and expressed as the percentage (%).

Immunofluorescence

Prostates from the control and EE/PRE groups were fixed in metacarn, subjected histological processing, embedded in Paraplast (Histosec, Merk), and sectioned into 5 μ m slices. The sections were subjected to immunofluorescence imaging in order to detect smooth muscle α actin. Antigenic recuperation was performed in citrate buffer (pH 6.0) at a high temperature (98 °C) for 1 hour, followed by three 5 min washes with PBS. Non-specific binding was blocked by incubation in 5% BSA in PBS for 1 hour. Sections were incubated with primary α actin antibody (1:100; mouse monoclonal IgG2a 1C4: sc-32251, Santa Cruz Biotechnology CA, USA) diluted in 1% BSA in PBS overnight at 4 °C. After this procedure, the sections were incubated with secondary antibody (1:200 goat anti- mouse IgG -FITC: sc-2010, Santa Cruz Biotechnology CA, USA) for 2 hours in the dark. After, we used a mounting means with DAPI (Ultra Cruz™ Mounting Medium: sc-24941, is with DAPI fluorescence, Santa Cruz Biotechnology CA, USA). The images were analyzed in a fluorescence microscope Zeiss Axio Imager M2.

Serum Hormonal Levels

The blood of male and female senile gerbils from the experimental groups was collected at the time of decapitation. The serum was centrifuged (3000 rpm for 20 min) and stored at -80 °C until analysis. Hormonal dosages were performed in duplicate by ELISA and using kits with high sensitivity (testosterone and 17 β -estradiol, Cayman Chemical Company, MI, USA) following the manufacturer's instructions. The detection limits for testosterone and 17 β -estradiol were 6 pg/ml and 19 pg/ml respectively. The readings were performed using the lector SpectraMax Plus 384 at 405 nm (Molecular Devices, CA, USA).

Transmission Electronic Microscopy

After removal, the prostate glands were cut into small fragments and fixed for 24 hours in 3% glutaraldehyde solution, 0.25% tannic acid, and 0.54% glucose in Milloning's buffer pH 7.3 [26]. After being washed, the fragments were postfixated with 1% osmium tetroxide for 2 hours, washed again, dehydrated in increasing concentrations of acetone, and then embedded in Araldite resin. Subsequently, the fragments were cut into ultrathin sections (50 nm) using diamond knives, and then contrasted with 2% uranyl acetate for 20 min and chumbo citrate for 6 min. The samples

were observed and evaluated using the LEO-Zeiss 906 transmission electronic microscope (Zeiss, Cambridge, UK).

Statistical Analysis

For statistical analysis of the biometric, morphometric, histopathological, immunohistochemistry, and serological data utilized the spreadsheets and graphics of GraphPad Instat. To prove the significance of the parametric results, we used the Tukey test and non-parametric data, and the Mann-Whitney test. The level of significance was 5% ($p \geq 0.05$) and was expressed as the mean \pm standard deviation.

Results

Table 1 show the biometric data of experimental groups of the male and female senile gerbils. In the control and EE/PRE groups, both sexes there were no significant differences between the groups. The serological data showed no significant differences in the levels of testosterone and estradiol between the males in the control and EE/PRE groups (Fig. A.2 and B). However, the EE/PRE female groups showed a significant increase in testosterone levels when compared to the control group (Fig B.2).

During analysis, we noted no anatomical differences between the testes, adrenal, and ventral prostate of the EE/PRE male group when compared to the control group. However, histologically, (Fig 3) the ventral prostate of EE/PRE male group showed the presence of acinar atrophy within the smallest lumen area (Fig C.3). Furthermore, we found the presence of stratified epithelium and an increase in the stromal density, which are characteristics of benign prostatic hyperplasia (BPH; Fig C.3). The epithelial height and the thickness of the muscle layer that surrounds the prostatic acini decreased significantly in EE/PRE group males when compared to control group males (Fig B.3, D.3, and F.3 and Fig A.4).

The morphological analysis of female prostates (Fig 5) revealed stratified epithelium and an increase in stromal densities, which are characteristics of BPH (Fig C.5). During the morphometric analysis, we found that the epithelial height and the thickness of muscle layer prostatic increased in the EE/PRE group females when compared to the control group (Fig B.4; Fig. B.5, D.5, and E.5).

EE exposure during the prenatal period also promoted the increase of prostatic intraepithelial neoplasia (PIN) in the glands of senile males (Fig D.3 and F, Fig 6) and females (Fig D.5 and F.5, Fig 6) when compared to their respective controls groups.

The Gömöri's reticulin technique allowed us to observe the stromal compartments of prostates from the EE/PRE male group. We noted changes in the arrangement of reticular fibers and collagen when compared to control groups (Fig A.7, B.7, D.7, and E.7). For the female prostate stromal compartments for the EE/PRE group, we noted changes in the arrangement of the reticular fibers (Fig C.7 and F.7).

Figure 8 shows immunofluorescence results for α -actin reaction surrounding the alveoli of the prostates from the males and females in the senile groups. In areas that had prostatic buds, the muscle layer exhibited ruptures, showing the absence immunoreactivity in the EE/PRE male group (Fig C.8). In the ventral male prostates from the EE/PRE group, we observed increased immunoreactivity in regions with stromal lesions (Fig D.8), while in the prostate of the EE/PRE female group we noted an increase in this immunoreactivity in regions with PIN (Fig E.8).

Table 2 shows the quantification (%) of prostatic epithelial cells in male and female that showed positive immunoreactivity for androgen receptors (AR) and p63 (basal cells). These results revealed increased immunoreactivity for AR in the prostates from the EE/PRE male group when compared to the control group (Fig A.9, B.9, G.9, and H.9). However, we observed decreased immunoreactivity for p63 in the prostates from the EE/PRE male group when compared to the control group (Fig E.9, F.9, G.9, and M.9). Immunohistochemistry for PCNA revealed an increase in immunoreactivity in the ventral prostates, especially in areas with PIN, from the EE/PRE male group when compared to the control group (Fig C.9, D.9, I.9, and J.9).

In the prostates from the EE/PRE female group, we did not note any significant differences in the frequency of cells positive for p63 and AR when compared with the control group, as shown in Table 2 and Figure A.10, B.10, G.10, and H.10. However, the immunoreactivity of PCNA was higher in the female prostates from the EE/PRE group when compared to the control group (Fig C.10, D.10, I.10, and J.10).

The immunoreactivity for estrogen receptors type alpha ($ER\alpha$) has been observed in the stromal cells of the ventral male prostate of control and EE/PRE group (Fig 11). However, in the group treated with EE during pregnancy, this immunoreactivity was intense in the cytoplasmic

region of prostatic epithelial cells of senile males (Fig C.11). Regarding ER α immunoreactivity has been noted an increase in the stromal cells of the prostate females in the EE/PRE group compared to control (Fig B.11, D.11).

The estrogen receptors of type beta (ER β) were observed primarily a nuclear immunoreactivity in the male prostate epithelial cells in the experimental groups (Fig 12). In the prostate of EE/PRE male group, this immunoreactivity was higher compared to control group (Fig A.12 and B.12). The immunoreactivity for ER β in the female prostate presented no different between the experimental groups (Fig B.12, C.12 and E.12).

In the ultrastructural analysis of ventral male prostate of gerbil senile it was observed columnar epithelial cells with the presence of dense nuclear chromatin condensation, nuclear mainly distributed in the periphery (Fig A.13). Under basal membrane it was observed in the prostatic stroma, collagen fibers and the presence of smooth muscle cells (Fig A.13).

Regarding the ultrastructure of male prostate of EE/PRE group there was an apparent increase of lipofuscin and organelles as the Golgi complex when compared to the control group and we observed the fusion of secretory vesicles (Fig D.13).

During the ultrastructural analysis of prostate female senile gerbil, it was observed highly secretory cells with large nucleus and the presence of lipofuscin deposits on prostate cytoplasm (Fig B.13 and C.13). In EE/PRE group evidenced the increase of lipid droplets in the cytoplasm prostatic, as well as the presence of vesicle fusion with the accumulation of secretion compared to control group. Further, we noticed a decrease in adhesion between epithelial cells prostatic of EE/PRE (Fig E.13).

Discussion

The present study shows that exposure to low levels of ethinylestradiol (15 μ g/kg/day) during the prenatal causes changes in the epithelium and stroma of the prostate of the gerbil males and females during aging senile females exposed to EE showed a significant increase in testosterone levels. The female prostate develops in an environment with high levels of endogenous estrogen [27], but the increase in testosterone stimulates growth and secretory activity of this gland [28]. In this study it was observed that exposure to EE interfered in the process of synthesis of steroid hormones (androgen), increasing hormone levels in the circulation

of rodent male. This fact was noted in the exposure to other synthetic estrogens like biphenol A (BPA) [29]. Early exposure to EE affect testosterone levels in senile females, resulting in increased of epithelial height and of the muscle layer of the prostate altering the morphology this gland in the senile phase.

Estrogen plays an important role in the development and growth of the prostate [30], however the exposure to synthetic estrogens during critical periods of development causes changes in prostate health [12, 31]. The EE exposure during the prenatal period resulted in increased prostatic intraepithelial neoplasia (PIN) in the ventral male prostate and female prostate of senile gerbil. Low doses of endocrine disruptors that act as estrogen agonist, exposed during development alters the prostate epigenome, this process is called estrogenic imprint, predisposing the animal to precursor lesions of prostate [8, 17]. The ethinylestradiol as an estrogen agonist which induces the estrogenic activity [7], exposed during development promotes permanent prenatal imprint consequences are accentuated during senescence.

The stromal-epithelial interaction is essential for normal prostate development [10, 32]. This interaction are mediate by $ER\alpha$, which is a receptor that acts in the muscle cells and fibroblasts, and influences the proliferation of epithelial cells [33]. Our data showed an increase in $ER\alpha$ immunoreactivity in stromal cells and the cytoplasm of epithelial cells in the prostates from male and female senile gerbils that were exposed to ED. One of the mechanisms of action of endocrine disruptors, or estrogen agonists, is the interference of estrogen signaling and the interaction with the estrogen receptors [34]. Interference of the action of $ER\alpha$ during development due to ED exposure promoted morphological changes in the structures of the prostatic stroma of senile males and females. We observed the disarrangement of collagen and reticular fibers in regions with the presence of PIN in the ventral male prostate. Alterations in the prostatic stroma contributes to the development of an invasive phenotype in the ventral male prostate and female prostate during senescence promoted by ED exposure during development of the prostate gland.

Similarly, to estrogen, androgen plays an important role in prostate development, in stromal-epithelial interactions, in epithelial cell proliferation, and in the development of prostatic buds [10, 35]. During immunohistochemistry analysis, we observed an increase in the frequency of AR in the senile ventral male prostate in the EE/PRE group. Studies with male mice exposed

to ED and DES (diethylstilbestrol) during the embryonic stage showed an increased in the frequency of AR in the prostate of the neonatal rodent [14, 36]. During the senile phase, the increase of AR in the male and female prostates of gerbils exposed to ED contributed to the development of alterations in stromal-epithelial interactions, including rupture of the muscular layer in invasive lesions and the increase in this layer in regions with PIN. Both ER α and the AR seem to contribute to the adverse effects on prostatic morphology in senile animals exposed to ethinylestradiol.

ED exposure during the prenatal period increased the proliferation of epithelial cells, resulting in the formation of neoplastic lesions in the male and female prostate in gerbils during aging. This change was shown by the increased in the PCNA immunoreactivity, mainly in regions with PIN. The expression levels of PCNA are not only associated with the pathways involved in cell cycle control and replication, but also with DNA repair processes. [37]. Chemicals can alter the levels of PCNA, interfering with cell cycle control pathways, thus promoting the increase of cell proliferation and the formation of neoplasms [37, 38].

The pattern of immunolocalization for p63 showed a decreased immunolocalization in basal cells in the ventral male prostates from the EE/PRE group. According Grisanzio and Signoretti [39], there is a progressive loss of the basal cell layer in high-grade PIN, which results in alterations p63 expression. Exposure to high doses of testosterone during the prenatal period decreases the immunoreactivity of p63 in regions with PIN in the ventral male prostate of the adult gerbil [16]. Even when using low doses, we noted that exposure to DE during development is enough to cause neoplastic lesions and the loss of the basal cell layer in the ventral male prostate in the senile stage.

While the functions of AR and ER α receptors are crucial during the early phases of prostate development [10, 30], the action of ER β on the prostate is more significant in adulthood, during which it has antiproliferative functions [40], while ER α plays, has proliferative functions [30]. According Rochel-Maia et al. [41], ER β decreases in the prostates of male and female gerbils during aging, predisposing these rodents to prostate proliferative diseases. Meanwhile, in the male ventral prostates from the EE/PRE senile group, we observed an increased in the immunoreactivity of ER β in epithelial cells. This increase in the male prostate may be an attempt of the gland to counteract the proliferative action exerted by ER α effects [30].

The structural analyzes of the ventral male prostate and female prostate of senile gerbils emphasized the morphological changes caused by exposure to EE during development. However, we also observed changes in the ultrastructure of the glands of the EE/PRE group for both sexes. The analyses revealed modifications to the secretory pattern of the prostatic epithelial cells, including the increase of vesicles containing secretions and organelles directly related to this function, such as the Golgi apparatus. The presence of lipofuscin deposits and changes in the arrangement of collagen fibers are characteristics observed in the prostates male [42] and female [43] senile gerbils. The exposure to EE during the prenatal period accentuated the alterations in the prostatic gland that are typical of senile gerbil.

Senescence is a period marked by the deregulation of steroid hormones [44], and this feature is one of the main factors for predisposition to prostate disease in senile males and females [42, 43]. Our study showed that exposure to EE during critical periods of development, such as prenatally, interferes with the normal morphology of male and female prostates in senile gerbils. The data suggest that exposure to EE caused an interference in epithelial-mesenchyme interactions, leading to emergence of PIN, widely modifying the prostate morphology.

The exposure to synthetic estrogen during development alters the levels of sex hormones, causing reproductive disorders through interference in the feedback that regulates the hypothalamic-pituitary-gonadal axis [29]. Changes, such as the increase of the proliferative index of epithelial cells, alterations in the expression patterns of steroid receptors, and potentiation of ultrastructural characteristics typical of senescence in gerbils caused by early exposure to EE. Even as changes in the morphological pattern of prostate, the exposure to EE increases the predisposition to emergence of pre-malignant lesions in males and females gerbils during senescence.

The male and female prostates of adult gerbils respond differently to exposure to estrogen disruptors during development. Additionally, the ventral male prostate is more sensitive to the action of ethinylestradiol than the female prostate, as well as the predisposition to the emergence of neoplastic lesions [11, 12]. The mammalian brain is extremely sensitive to the action of hormones during developmental phases (pre-natal, puberty), and differences in brain development between males and females may result in different effects due to DE exposure [34, 45]. The action of estrogen disruptors during the critical phases of development exacerbates the

development of precancerous lesions in the male prostate, damaging the health this gland during the senile phase.

Declaration of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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Table and Figures

Figure 1. Schematic of the experimental design. Males and females in EE/PRE group received 15 µg/kg/day of ethinylestradiol (EE) during the embryonic period (E). The animals were killed at 12 months of postnatal life (P). In the control group (C), the pregnant females were not treated.

Table 1. Biometric data of the experimental male and female groups of senile gerbils.

Values are expressed as the mean ± standard deviation. EE/PRE: Senile male exposed to ethinylestradiol during the prenatal period (n=5).

Figure 2. Serological data for the male and female of senile gerbils from the experimental groups. *A.* Testosterone levels (ng/ml). *B.* Estradiol levels (pg/ml).

Values expressed as mean ± standard deviation (n=5). *Significant difference between the groups with $p \leq 0.05$.

Figure 3. Histological sections of the ventral male prostate from senile gerbils stained by hematoxylin and eosin (HE). *Control group: A-C.* Prostatic acini surrounded by a simple prismatic epithelium (Ep) with presence of luminal region (Lu), smooth muscle layer (SM), and stroma (St) with vessels (Vs). Detail of the epithelial height (arrow). *EE/PRE group: D-G.* Acinar atrophy (at). Presence of prostatic intraepithelial (PIN) and atypical nuclei (broad arrow). Decrease of epithelial height (dashed arrow).

Figure 4. Morphometric of epithelial heights and thicknesses of the muscle layers of the ventral senile male prostate and female prostate of the experimental groups.

Values expressed as the mean ± standard deviation. EE/PRE: exposure to ethinylestradiol during the prenatal period.

Figure 5. Histological sections of the female prostate from senile gerbils stained by hematoxylin and eosin (HE). *Control group: A-B.* Epithelium (Ep) that coats the prostatic acini surrounded by smooth muscle (SM) and vascularized stroma (vs). Height of the prostatic epithelium (arrow) and muscle layer (SM). *EE/PRE group: C-F.* Presence of smaller acini (ac). Increase in the muscle

layer (*) and epithelial stratification (dashed arrow). Prostatic intraepithelial neoplasia (PIN). An increase of epithelial height (broad arrow) has been shown. Detail of the nuclear atypia (arrowhead).

Figure 6. Multiplicity (specific number) of prostatic neoplasia intraepithelial (PIN) for each experimental senile male and female. Values are expressed as the mean \pm standard deviation (n=5). *Significant difference between the groups with $p \leq 0.05$.

Figure 7. Histological sections of the ventral male prostate and female prostate from senile gerbils stained by Gömori reticulin. *Control group: A-C.* Elements of prostatic stroma: collagen (white asterisks) and reticular fibers (arrows). *EE/PRE group: D and E.* Disorder of arrangement of reticular fibers (large arrow) and collagen fibers (*). *F.* Apparent increase in collagen fibers (dashed arrow) and disturbance in the arrangement of reticular fibers (arrowhead) in the regions rich in blood vessels (vs).

Figure 8. Histological sections of the ventral male prostate and female prostate from senile gerbils subjected to immunofluorescence for α -actin of smooth muscle. *Control group: A and B.* Immunoreactivity in the prostatic muscle layer (dashed arrows) and in the vessels (vs). *EE/PRE group: C-E.* Region with prostatic buds noted absence of this immunoreactivity (arrow). Observed an increase in the α -actin immunolocalization in lesions in the stroma (*) and in the regions with PIN (large arrow).

Figure 9. Histological sections of the ventral male prostate subjected to immunohistochemistry of the androgen receptor (AR). *Control group: A and B.* We observed immunoreactivity in the prostatic epithelial cells (Ep) (arrows). Presence of the prostatic stroma (St). *EE/PRE group: G and H.* Increase in AR immunoreactivity in the epithelial cells (arrowhead). *I.* PCNA immunohistochemistry. *Control group: C and D.* Marcation in the prostatic epithelial cell (dashed arrows). *EE/PRE group: I and J.* Increase of the PCNA immunolocalization in regions with PIN (large arrow). Immunohistochemistry for p63. *Control group: E and F.* Labeling of

basal cells (large filled arrows). *EE/PRE* group: *L* and *M*. Decrease in the p63 immunoreactivity (large arrow).

Figure 10. Histological sections of the female prostate subjected to immunohistochemistry for the androgen receptor (AR). *Control* group: *A* and *B*. Marcation in the epithelial cells (arrows). *EE/PRE* group: *G* and *H*. Evidence for AR immunoreactivity in the prostatic epithelial cell (arrowhead). Immunohistochemistry for PCNA. *Control* group: *C* and *D*. *EE/PRE* group *I* and *J*. Immunoreactivity of cellular proliferation markers (dashed arrows). p63 immunohistochemistry. *Control* group: *E* and *F*. Details of the immunoreactivity in the prostatic basal cells (large filled arrow). *EE/PRE* group: *L* and *M*. Increase of p63 immunoreactivity (large arrows).

Table 2. Frequency of cells labeled by AR and p63 immunohistochemistry in the male and female prostates from senile gerbils in experimental group.

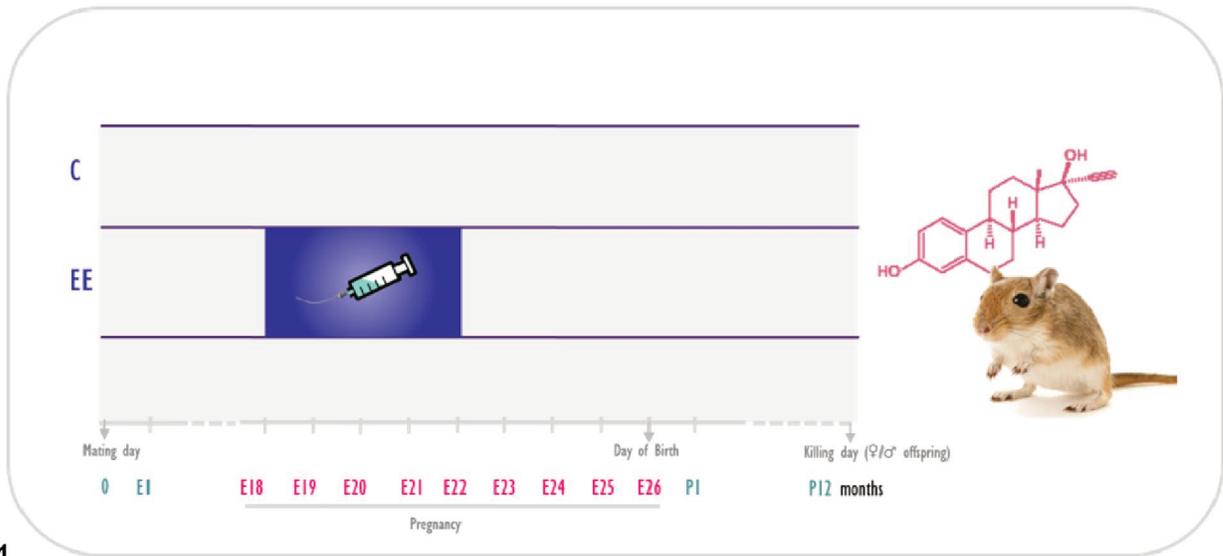
Values expressed as the mean \pm standard deviation (n=5). *Significant difference between the groups with $p \leq 0.05$.

Figure 11. Histological sections of the ventral male prostate and female prostate from senile gerbils subjected to immunohistochemistry for ER α . *Control* group: *A* and *B*. Presence of immunoreactivity in the prostatic stroma (St) cell (arrows). Epithelium (Ep). *EE/PRE* group: *C*. Cytoplasm labeling (*) and epithelial cell (arrow). *D*. We noted an increase of ER α immunoreactivity in the stromal cell (large arrows).

Figure 12. Histological sections of the ventral male prostate and female prostate of senile gerbils subjected to immunohistochemistry for ER β . *Control* group: *A* (male) and *B-C* (female). Details of the nuclear immunoreactivity in the epithelial cells (large arrows). Cytoplasmic marcation (dashed arrow). *EE/PRE* male group: *D*. Increase in nuclear immunoreactivity in the epithelial cell (arrowhead). *EE/PRE* female group *E*. Labeled epithelial cell (arrow)

Figure 13. Ultrastructural aspects of ventral male prostate and female prostate of senile gerbil. *Control* group: *A* (male), *B* and *C* (female). We observed large nuclei (n) and deposit of

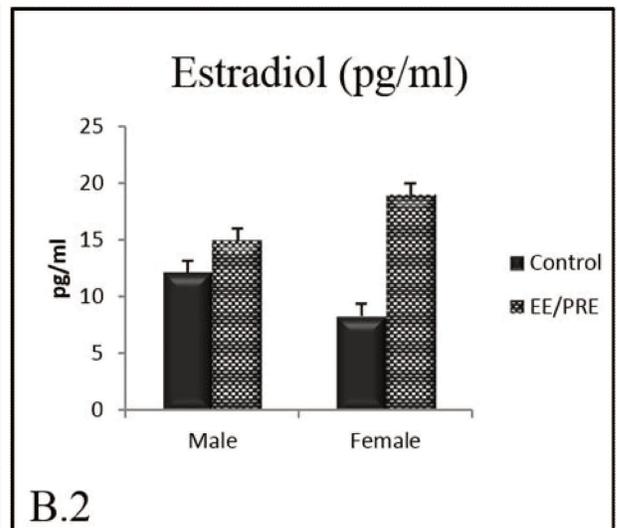
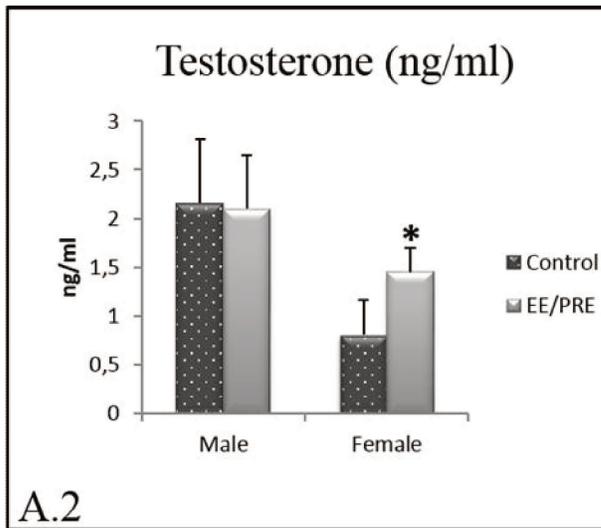
lipofuscin in the cytoplasm (large white arrow) of the epithelium. Basal membrane (bm). Dense stroma with abundant collagen fibers (co), and smooth muscle cells (smc). *EE/PRE group: D (male)*. Increase of lipofuscin deposit (lp), amount of vesicle (v), Golgi complex (G) in the cytoplasm. *E (female)*. Observed the presence of vesicle (dashed white arrow) in the secretory cells (sc). Note the presence of fusion of the vesicles (fv) and alterations in the cellular adesion (large arrow). A: 7750X; B: 3597X; C, D: 6000X; E: 4646X.



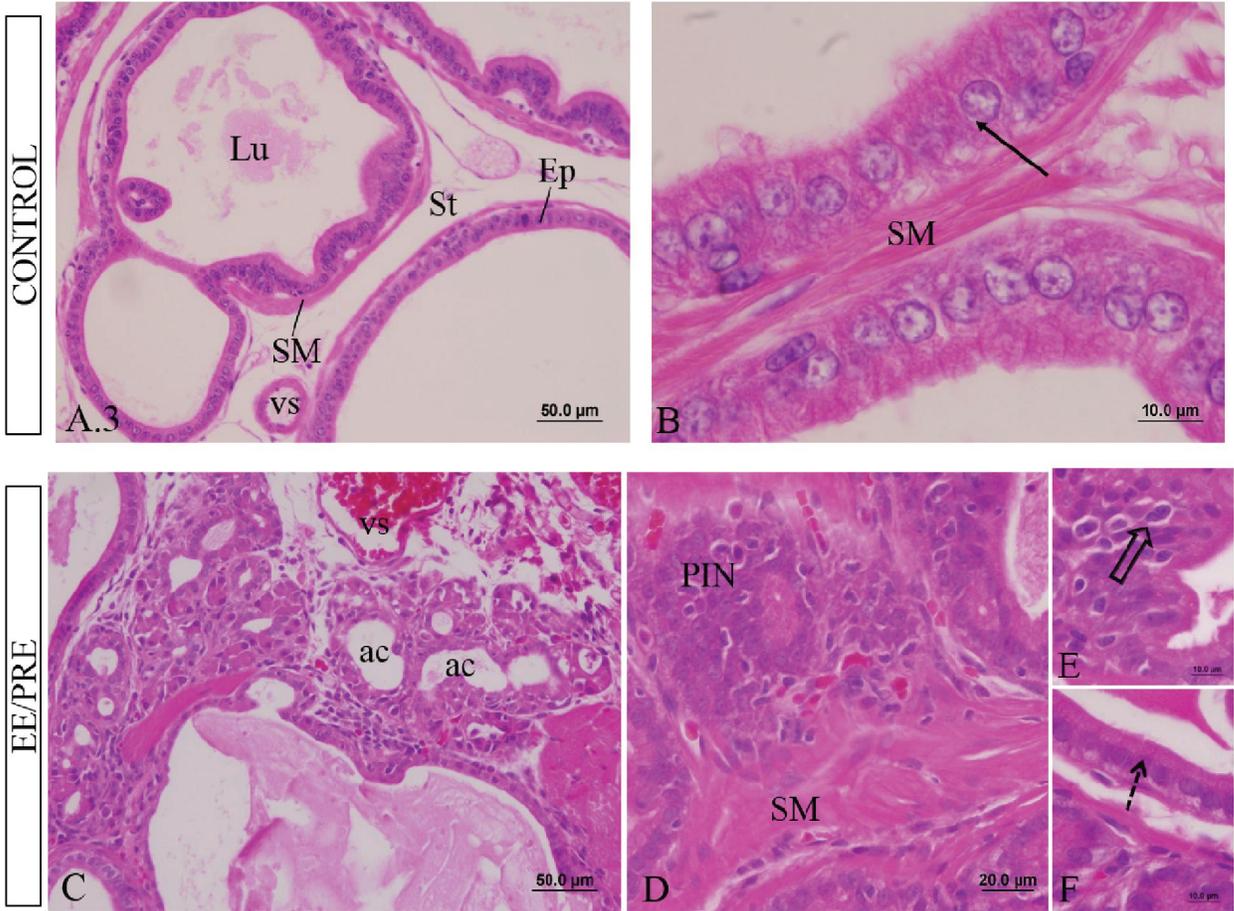
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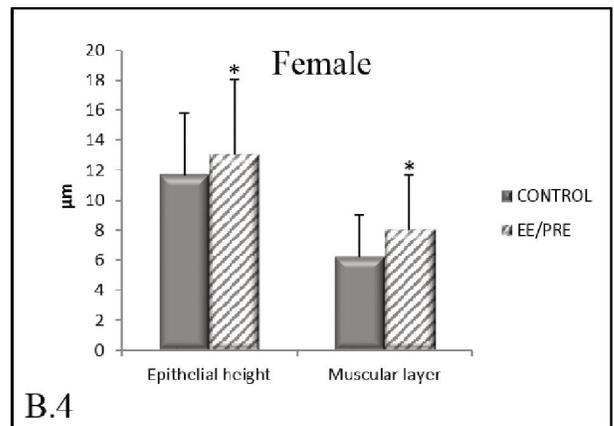
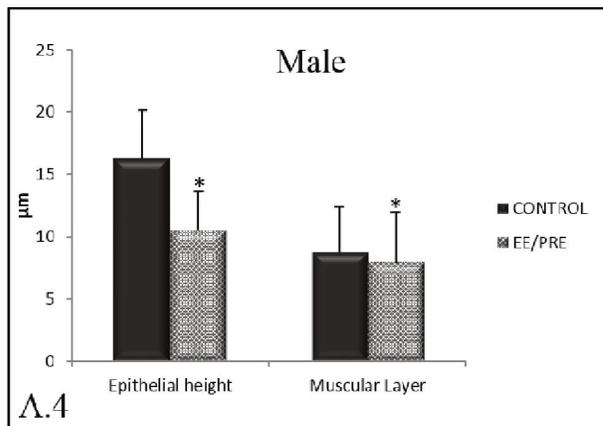
Table 1.

<i>PARAMETERS</i>	<i>EXPERIMENTAL GROUPS</i>	
	<i>MALE</i>	
	<i>CONTROL</i>	<i>EE/PRE</i>
Body Weight (g)	90.44 ± 14.38	87.11 ± 10.30
Prostatic complex (g)	1.06 ± 0.23	1.06 ± 0.11
Ventral prostate (g)	0.024 ± 0.006	0.023 ± 0.006
Relative prostate of ventral prostate (x10 ⁻³)	0.26 ± 0.07	0.26 ± 0.04
Right testis (g)	0.65 ± 0.06	0.62 ± 0.06
Left testis (g)	0.64 ± 0.05	0.61 ± 0.05
Adrenal (g)	0.05 ± 0.01	0.05 ± 0.01
Anogenital distance (AGD) (mm)	12.50 ± 1.50	12.31 ± 1.40
	<i>FEMALE</i>	
	<i>CONTROL</i>	<i>EE/PRE</i>
Body Weight (g)	66.20 ± 6.54	72.20 ± 7.00
Urethra and Prostate (g)	0.03 ± 0.01	0.02 ± 0.01
Relative prostate (x10 ⁻³)	0.44 ± 0.22	0.34 ± 0.08
Ovary (g)	0.04 ± 0.01	0.07 ± 0.02
Adrenal (g)	0.04 ± 0.01	0.04 ± 0.01
Anogenital distance (AGD) (mm)	3.92 ± 0.55	4.21 ± 0.40

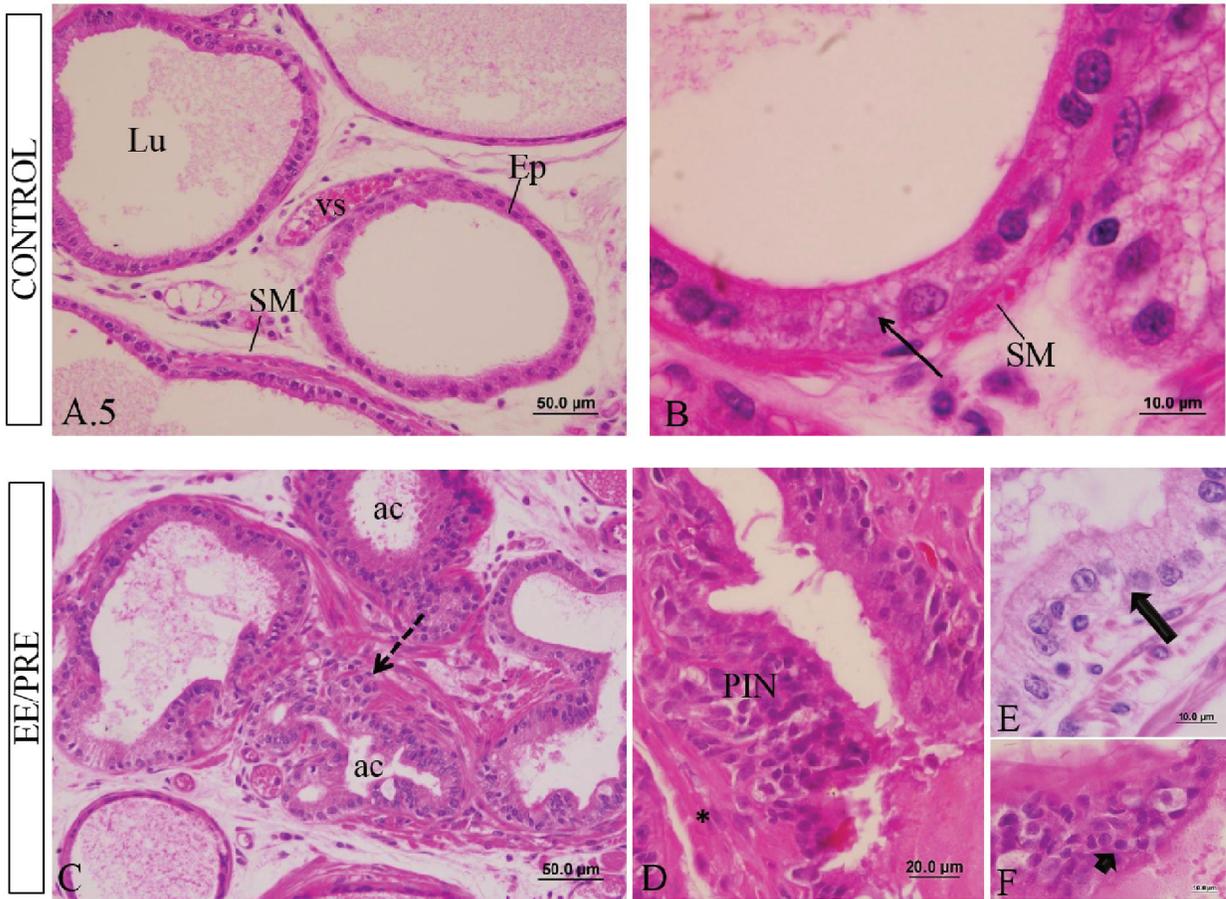


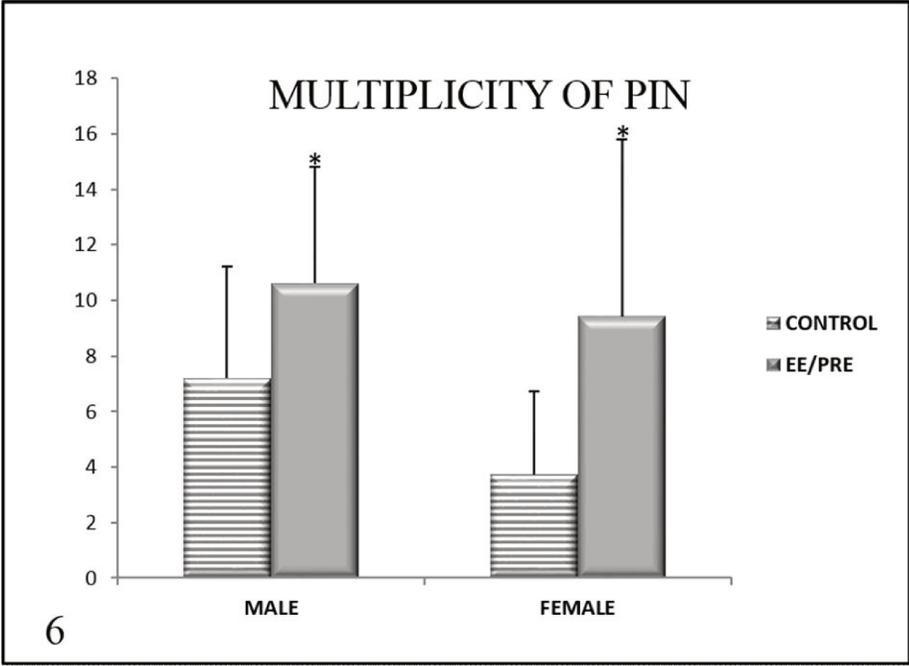
MALE: HE





FEMALE: HE

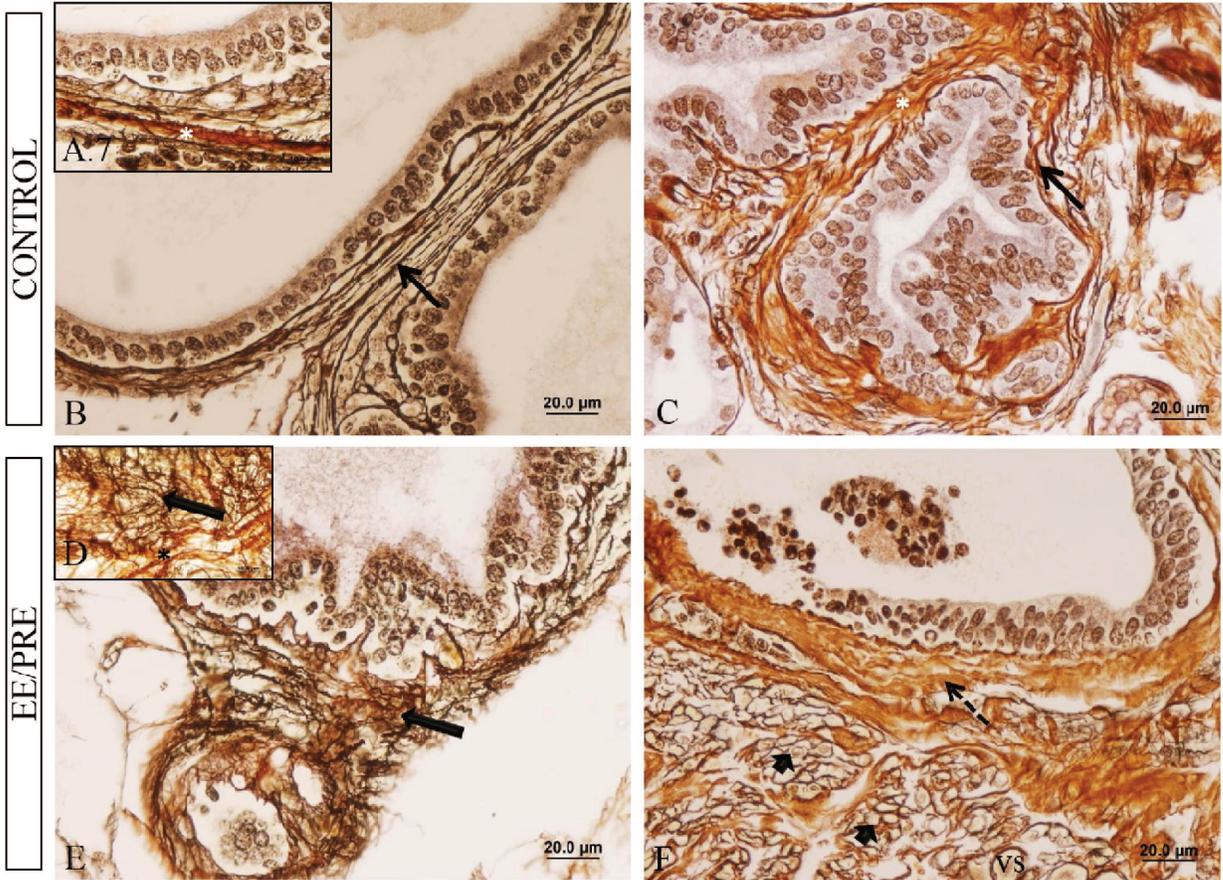


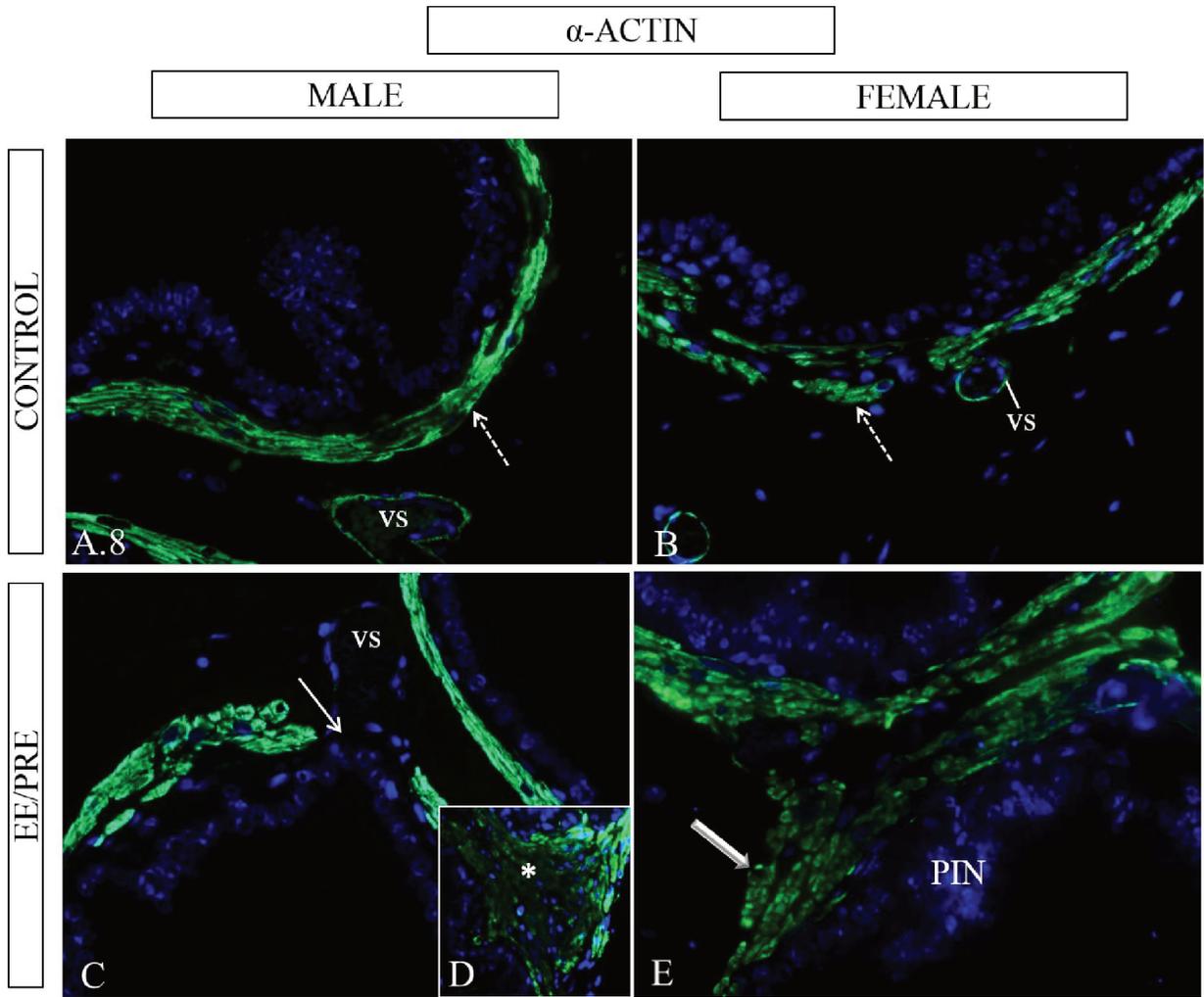


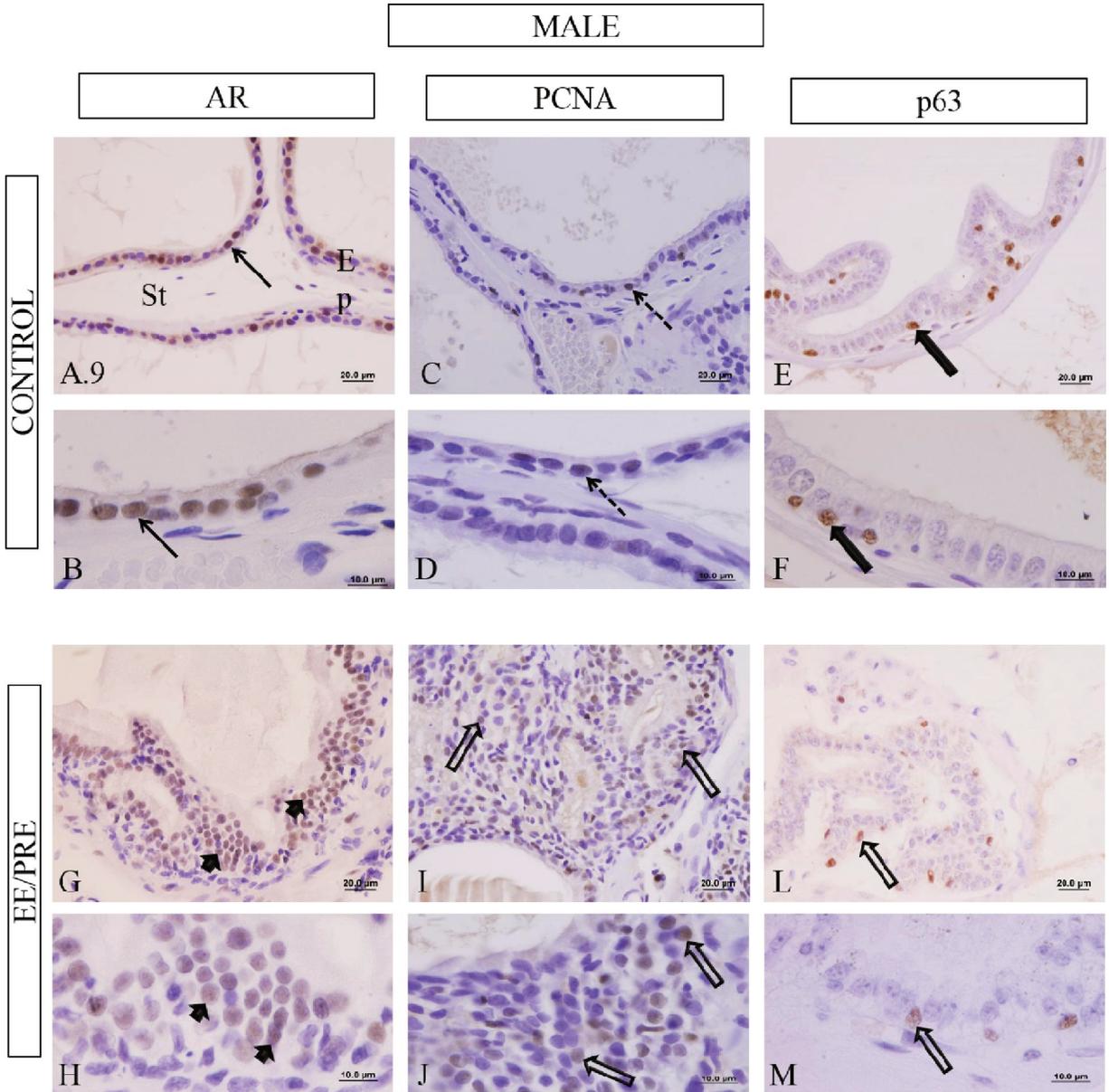
GÖMÖRI'S RETICULIN

MALE

FEMALE







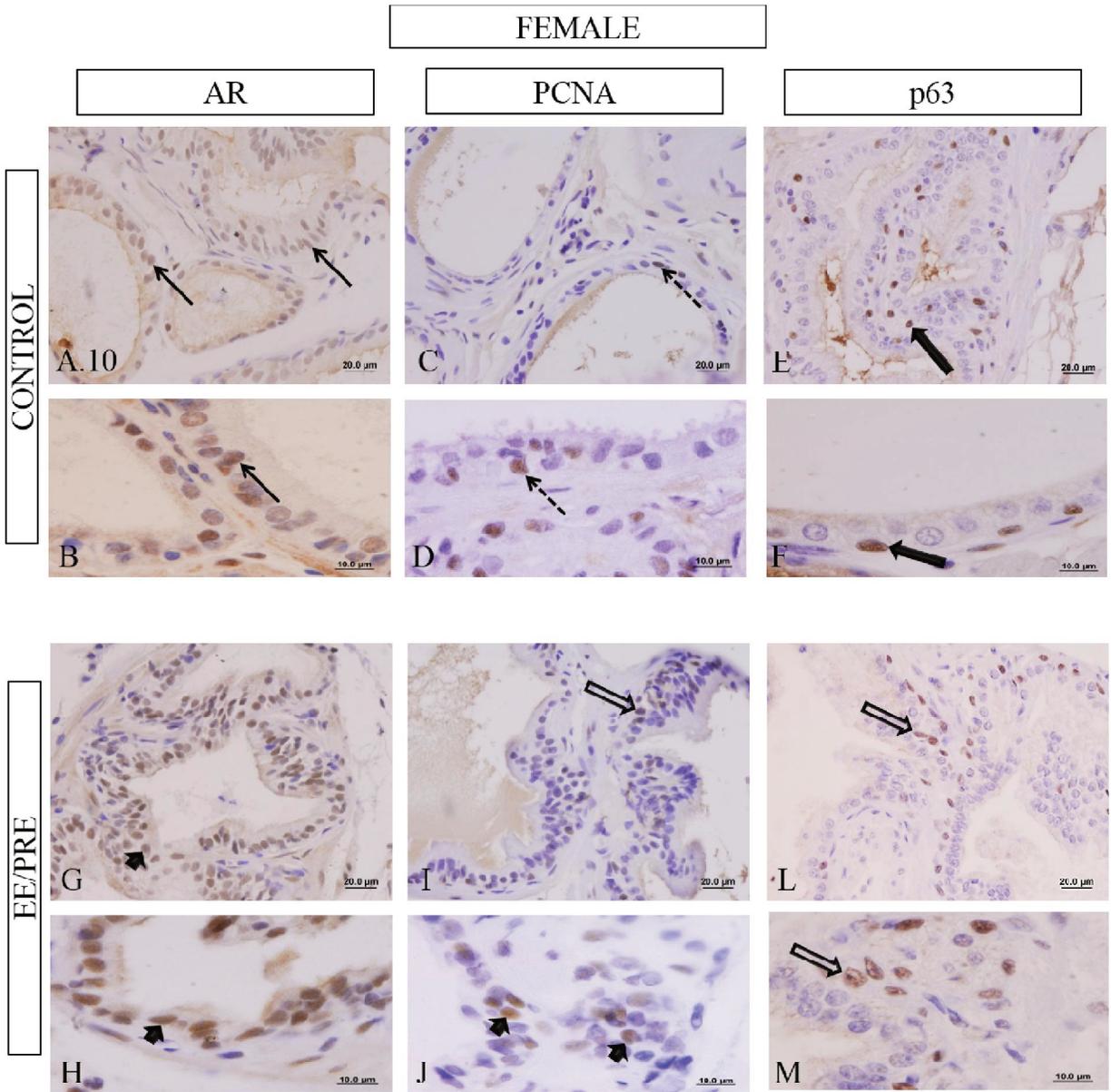
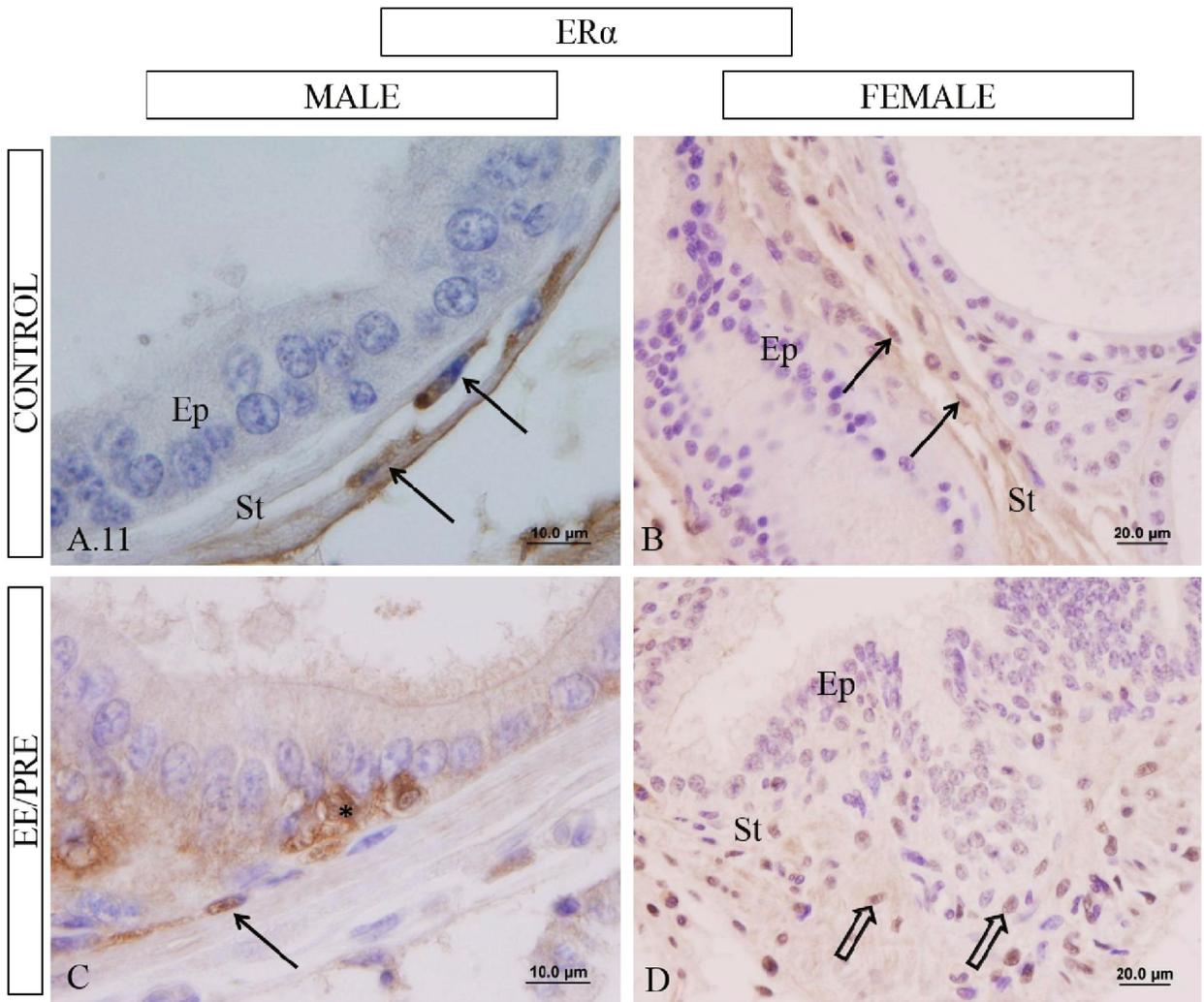
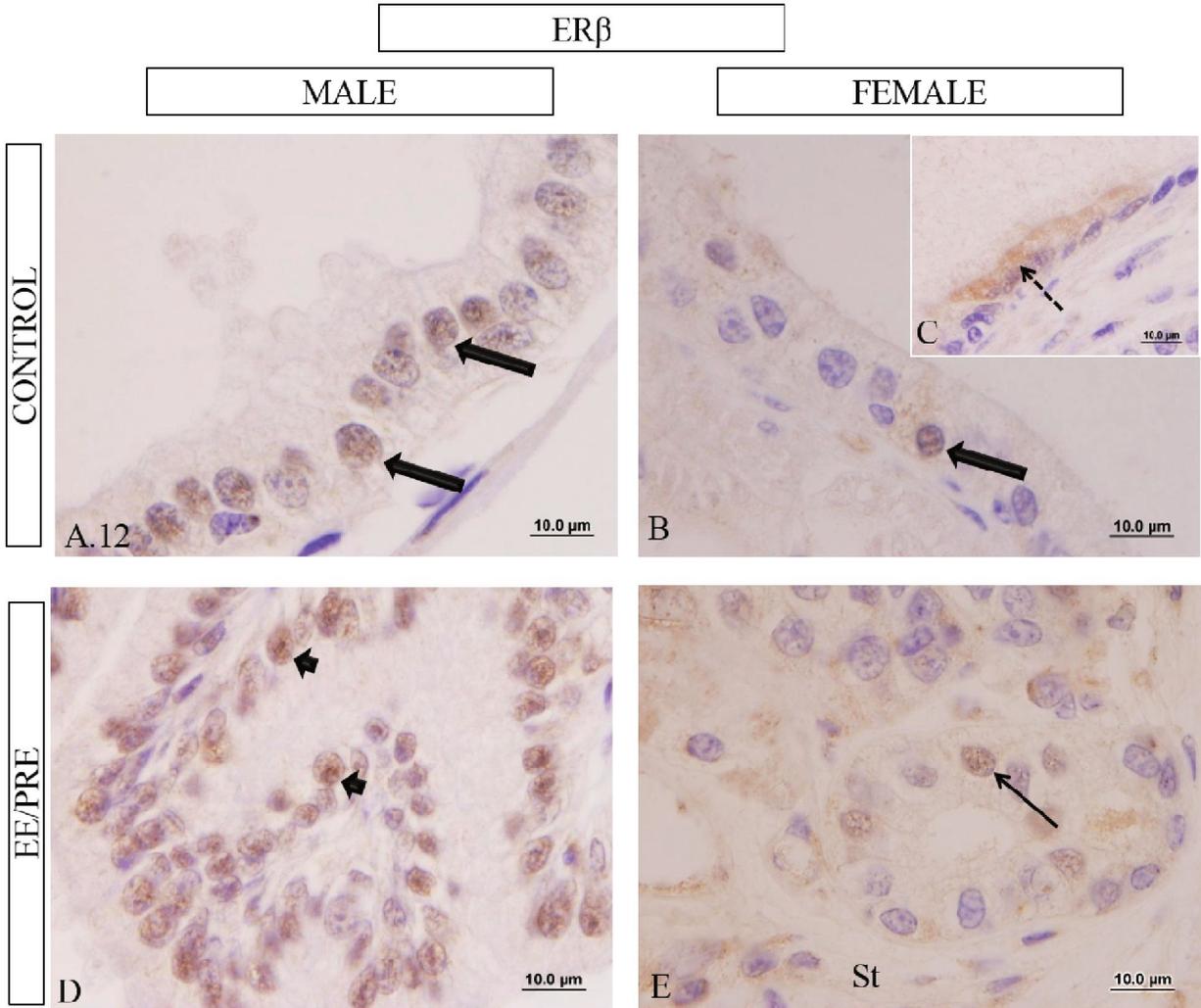
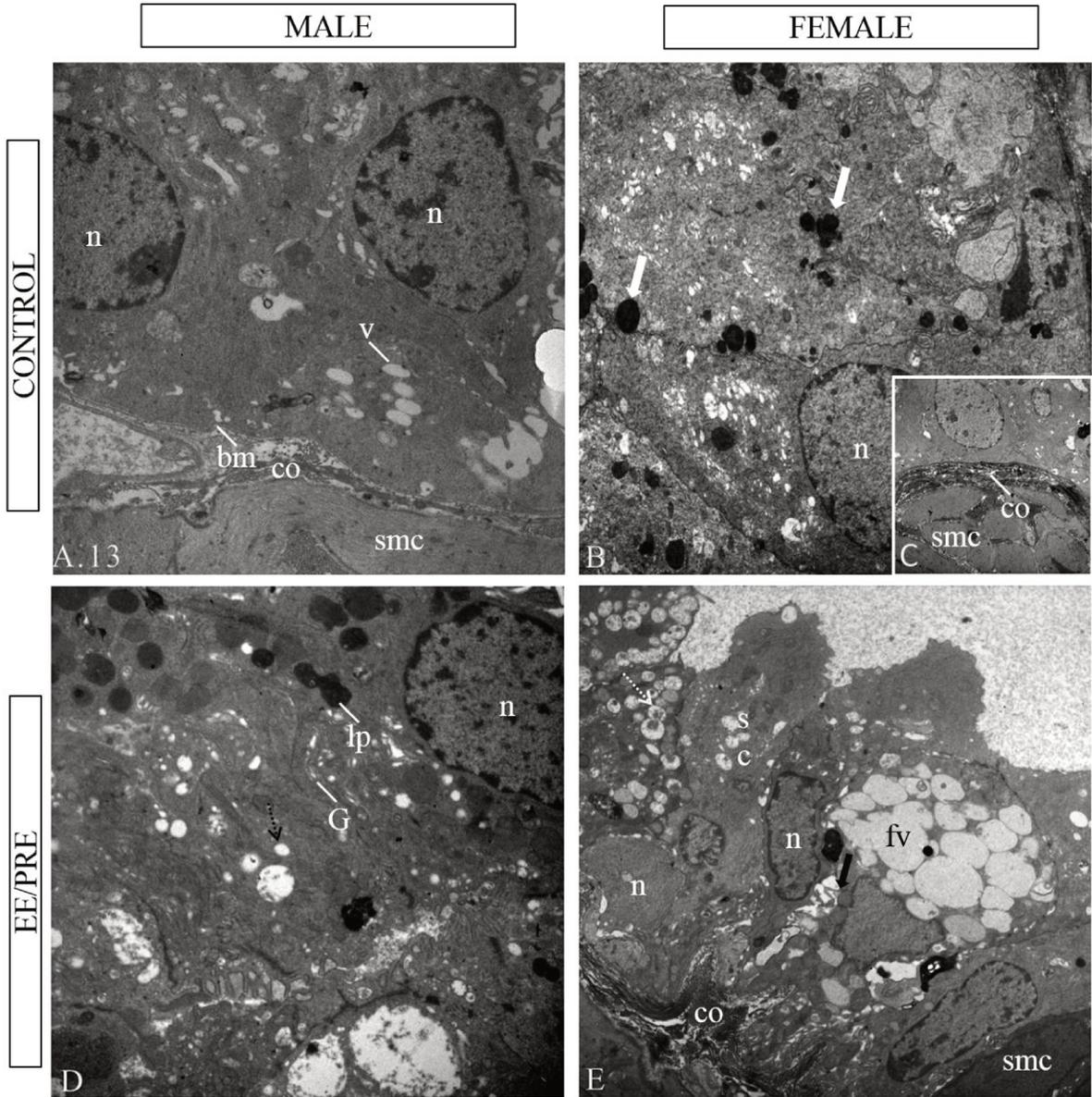


Table 2.

<i>EXPERIMENTAL GROUP</i>		
<i>% EPITHELIAL CELLS</i>		
	<i>CONTROL</i>	<i>EE/PRE</i>
AR	♂ 51.17 ± 14.26	72.53 ± 10.14*
	♀ 80.00 ± 15.44	83.22 ± 12.66
p63 (basal cell)	♂ 11.23 ± 3.25	8.61 ± 3.60*
	♀ 14.00 ± 5.24	18.03 ± 7.60







Artigo 2

**PUBERTY AS CRITICAL PHASE, IN THE EXPOSURE TO ETHINYLESTRADIOL ON
MALE AND FEMALE PROSTATE OF SENILE GERBIL.**

PUBERTY AS CRITICAL PHASE, IN THE EXPOSURE TO ETHINYLESTRADIOL ON MALE AND FEMALE PROSTATE OF SENILE GERBIL.

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Running Title:

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Abstract

Male and female rodent exposed to synthetic estrogens during critical time points of development, such as prenatal and neonatal as well as puberty, affect the prostatic health. Thus, this study examined by serologic, morphologic and immunohistochemistry analysis the effects of exposure to 17 α -ethinylestradiol (EE) during puberty on ventral male and female prostate of senile gerbil. For this, male and female gerbils (n=5) received 15 μ g/Kg/day of EE of the 42th to 49th postnatal life (EE/PUB group) and the animals control group was not exposed. Prostate glands of both sexes were analyzed in senescence. Exposure to EE in the puberty reduced testosterone levels of senile male and decreased the prostatic height epithelial and thickness muscle layer. Additional, were observed increase of multiplicity PIN and prostatic inflammation. In the senile female of EE/PUB group, increased testosterone levels and enhanced the prostatic height epithelial and thickness muscle layer. Immunohistochemistry analysis reported increase of positive cells (%) for AR and PCNA in the male prostate and increase positive basal cell for p63 in the female prostate of EE/PUB. These alterations, thus as increase of immunoreactivity for smooth alpha actin muscle were observed mainly in regions with PIN and invasive lesions, as adenocarcinoma. Exposure to EE during puberty presented an inhibitory action on ventral male prostate and anabolic effect on female prostate of senile gerbil. This study revealed that puberty is a critical period in the exposure to EE during developing prostatic lesions in male and female in the senescence.

Keywords: Puberty, Senescence, Male prostate, Female prostate, ethinylestradiol.

Introduction

Pubertal period is characterized by activation of hypothalamic-pituitary-gonadal axis and stimulates gonadal secretion of sex steroids hormones (androgen and estrogen) (Romeo, 2003). According to Siegford and colleagues (2003), male and female gerbil (*Meriones unguiculatus*) experimental model utilized of this work, presented high testosterone levels with 43 days postnatal life, important event of puberty. The regulations androgen (Pu et al., 2007) and estrogen levels (McPherson et al., 2008) are crucial for prostate development, during rodent puberty, occurs prostate maturation. There is also an additional increase of weight prostate and enhance of ductal branches (Cunha et al., 2004).

Studies show exposure to synthetic estrogen as ethinylestradiol (EE) and bisphenol-A (BPA) during pubertal phase affects the sexual behavior in male rat (Seta et al., 2006) and increases the risk of precocious onset of puberty in female mice (Nah et al., 2011).

Unlike pubertal period, in aging there is a decline steroids hormones levels (Chahal and Drake, 2007) and promotes development of prostatic lesions in gerbil (Campos et al., 2008; Custódio et al., 2010). Process of reproductive aging is regulated by environmental factors, as the exposure to endocrine disruptors chemicals (EDCs) (Diamanti-Kandarakis et al., 2009) during developmental period may disturb reproductive function and result premature reproductive aging (Walker et al., 2013) and predispose to prostate carcinogenesis in rodents (Prins et al., 2007; Prins, 2008) and human (Hu et al., 2012).

EE is compound important of contraceptive oral, named as 17 α -ethinylestradiol (Helles, 1971). These synthetic estrogens act as endocrine disruptors (ED) because block or mimic steroids hormone action (De Coster and van Larebeke, 2012; Schug et al., 2011). Exposure to this estrogen during prenatal period affects health of prostate gland (Thayer et al., 2001; Timms et al., 2005), contributing to an increase of prostatic intraepithelial neoplasia (PIN) in male and female of adult gerbil (Perez et al., 2011; 2012).

Several studies report that male and female gerbil prostate are sensible to steroid hormones actions, independent of treatment phase can be adult phase (Biancardi et al., 2010; Da Silva et al., 2013; Santos et al, 2006) or prenatal period (Biancardi et al., 2012; Perez et al.,

2012). However the environment, which occurs the prostatic development, is crucial for normal prostate function (Cunha et al., 2004) mainly during senescence where there is the increase to risk of prostate disease (Campos et al., 2008; Custódio et al., 2010). Thus, aim of this study was analyze by morphologic, immunohistochemistry and serologic parameters, which would be the effects of exposure to ethinylestradiol during puberty on the ventral male prostate and female prostate in the senescence.

Material and Methods

Animals and Experimental Design

The animals in this study were provided by the São Paulo State University (UNESP) (São José do Rio Preto) and maintained in polyethylene cages under controlled conditions of light (12 h dark/12 h light) and temperature, provided with filtered water in glass bottles and rodent food *ad libitum*. Animal handling and experiments were in accordance with ethical principles of animal research and were approved by Committee for Animal Research (CEEAA) of UNESP.

We utilized 8 senile female gerbils and 8 senile males gerbils (*Meriones unguiculatus*, Muridae: Gerbillinae). The females and males with 42 days of postnatal life received by gavage 15µg/kg/day of ethinylestradiol (EE, Formula: C₂₀H₂₄O₂, Form: solid) (17α-ethinylestradiol; E4876 Sigma, St. Louis, MO, USA) diluted in 100µl of mineral oil Nujol® (CAS 8020-83-5; Sigma-Aldrich, St Louis, MO) during 1 week. These animals received the supplementation of EE during the puberty period (Siegford et al., 2003), named as EE/PUB group and the control group the females and males did not receive this treated.

The male and female of experimental groups were killed with 12 months of age (senile phase). Before this procedure, the females were cycled and they were all in the proestrus phase (Nishino and Totsukawa, 1996).

Biometry

After sacrificing animals, we collected the male organs: ventral prostate, testis, adrenal gland and female organs: prostate, ovary and adrenal gland. All these organs were weighted. Prior to the death, the senile animals were weighted and submitted to anogenital distance (AGD) measurement and utilized a caliper rule King Tools Digital Caliper (0-300 mm).

Serum Hormonal Levels

The senile animals' blood of EE/PUB and control groups were collected and the serum was centrifuged (3000 rpm, 20 min) and stored to -80°C prior to samples analysis of. The hormonal levels were performed in duplicate and we used the Elisa kits (testosterone and 17β-estradiol, Cayman Chemical Company, MI, EUA), following the manufacturer's protocol. The limit of detection for testosterone and 17β-estradiol was respectively 6 pg/mL and 19 pg/mL. The readouts were made in the SpectraMax Plus (384 to 405 nm) lector (Molecular Devices, CA, USA).

Morphologic and Morphometric Analysis

Ventral male prostate and female prostate of experimental groups were fixed in paraformaldehyde buffered to 4% or methacarn (1:3:6 acetic acid, chloroform and methanol), submitted to histological steps and imbibed in Paraplast (Histosec, Merk). After, the glands were sectioned (5µm) and stained with Hematoxylin and Eosin (HE) for morphologic and morphometric comparison of the prostates of experimental groups and for the analysis of reticular and collagen prostatic fibers, we utilized the Gömöri's reticulin technique.

To perform morphometric analyze, were collected 200 measures of prostates of the each senile male and female of experimental groups (n=5). These data refer to thicknesses (µm) of epithelial height and muscle layer of prostates.

The images for these analysis were captured by a microscope Zeiss Jenaval-(Jena, Germany) and the morphometric data were made in the Image-Pro Plus software version 4.5 for Windows (Media Cybernetics, Inc., Silver Spring, MA, USA).

Histopathological analysis

Male ventral prostate and female prostate of EE/PUB and control groups were fixed in paraformaldehyde 4% or methacarn, histologically processed and imbedded in Paraplast (Histosec, Merck). The sections (5µm) of prostatic glands of 5 animals each group were histopathologically analyzed. Through of the multiplicity of inflammatory foci and prostatic intraepithelial neoplasia (PIN), according to the classification of lesions of Shappell and

colleagues (2004). The multiplicity was analyzed according to the number of prostatic lesions found in each section observed in optic microscope.

Immunohistochemistry

The prostates of experimental groups were fixed in paraformaldehyde 4% or methacarn. The glands were performed to histological procedure (dehydration, clarification and infiltration) and imbedded in Paraplast (Histosec, Merk). The sections (5 μ m) were submitted to immunohistochemistry technique for detection of vimentin, PCNA (proliferation cellular of nuclear antigen), p63 protein, AR (androgen receptor), ER α (estrogen receptor alpha) ER β (estrogen receptor beta) and smooth muscle α -actin. Retrieval antigenic was realized in citrate buffer (pH 6.0) at 98°C, after the sections were washed with PBS (Phosphate buffer saline) and for peroxidase blocked utilized H₂O₂ (12%) in methanol. All the antibodies were diluted in BSA 1% (bovine serum albumin) (Sigma, St. Louis, MO, USA) in PBS and incubated at 4°C overnight. Then the sections were incubated with biotinylated anti-rabbit (ABC kit, sc-2018, Santa Cruz Biotechnology CA, USA) (AR) and polymer (Novolink, Leica Novocastra) After the sections were revealed with DAB (diaminobenzidine, Sigma, St. Louis, MO, USA) and counterstained with Harris hematoxylin.

In the immunohistochemistry for PCNA, p63 and AR were performed the count of positive nuclei of prostatic epithelial cells and basal cells of experimental groups. For this count, we used fields of microscopy image (400x) (Olympus BX60) of prostate each group (n=3), totaling 2000 cells. The values of positive nuclei were divided by total number of cells counted, expresses in percentage (%).

Statistical Analysis

The statistical analysis of biometric, morphometric, histopathology, immunohistochemistry and serology data has been performed in spreadsheets and graphic of GraphPad Instat. For the parametric results, Tukey test was used and no parametric results utilized o Mann-Whitney test. The significance level employed was 5% ($p \leq 0.05$) express as media and standard deviation (\pm).

Transmission Electronic Microscopy

After collecting prostatic glands, we sectioned them in small fragments, fixed for 24h in solution: glutaraldehyde 3%, tannic acid 0,25% and glucose 0,54%, diluted in Milloning' buffer pH 7.3 (Cotta-Pereira et al., 1976). The fragments were postfixed with osmium tetroxide 1% during 2h, dehydrated in series increasing of acetone and imbedded in Araldite resin. Posteriorly, the fragments were sectioned in ultrathin sections (50nm) and contrasted with uranyl acetate 2% for 20 min and in chumbo citrate for 6 min. The samples were observed by using the transmission electronic microscope LEO-Zeiss 906 (Zeiss, Cambridge, UK).

Results

The table 1 shows the biometric data of senile male and female gerbil of EE/PUB and control groups. During the biometric analysis, we did not observe significant differences between the experimental groups studied. Meanwhile, the serology analysis seen in figure 2 presented significant differences in testosterone levels. These levels decreased in the senile male of EE/PUB group compared to control group, whereas in the senile female, the testosterone levels increased in the EE/PUB group compared to control group.

The exposure to EE during the puberal phase altered the morphology and morphometry of male and female prostate of senile gerbil (Fig. 3 and 4). In the EE/PUB, senile male group was observed changes in the prostate glandular structure as acinar atrophy with small luminal compartment and presence of secretion eosinophilic (Fig. 3D). Prostate EE/PUB group presented a significant decrease of epithelial height and thickness of muscle layer compared to observed in the prostate of control group (Fig. 3C, F, G and 4). The presence of inflammatory cells within prostatic stroma and epithelium is a characteristic of prostatitis (Fig 3D and E). There was also in the male prostate of EE/PUB group presence of prostatic intraepithelial neoplasia (PIN) and invasion of epithelial cells in direction to prostatic stroma (Fig. 3D, E, F).

In the EE/PUB female group, we also viewed alterations in the prostate glandular structure as acinar atrophy, with small luminal region and papillary cells (Fig. 3L, M) compared to morphology observed in the prostate of control group (Fig. 3H-J). The epithelial height and

thickness of layer muscle this gland increased compared to control group (Fig. 3I and L; 4B). There was presence of PIN with invasion of epithelial cells in the stromal region (Fig. 3L, O). The exposure to EE promoted increasing of multiplicity of prostatic lesions in senile gerbil male and female. We observed a significant increase of PIN in both sexes and increment of prostatic inflammation in the male of EE/PUB group compared to its respective control group (Fig. 3P, Q).

In the prostate stromal compartment, we observed by immunohistochemistry technique, increase of immunoreactivity for smooth muscle alpha actin in regions where invasive prostatic lesions was noted in the EE/PUB male group compared to control group (Fig 5A, B). The female prostate of EE/PUB group presented increase of this immunoreactivity in area with micro invasive lesions compared to prostate of control group (Fig. 5C, D).

In addition, we observed an increase of immunoreaction of vimentin in the cytoplasm of epithelial cells in neoplastic lesions and increments this immunoreactivity in the inflammatory foci present in the prostate of EE/PUB male group (Fig. 5E-F). Whereas, in the EE/PUB female group was not observed increase this immunoreactivity in the prostatic gland (Fig. 5J-L).

The immunohistochemistry for ER α showed different in the male and female prostate of EE/PUB group compared to control group (Fig.6). In the ventral male prostate of senile gerbil was observed immunoreactivity of this receptor mainly in stromal cells (Fig. 6A, B), however in the prostate of EE/PUB group was verified an increase of this immunoreactivity in the prostatic stroma (Fig. 6C, D). In the female prostate, the immunoreaction for ER α was viewed in the epithelial and stromal cells (Fig. 6E, F). Meantime, in the gland of EE/PUB this immunoreaction was bigger, mainly in regions with PIN (Fig. 6G, H).

Other estrogen receptor analyzed in the immunohistochemistry was the ER β , which we verified an increase of immunoreactivity in the nuclei and cytoplasm of epithelial cells of male and female prostate of EE/PUB group, mainly in regions where we observed pre-malignant prostatic lesions (Fig. 6I-P).

In figure 7 is given the immunohistochemistry for AR and PCNA, in which was noted increase of frequency (%) positive epithelial cell in the ventral male prostate of EE/PUB compared to control group. Regarding the immunohistochemistry for p63, we observed decrease this immunoreactivity in the basal cell layer where noted invasive lesions present in the male prostate of EE/PUB group (Fig. 7F).

The exposure to EE during puberal phase did not alter the frequency of positive cells for AR and PCNA in the female prostate (Fig. 8). On the other hand, in the immunohistochemistry for p63 was verified frequency raise of basal cell in the female prostate of EE/PUB compared to control (Fig. 8E-G).

During the ultrastructural analysis, morphologic changes as increase of lipofuscin deposit in the cytoplasm, loss of cellular adhesion between the epithelial cells, alterations in the secretory activity, disarrangement of collagen fibers in the stroma, were observed in the prostate of senile male and female gerbil exposure to EE in the puberty (Fig. 9).

Discussion

Exposure to low dose (15 µg/Kg/day) of EE during puberty, was considered a critical period for prostatic functionally induces the development of prostatic lesions and alters the morphology of male and female prostate during the senescence.

Studies have showed that exposure to EE (20µg/kg/day) during the gestation period increased the male rat AGD in the postnatal life (Tinwell et al., 2002) and malformation of female genitals were found in young and adult exposure to high doses of EE during the prenatal phase (Mandrup et al., 2013). The exposure to EE in the puberty did not alter the senile male and female AGD, thus this estrogen-induced did not influence the gerbil external sexual characteristic in aging.

Synthetic estrogens exposure in critical period affects expression levels of steroids hormones and alters hypothalamic-pituitary-gonadal (HPG) axis may cause changes in gonadal steroidogenesis and sex hormone production (Xi et al., 2011). EE exposed in the pubertal period may be interfered in feedback regulatory mechanism of the HPG-axis decreasing testosterone levels in male and increasing the levels this hormone in female during senescence. Balance between testosterone and estrogen is crucial for development and functionality of prostatic gland (Cunha et. al, 2004; Ellem and Risbridger, 2009). Exposure to EE in pubertal period altered the testosterone estrogen ratio and contributed to changes in the morphometry of prostatic gland normal, as decrease and increase respectively of prostatic epithelium and muscle of male and female during aging.

Low dose of bisphenol-A (BPA), which presented estrogenic activity, exposed in the prenatal period enhanced the smooth muscle cells ratio and altered the differentiation pattern of stromal cells in the prepubertal ventral male prostate (Ramos et al., 2001). EE exposed during puberty altered the stromal-epithelial interaction in senescence as were demonstrated by raise of immunoreactivity for smooth muscle alpha actin and vimentin gerbil prostate, mainly in regions where were observed inflammatory foci and PIN. Vimentin maintains the cytoarchitecture and integrity tissue (Franke et al., 1982), it was found overexpressed in prostate cancer (Satelli and Li, 2011) Alterations in the stromal compartment may increase the invasive potential as was observed in the senile ventral male prostate exposed to EE in the pubertal phase, this data was not peculiar of female prostate. Exposure to EE during the prostatic development changes the epithelial-stroma interactions and it contributed for progress of prostatic lesions in the male gerbil.

ER α is responsible for the basic structure of prostate gland before puberty (Omoto et al., 2005). However, exposure to synthetic estrogens during critical period of prostatic development alters the DNA methylation of genes involved in the signaling pathway of prostatic cells, mechanism named as estrogen imprint (Ho et al., 2006; Prins et al., 2007). Our study showed enhance of immunoreactivity for ER α in the stromal cells of male and female prostate and epithelial cell of female prostate of senile gerbil. The ER α signaling in both stroma and epithelial compartment may be disrupted by neonatal exposure with estrogen (Omoto et al., 2005; Prins et al., 2001a) promoted alterations of stromal compartment and enhanced the development of PIN in aging.

Other receptor estrogen analyzed, was ER β by immunohistochemistry technique, this receptor plays an important role in the antiproliferative acts on the prostate (Weihua et al., 2002). In the senile male exposed to EE, presented low testosterone levels and was observed a significant increase in the multiplicity of prostatic inflammation. The ER β is on positive control of androgen and this receptor mediates the anti-inflammatory acts on prostate when the testosterone levels are high (Harris et al., 2003). Senile female was also exposed to EE during puberty presented high testosterone levels and was noted increase of immunoreactivity for ER β , but was not observed enhance of prostatic inflammatory foci. Thus can report that ER β had anti-inflammatory acts on prostate female senile exposed to EE in the pubertal period.

Study with rodents demonstrated that the exposure to synthetic estrogens during critical phases of prostatic development blocked the paracrine communication between stromal and epithelial cells (Chang et al., 1999). Exposure to EE may have altered the androgen-stimulates process for autocrine mechanism (Gao et al., 2001), thus being responsible by development prostatic carcinogenesis in the adult phase (Prins et al., 2007; Prins, 2008). This exposure influenced the increase of positive nuclei frequency of AR observed in prostatic epithelial cells of senile male, contributing for alterations morphometric and morphologic prostatic during the aging.

Exposure to EE during crucial periods of prostatic development, as the puberty, phase characterized by actions of various neuroendocrine events leads the sexual maturation (Romeo, 2003) promoted some alterations in the stromal and prostatic epithelial of senile gerbil. Other immunohistochemistry techniques were performed to clear these results, as the increase of positive cells frequency of PCNA in the senile male prostate. Proliferation of epithelial cells are induced by exposure to synthetic estrogen and predisposes the development of invasive prostatic lesions in rodent (Prins et al., 2008). Then, the exposure to EE during the puberty is associated to increase proliferation of prostatic epithelial cells in male and with the process of DNA repairs play by PCNA (Zhang et al., 2011) during senescence, in female these actions were not observed.

Another technique used for diagnostic of senile prostate exposure to EE was the immunohistochemistry for p63. This protein found in basal cell, is associated to differentiation, development and proliferation of epithelial cells, but in local, where there is evidence of high-grade PIN and invasive lesions, occurs alterations in the p63 expression (Grisanzio and Signoretti, 2008). This data was evidenced through of decrease in the immunoreactivity for p63 observed in adenocarcinoma regions in the senile prostate of male expose to EE. Meanwhile, the ER α mediates the effects of exposure to synthetic estrogens in stimulates of basal layer proliferation, which cause the multi-layering of the basal cells (Yatkin et al., 2009) data observed in senile prostate of female exposed to EE.

The EE actions on prostate accentuated the ultrastructure aspects observed this of senile male (Campos et al., 2008) and female prostate gland (Custódio et al., 2010). The alterations in testosterone levels observed in the senile male and female was considered the crucial points of exposure to EE during the puberty, thus as altered seen in the immunoreactivity of ER α , ER β and

AR in senile prostate. Signaling pathways this receptors are changed by EE exposure actions on prostate (De Coster and van Labereke, 2012, Prins et al., 2001b). These changes may be associated to EE play as predisposing factor to lesions severe development in male and female prostate in senescence. As pubertal period is associated to enhance steroids hormones levels (Siegford et al., 2003, Vilamaior et al, 2006). The exposure to EE in puberal period possibly altered the mechanisms of steroid hormone production during of this period and as consequence, the sexual hormone levels changed in the senescence, contributing for significant morphologic changes in male and female prostate of senile gerbil.

Different of prostate gland development in humans (Timms, 1997), in the rodents bud initiation commences in late fetal life and neonatal phase occurs the branching morphogenesis (Cunha et al., 2004). The main molecular signaling pathways that regulate developing rodent prostate were reported in these periods (Prins and Putz, 2008). Thus, the rodent prostate as gerbil is a good model to study the effects of exposure to EE during prenatal period on adult phase (Perez et al., 2011; 2012). In the pubertal period, occurs final growth and maturations of prostate gland (Staack et al., 2003). Our present results revealed that the exposure to EE during the puberty affect the structural and ultrastructural morphology of ventral male prostate and female prostate and increase developing prostatic lesions as PIN and prostatic inflammation during senescence. Exposure to EE, showed puberty can be considered a critical phase developing male and female prostate, as the prenatal (Perez et al., 2011; 2012) and neonatal (Putz et al., 2001) periods.

Conclusion

The present study revealed effects different of exposure to EE during the puberty on ventral male prostate and female prostate in senile gerbil. Our further studies showed adult ventral male prostate is more sensible than female prostate when exposed to EE during prenatal period (Perez et al., 2011; 2012). Estrogen-exposure in the puberty reduced testosterone levels in senile male, decrease the prostatic epithelium and muscle layer and increase the development of PIN and prostatic inflammation. Whereas, in the senile female this exposure raised testosterone levels, enhanced the prostatic epithelium and muscle layer and increased the multicity of PIN. These results different observed between the sexes showed the exposure to EE acts as inhibitor of

prostatic glandular structure and contributed for development of invasive lesions in senile male prostate. In the female, the EE has anabolic effects on prostate and increase the emergence of neoplastic lesions of this gland. Thus, puberty may be considered a critical period during the exposure to ethinylestradiol on male and female prostate of gerbil in the senescence.

Declaration of interest

The authors declare that there is no conflict of interest associated with this manuscript.

Acknowledgements

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Table and Figure

Figure 1. Schematic representation of experimental groups. Male and female gerbil were exposure to EE (17α -ethinylestradiol) of the 42th to 49th days of life during puberty (EE/PUB group). In the control group (C), the gerbils were not exposed to EE. These gerbils (n=5) were killed with 12 months (360 days) of life, in the senescence.

Table 1. Biometric data of male and female of senile gerbil of experimental groups.

Values expresses as media \pm standard deviation (n=5).

Figure 2. Estradiol levels (pg/ml) (A) and testosterone levels (ng/ml) (B) of male and female of senile gerbil of experimental group.

*Significant difference between the groups ($p \leq 0.05$). Values expresses as media \pm standard deviation (n=5).

Figure 3. Morphology of ventral male prostate and female prostate of senile gerbil of experimental group stained with hematoxylin and eosin. *Control*: A-C. (σ). Prostatic acini with large lumen (Lu), coat by epithelium (Ep) and surrounded by stroma (St) with smooth muscle (Mu). *EE/PUB* (σ): D-G. Presence of acinar atrophy (at) with small lumen and eosinophilic secretion (*) and observed inflammatory cells into prostatic stroma, inflammation (IF). Regions with prostatic intraepithelial neoplasia (PIN) and invasion of epithelial cells in the stroma (large arrow), rupture of muscle layer (**). Decrease of height prostatic epithelial (arrow). *Control* (φ): H-J. Epithelium (Ep) line the prostatic acini with large lumen (Lu) and surrounded by smooth muscle (Mu) in the stroma (St). *EE/PUB* (φ): L-O. Noted the presence of acinar atrophy (at) with papillary cells and small lumen (**). Increase of layer muscle (double arrow) and of epithelial height (arrow) of prostate and progression of epithelial cell in the stromal region (large arrows). Detail for secretory activity with presence of blebs (arrowhead). P-Q. Multiplicity of PIN and prostatic inflammation in the male and female prostate of experimental group. *Significant difference between the groups ($p \leq 0.05$). Values expresses as media \pm standard deviation (n=5).

Figure 4. Morphometric data of male (A) and female (B) senile gerbil prostate of experimental group.

*Significant difference between the groups ($p \leq 0.05$). Values expresses as media \pm standard deviation (n=5).

Figure 5. Histological sections of ventral male prostate and female prostate submitted to immunohistochemistry for smooth muscle α -actin and vimentin. *Control*: A, C. Immunoreactivity for α -actin (arrows). Prostatic epithelium (Ep) and lumen (Lu). *EE/PUB*: B, D. Increase of

immunoreactivity for α -actin (large arrows) in invasive (**) and microinvasive (*) prostatic lesions foci. Presence of stromal cells in the prostatic lesions (double arrow). *Control*: E, F, I, J. Immunoreaction for vimentin in the prostatic stroma (St) (large arrows). *EE/PUB*: G, H. In the male prostate was observed an increase of this immunoreaction in inflammatory foci (dashed arrow) (IF) and local with PIN (arrowhead). L, M. In female prostate was seen immunoreactivity for vimentin in vessels (double arrows) and in the prostatic stroma (arrow).

Figure 6. Histological sections of ventral male prostate and female prostate submitted to immunohistochemistry for ER α and ER β . *Control*: A, B, E, F. Presence of Immunoreactivity for ER α in the stromal cells (arrows) of prostate (σ and φ) and prostatic epithelial cells (φ) (arrowhead). *EE/PUB*: C, D, G, H. There was increase of this immunoreactivity in the prostatic stromal (σ and φ) (dashed arrow) and in the epithelial cells of prostate (φ), mainly in regions with PIN. *Control*: I, J, N, O. Note the immunoreactivity for ER β in the prostatic epithelial cells (σ and φ) (arrows). *EE/PUB*: L, M (σ). Detail of this immunoreaction (double arrow) in locals with adenocarcinoma in situ (**). P, Q (φ). Increase of immunoreactivity in the prostatic epithelial cells and it was observed in regions with presence of PIN (large arrows).

Figure 7. Histological sections of ventral male prostate submitted to immunohistochemistry for AR, PCNA and p63. *Control*: A. Positive epithelial (Ep) cells for AR (arrow). *EE/PUB*: B. Increase this immunoreactivity in the epithelial cells and in locals with PIN (large arrow). *Control*: C. Positive PCNA in the prostatic epithelial (dashed arrow). Muscle layer (Mu). *EE/PUB*: D. Increase of this positive cells for PCNA (arrow) in the invasive lesions (**). presence of vessels (vs). *Control*: E. Note the positive basal cells for p63 (large arrows). *EE/PUB*: F. Decrease of immunoreactivity for p63 (double arrows) in malignant lesions (**). G. Frequency (%) positive cells for AR, PCNA and p63 in the ventral male prostate of experimental group.

*Significant difference between the groups ($p \leq 0.05$). Values expresses as media \pm standard deviation (n=5).

Figure 8. Histological sections of female prostate submitted to immunohistochemistry for AR, PCNA and p63. *Control: A. and EE/PUB: B.* Positive epithelial (Ep) cells for AR (arrow). *Control: C and EE/PUB D.* Positive PCNA in the prostatic epithelial (dashed arrow). *Control: E.* Observe the positive basal cells for p63 (large arrows). *EE/PUB: F.* Increase of immunoreactivity for p63 in the basal cell layer of prostate (double arrows). *G.* Frequency (%) positive cells for AR, PCNA and p63 in the female prostate of experimental group.

*Significant difference between the groups ($p \leq 0.05$). Values expresses as media \pm standard deviation (n=5).

Figure 9. Ultrastructural analysis of ventral male prostate and female prostate of senile gerbil. *Control: A (♂).* Observe the epithelial cells with nuclei voluminous (n), presence of lipid droplets in the cytoplasm (black large arrow). Secretory activity with presence of vesicles (*). In the prostatic stromal region evidence of fibroblast (fb), collagen (co) and smooth muscle (sm). *B (♀).* We also observed the presence of epithelial cells with nuclei large (n). Detail for Golgi apparatus (G) and vesicles (*). Cellular adhesion between the epithelial cells (white large arrow). Osmiophylic membranous structures along the lateral membrane and in cytoplasm (arrowhead) *EE/PUB. C-D (♂ and ♀).* Loss of cellular adhesion between the epithelial cells (double arrows). Detail for vesicles (arrow). Disarrangement of collagen in stroma (dashed arrow). High amount of Golgi apparatus (large arrow) and Osmiophylic membranous structures in cytoplasm (white arrows). A: 3597x, B: 6000x, C and D: 7750x.

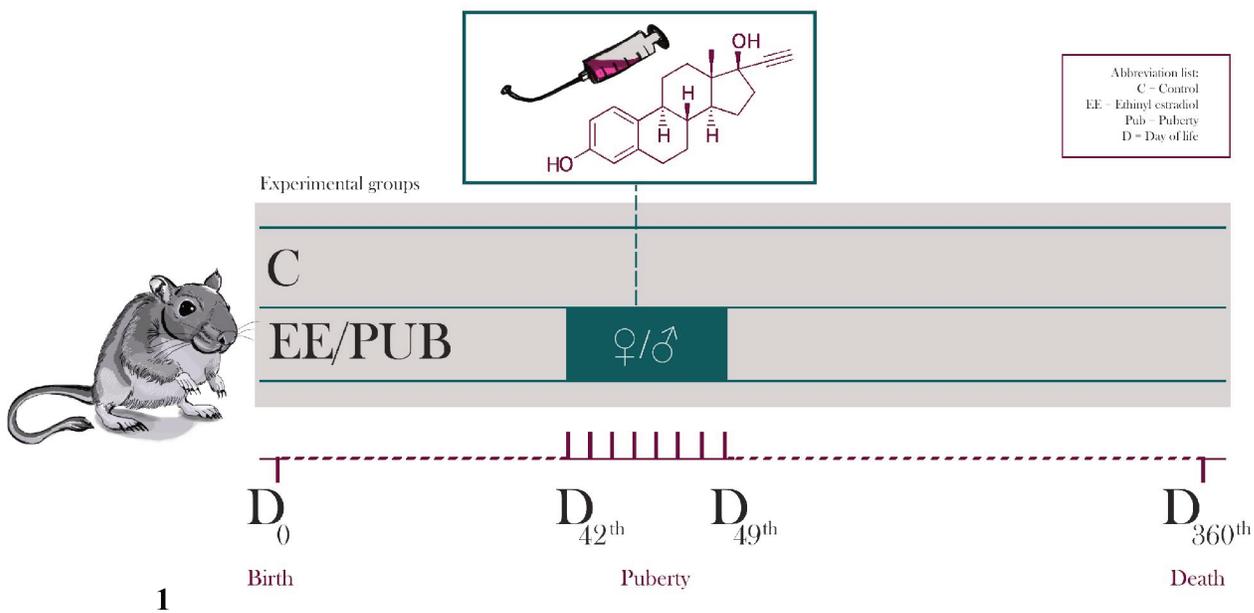
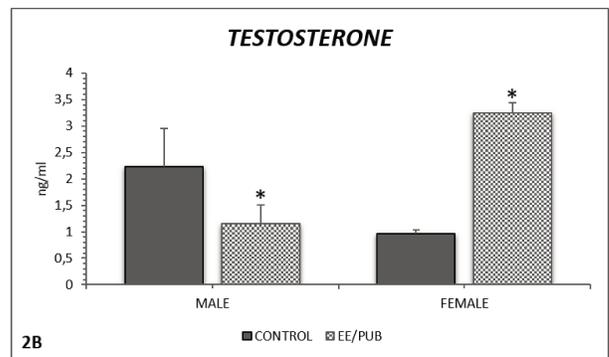
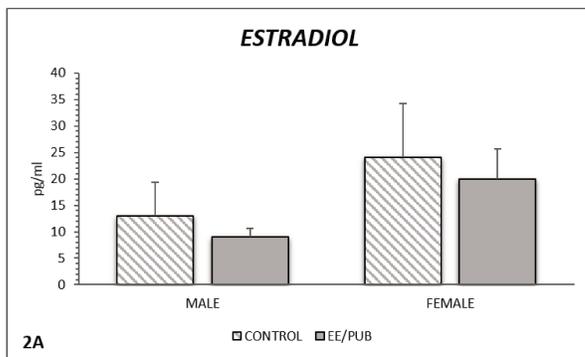
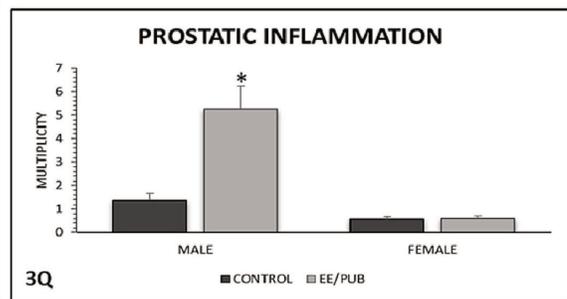
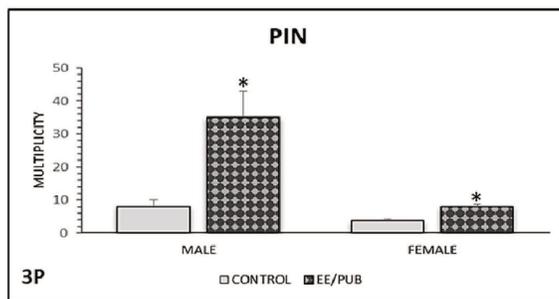
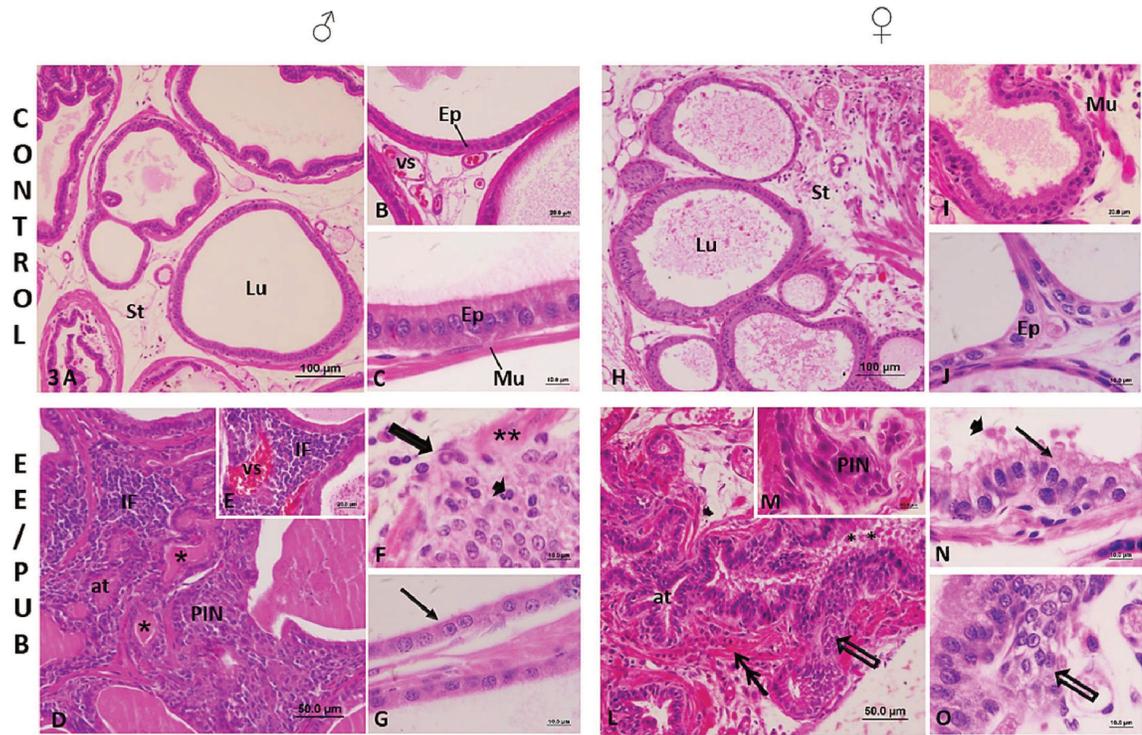
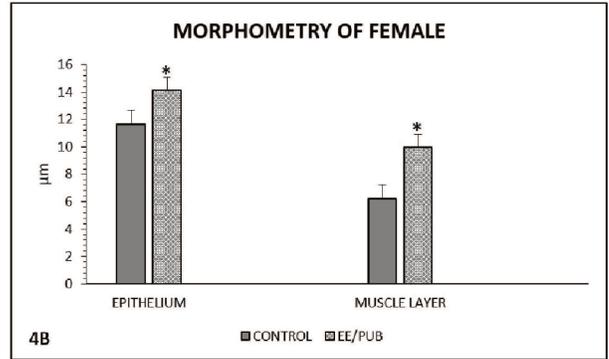
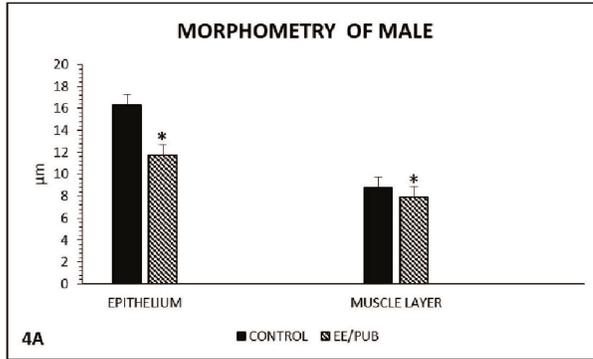


Table 1.

	<i>MALE</i>		<i>FEMALE</i>		
	<i>CONTROL</i>	<i>EE/PUB</i>	<i>CONTROL</i>	<i>EE/PUB</i>	
Body Weight (g)	90.44 ± 14.40	78.44 ± 6.69	Body Weight (g)	68.60 ± 7.12	67.20 ± 9.81
Ventral Prostate (g)	0.02 ± 0.01	0.02 ± 0.01	Prostate + urethra (g)	0.04 ± 0.01	0.03 ± 0.01
Relative Weight of Ventral Prostate (x10 ⁻³)	0.25 ± 0.07	0.29 ± 0.09	Relative Weight of Prostate + Urethra (x10 ⁻³)	0.43 ± 0.22	0.46 ± 0.07
Testis (g)	1.30 ± 0.11	1.23 ± 0.10	Ovaries (g)	0.04 ± 0.01	0.07 ± 0.04
Adrenal (g)	0.04 ± 0.01	0.05 ± 0.01	Adrenal (g)	0.04 ± 0.01	0.04 ± 0.01
AGD (mm)	12.48 ± 1.49	11.26 ± 1.88	AGD (mm)	3.93 ± 0.49	3.24 ± 1.15

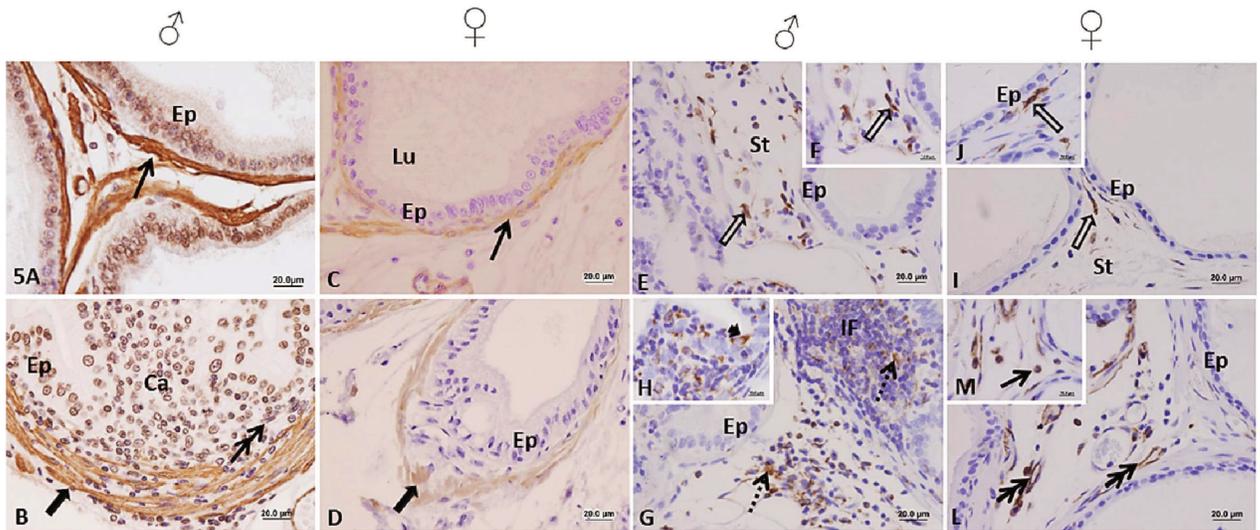


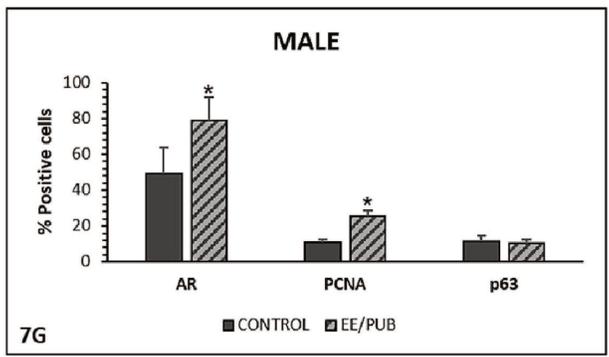
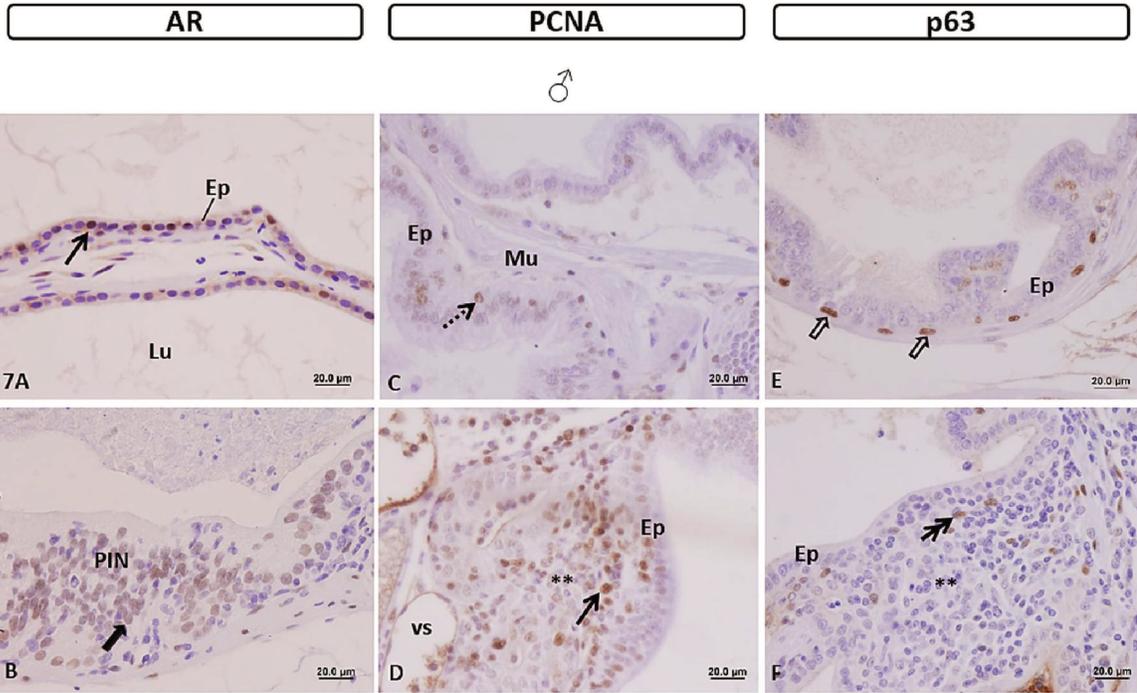




α-actin

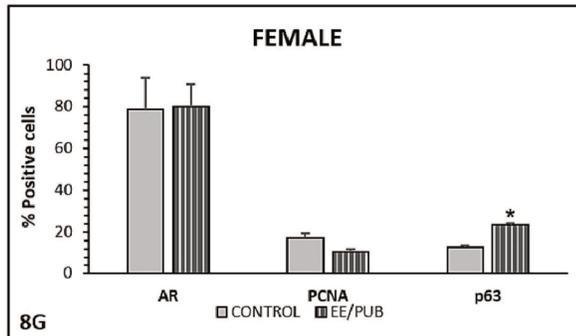
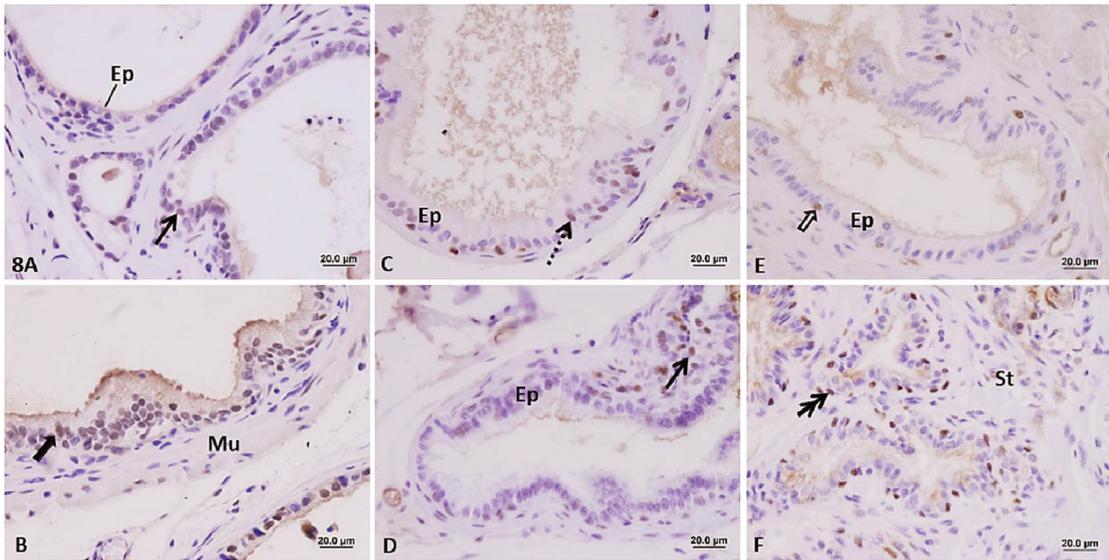
Vimentin





AR
PCNA
p63

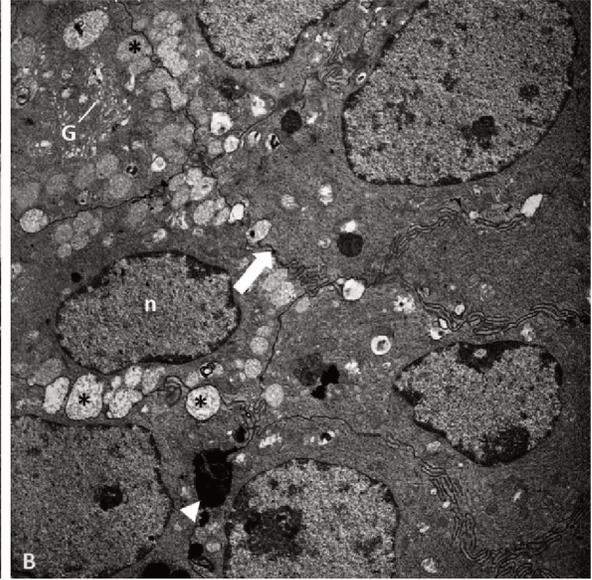
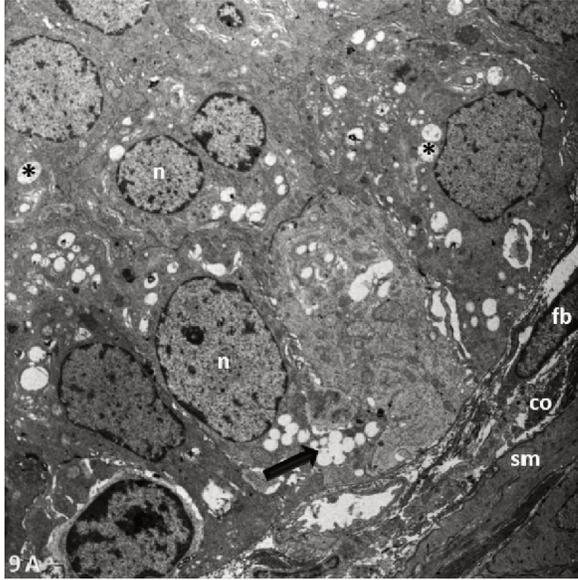
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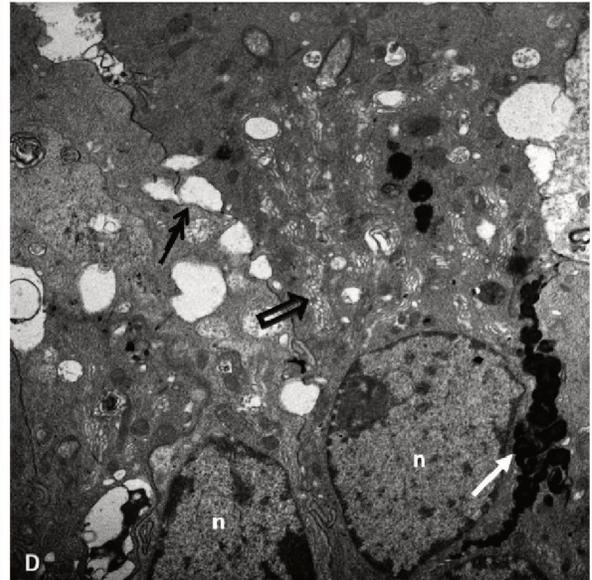
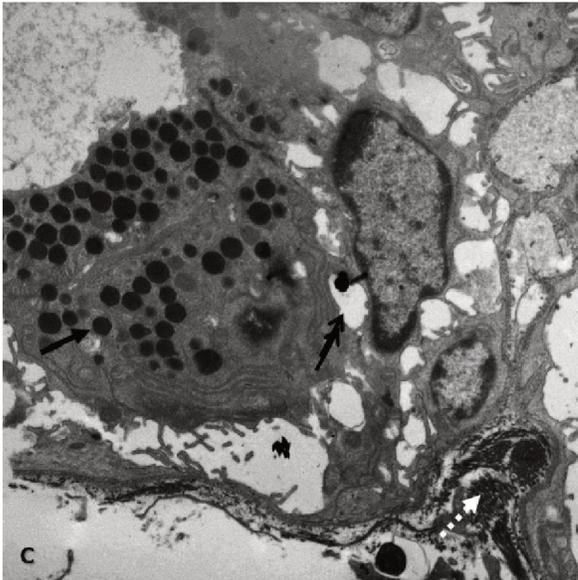
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Artigo 3

17 α -ETINILESTRADIOL EXPOSTO DURANTE OS PERÍODOS PRÉ-NATAL E PUBERAL ALTERA A ESTRUTURA GLANDULAR PROSTÁTICA DE GERBILOS MACHOS E FÊMEAS SENIS.

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Resumo

Esse estudo teve como objetivo analisar como seria a resposta da glândula prostática masculina e feminina quando gerbilos senis fossem expostos à baixa dose de 17 α -etinilestradiol (EE) (15 μ g/kg/dia) nos períodos pré-natal e puberal (grupo EE/PRÉ-PUB). Para isso a próstata ventral masculina e a próstata feminina foram submetidas às análises morfométricas, morfológicas, imunohistoquímicas. E os animais senis dos grupos experimentais, submetidos à análise sorológica. Nossos resultados mostraram que a exposição ao EE durante o desenvolvimento prostático diminuiu os níveis de testosterona nos machos senis do grupo EE/PRÉ-PUB. Enquanto que nas fêmeas senis do grupo EE/PRÉ-PUB foi observado aumento nos níveis de estradiol, comparado aos seus respectivos controles. Exposição à estrógenos sintéticos durante fases do desenvolvimento interfere nas vias de produção dos hormônios sexuais, esse fato pode ter contribuído para as alterações nos níveis hormonais durante o envelhecimento, mostrado em nosso estudo. O etinilestradiol, como agonista de estrógeno exposto durante o período pré-natal e puberal contribuiu para as alterações na morfometria e morfologia prostática de machos e fêmeas senis. A imunorreatividade de AR na próstata ventral masculina do grupo EE/PRÉ-PUB diminuiu e a imunorreação de ER α aumentou nas células epiteliais prostáticas dos machos e das fêmeas senis. Alterações nessas vias podem ter contribuído para o desenvolvimento de lesões prostáticas neoplásicas e invasivas observadas nas próstatas de ambos os sexos. A exposição ao EE durante períodos importantes, como o pré-natal e puberal interfere na estrutura glandular prostática na senescência, este fato mostra que o consumo ao EE prejudica a saúde prostática masculina e feminina, tendo o gerbilo da Mongólia como um ótimo modelo de estudo sobre a ação desse disruptor endócrino.

Materiais e Métodos

Delineamento experimental

Para a realização do presente experimento foram utilizadas 5 fêmeas de gerbilo (*Meriones unguiculatus*) virgens adultas (90 a 120 dias). Cada uma destas fêmeas foi mantida com um macho da mesma idade para a formação de diferentes famílias. Esses animais receberam ração *ad libitum* e água filtrada em garrafas de vidro e foram mantidos em um biotério na UNESP/IBILCE (São José do Rio Preto - SP) de acordo com os princípios éticos da experimentação animal e aprovado pelo Comitê de ética na experimentação animal (CEEA) do instituto.

As famílias formadas foram separadas em dois grupos diferentes. No grupo EE/PRÉ-PUB, as fêmeas grávidas receberam por meio de gavagem 15 µg/kg/dia de etinilestradiol (EE, 17 α -ethinylestradiol; Sigma, St. Louis, MO, USA) diluído em 100 µl de óleo mineral Nujol® (CAS 8020-83-5; Sigma-Aldrich, St Louis, MO), segundo modificações do método de Thayer e colaboradores (2001). A exposição ao EE ocorreu do 18º ao 22º dia de gestação, período no qual ocorre a morfogênese prostática em gerbilos (Sanches et al., 2013). Os filhotes machos e fêmeas que foram expostos ao EE no período gestacional também receberam a mesma suplementação de EE, do 42º ao 49º dia de vida pós-natal, período da puberdade em gerbilos (Siegford et al., 2003). Os machos e as fêmeas do grupos EE/PRÉ-PUB e controle foram mortos quando completaram 12 meses de vida, na senescência (Fig. 1).

Biometria

Após a morte, foram medidos os pesos corpóreos dos gerbilos machos e fêmeas senis dos grupos experimentais. Assim como, o complexo prostático, a próstata ventral, dorsal e dorso lateral, os testículos e as adrenais dos machos. Nas fêmeas, foram coletados e pesados a próstata juntamente com a uretra, os ovários e as glândulas adrenais. Antes das fêmeas serem mortas, estas foram cicladas, estando todas na fase proestro (Nishino e Totsukawa, 1996), já que a próstata feminina tem a sua morfometria e morfologia alterada nas diferentes fases do ciclo estral (Fochi et al., 2008).

Anterior à coleta dos órgãos, os machos e as fêmeas dos grupos experimentais foram submetidos à mensuração da distância anogenital (DAG), utilizando-se um paquímetro King Tools Digital Caliper (0-300 mm), segundo modificações do método de Swan e colaboradores (2005).

Níveis séricos hormonais

O sangue dos gerbilos senis machos e fêmeas dos grupos EE/PRÉ-PUB e controle foi coletado no momento da decapitação. O soro foi centrifugado (3000 rpm, 20 min) e estocado à 80°C até o momento da análise. As dosagens hormonais foram realizadas em duplicata com o uso de ELISA e kits de alta sensibilidade (testosterona e 17 β -estradiol, Cayman Chemical Company, MI, EUA), seguindo as instruções dos fabricantes. O limite de detecção para a testosterona e para o 17 β -estradiol foi de 6 pg/mL e 19 pg/mL, respectivamente. As leituras foram realizadas no leitor SpectraMax Plus 384, a 405 nm (Molecular Devices, CA, EUA).

Morfologia e Morfometria

A próstata ventral masculina e a próstata feminina dos grupos experimentais foram fixadas em paraformol tamponado a 4% ou metacarn (na proporção 1: 3: 6 respectivamente de solução de ácido acético, clorofórmio e metanol), submetidas às etapas histológicas e incluídas em Paraplast (Histosec, Merk). Posteriormente, os órgãos foram seccionados a 5 μ m e corados com Hematoxilina-Eosina (HE) para estudos morfológicos e morfométricos das próstatas (Behmer et al., 1976) e pela técnica de reticulina de Gömöri para análise do compartimento estromal prostático (fibras reticulares e colágenas prostáticas).

Durante a análise morfométrica, foram coletados 200 medidas de cada próstata ventral masculina e próstata feminina dos grupos experimentais (n=5). As medidas coletadas (μ m) foram da altura epitelial e da espessura da camada muscular das próstatas analisadas. A captura das imagens para a análise morfométrica e morfológica das próstatas foram realizadas, utilizando-se o programa Image Software Pro-Plus versão 4.5 para Windows.

Imunohistoquímica

Para a realização das técnicas de imunohistoquímica, as próstatas dos grupos experimentais foram fixadas em paraformol tamponado a 4% ou metacarn, submetidos às etapas de processamento histológico (desidratação, diafanização e infiltração), incluídos em Paraplast (Histosec, Merck) e seccionados à 5µm. Os cortes foram submetidos à imunohistoquímica para detecção de PCNA (antígeno nuclear de proliferação celular), p63 (marca células basais), alfa actina de músculo liso e dos receptores AR (receptores de andrógenos), ER α e ER β (receptores de estrógeno alfa e beta, respectivamente), a tabela 1 mostra a descrição detalhada de cada anticorpo utilizado. A recuperação antigênica foi realizada em tampão citrato (pH 6,0) à alta temperatura (98°C) por 45 min, seguida por três lavagens de 5min com fosfato salina tamponado (PBS). Durante o bloqueio de peroxidase, utilizou-se H₂O₂ a 12% em metanol por 20 min. Posteriormente, todos os cortes foram submetidos ao bloqueio de proteínas inespecíficas (leite em pó diluído em PBS por 30 min) e os anticorpos foram diluídos (tabela 1) em BSA 1% em PBS e os cortes foram incubados no dia seguinte, overnight (4°C). Os cortes foram incubados com Polímero (Novolink, Novocastra) a 37°C por 45 min, em seguida revelados com diaminobenzidina (DAB, Sigma, St. Louis, MO, USA) e contra corados com Hematoxilina de Harris.

Análise Estatística

As análises estatísticas dos dados biométricos, morfométricos e sorológicos foram realizadas em planilhas e gráficos do GraphPad InStat. Para a comprovação da significância dos resultados paramétricos foram utilizados o teste Tukey. Para os dados não-paramétricos utilizou-se o teste Mann-Whitney. O nível de significância adotado foi de 5% ($p \leq 0,05$) expressos em média e desvio padrão (\pm).

Resultados

Biometria e Sorologia

Os dados biométricos dos machos e fêmeas senis representados na tabela 2, mostram que o peso corpóreo, peso da próstata masculina e feminina, peso das gônadas e das adrenais de

ambos os sexos, assim como a medida da distância anogenital não foram alterados significativamente quanto à exposição ao EE durante as fases pré-natal e puberal.

Entretanto durante a análise sorológica, foram observadas alterações significativas nos níveis dos hormônios esteroides (testosterona e estradiol) dos machos e fêmeas senis do grupo EE/PRÉ-PUB comparado ao grupo controle (tabela 3). Nos machos do grupo EE/PRÉ-PUB, os níveis de testosterona diminuíram, comparado ao grupo controle. Enquanto que, nas fêmeas do grupo EE/PRÉ-PUB observou-se aumento nos níveis de estradiol comparado ao grupo controle.

Morfologia e Morfometria

A exposição ao EE durante as fases pré-natal e puberal promoveu evidentes alterações na morfologia (Fig. 2) e morfometria (tabela 3) das próstatas de machos e fêmeas senis. Na próstata dos machos do grupo EE/PRÉ-PUB observou-se uma região luminal ampliada e a presença de regiões com adenocarcinoma mucinoso, presença de mucina no citoplasma das células epiteliais, característica que não foi evidente na próstata controle (Fig. 2A-E). A altura epitelial e a espessura da camada muscular da glândula prostática do grupo EE/PRÉ-PUB apresentaram-se maiores do que a medida observada no grupo controle (tabela 3).

Na próstata das fêmeas senis do grupo EE/PRÉ-PUB, notou-se a presença de microacinos com o compartimento luminal reduzido comparado ao grupo controle e regiões com neoplasia intraepitelial prostática (NIP) (Fig. 2F, G, I). Em ademais, evidenciou-se no citoplasma epitelial prostático das fêmeas expostas ao EE, pequenas vesículas, caracterizando a presença de metaplasia mucinosa (Fig. 2H) e aumento da altura epitelial dessa glândula comparada ao grupo controle (tabela 3).

Em relação à morfologia do compartimento estromal prostático, observou-se a ruptura das fibras reticulares subepitelial em locais onde se observou a presença de adenocarcinoma na próstata ventral masculina do grupo EE/PRÉ-PUB. Na próstata feminina do grupo EE/PRÉ-PUB relatou-se um desarranjo das fibras reticulares localizadas na região subepitelial.

Imunohistoquímica

Durante a análise imunohistoquímica observou-se a imunorreatividade dos receptores AR, ER α e ER β na próstata ventral masculina e na próstata feminina de gerbilos senis dos grupos EE/PRÉ-PUB e controle (Fig. 4). Com essa técnica foi possível observar a diminuição da imunorreatividade do AR nas células epiteliais da próstata ventral masculina do grupo EE/PRÉ-PUB comparada ao grupo controle. Na próstata feminina, não se observou essa alteração (Fig. 4A-D).

Na imunohistoquímica para ER α , constatou-se aumento da imunorreatividade nas células epiteliais da próstata ventral masculina e da próstata feminina do grupo EE/PRÉ-PUB comparado ao grupo controle (Fig. 4E-H). Em relação à imunohistoquímica para ER β , observou-se diminuição da imunorreação desse receptor nas células epiteliais da próstata ventral masculina do grupo EE/PRÉ-PUB e aumento dessa imunorreatividade no citoplasma das células epiteliais da próstata feminina do grupo EE/PRÉ-PUB, comparado aos seus respectivos controles (Fig. 4I-M).

Em ademais, na próstata ventral masculina do grupo EE/PRÉ-PUB verificou-se aumento da imunoreatividade do PCNA, principalmente em regiões com focos de NIP (Fig. 5A, B). A glândula prostática feminina também apresentou este aumento na imunoreatividade de PCNA comparado ao grupo controle (Fig. 5C, D).

A camada basal prostática dos machos e fêmeas senis dos grupos experimentais foi analisada por meio da técnica de imunohistoquímica para p63. Assim, foi possível observar uma diminuição da imunoreatividade de p63, principalmente em regiões onde verificou-se lesões invasivas como adenocarcinoma na próstata ventral masculina do grupo EE/PRÉ-PUB (Fig. 5E, G). Entretanto, na próstata feminina não se observou alterações nesta imunoreatividade (Fig. 5F, H).

imunoreatividade de α -actina em regiões onde foi identificado adenocarcinoma prostático nos machos senis do grupo EE/PRÉ-PUB (Fig. 5I-L). Enquanto que nas fêmeas do grupo EE/PRÉ-PUB, essa imunoreatividade mostrou-se aumentada próxima de focos de PIN (Fig. 5J-M).

Discussão

A exposição ao EE durante fases críticas do desenvolvimento como pré-natal e puberal alterou o equilíbrio da razão testosterona e estradiol, promovendo alterações significativas na morfologia e imunohistoquímica de machos e fêmeas durante a senescência.

Os níveis de testosterona diminuíram nos machos senis e aumentaram, nas fêmeas senis do grupo EE/PRÉ-PUB. Uma provável explicação reside no fato de que, a exposição à compostos sintéticos com atividade estrogênica altera as vias do eixo hipotálamo-pituitária-gonadal (HPG), interferindo na produção de hormônios esteroides como a testosterona e o estradiol (Frye et al., 2012). O que contribuiu para a diminuição da altura epitelial e da espessura da camada muscular da próstata masculina e aumento da próstata feminina de gerbilos senis expostos precocemente ao EE.

Estudos mostram que a exposição aos estrógenos sintéticos durante o desenvolvimento altera a metilação do DNA de genes que estão relacionados com vias de sinalização nas células prostáticas, processo denominado *imprint* estrogênico (Ho et al., 2006; Prins et al., 2007). O nosso estudo permitiu observar a presença de focos de lesões prostáticas como PIN e adenocarcinoma na próstata ventral masculina e, PIN e metaplasia na próstata feminina de gerbilos senis expostos ao EE.

O *imprint* estrogênico promovido pela exposição ao EE durante o período pré-natal e puberal pode ter alterado a interação epitélio-estroma, contribuindo para a criação de um microambiente adequado para a formação de inflamações e lesões neoplásicas na próstata de gerbilo na fase senil. Na próstata ventral masculina e na próstata feminina do grupo EE/PRÉ-PUB, observou-se alterações nos arranjos das fibras reticulares e colágenas. Estudo mostra que a exposição à estrógeno sintético inibem a ação do ER β (Pellegrini et al., 2013), este receptor apresenta ação antiproliferativa (Morani et al., 2008) e a diminuição dos seus efeitos sobre a glândula prostática pode levar ao desenvolvimento de lesões prostáticas e alterações na interação epitélio-estroma como foi observado na próstata de machos e fêmeas de gerbilos senis.

O etinilestradiol é considerado um disruptor endócrino (DE) com atividade agonista, no qual ativa a ação estrogênica (De coster e Larebeke, 2012). A exposição ao EE durante períodos cruciais do desenvolvimento, como a puberdade, fase caracterizada pela ação de vários eventos neuroendócrinos que levam a maturação sexual (Romeo, 2003), promoveu severas alterações na

próstata de gerbilos senis. A proliferação das células epiteliais é altamente induzida pela exposição aos estrógenos sintéticos, promovendo o aparecimento de lesões invasivas (Prins et al., 2008), dado mostrado no aumento da imunorreatividade de PCNA nas próstatas masculinas e femininas do grupo EE/PRÉ-PUB. Outro diagnóstico utilizado para analisar a morfologia prostática foi o p63. Na próstata dos machos senis do grupo EE/PRÉ-PUB a imunorreatividade de p63 diminuiu principalmente em locais com NIP, segundo Grisanzio e Signoretti (2008) a diminuição ou ausência da expressão do p63 é diagnóstico para identificar o grau da lesão estudada. Os resultados revelaram que a ação agonista do EE durante os períodos pré-natal e puberal, prejudica principalmente a próstata ventral masculina de gerbilo senil.

A próstata masculina e a feminina de gerbilos adultos respondem de maneiras diferentes à exposição aos disruptores estrogênicos durante o desenvolvimento, sendo que a próstata ventral masculina é mais sensível à ação do etinilestradiol do que a próstata feminina (Perez et al., 2011; 2012). O cérebro de mamíferos é extremamente sensível à ação dos hormônios durante as fases do desenvolvimento (pré-natal, puberdade), e diferenças no desenvolvimento do cérebro entre o sexo masculino e feminino pode resultar diferentes efeitos à exposição aos DE (Gore, 2008; De Coster e van Larebeke, 2012). A exposição ao EE durante a puberdade e o período pré-natal promove efeitos diferentes entre a próstata masculina e a feminina, pelo fato que na próstata de gerbilo macho, a predisposição ao surgimento de inflamações e lesões neoplásicas é maior do que na próstata de fêmea de gerbilo senis.

O etinilestradiol exposto durante o desenvolvimento alterou a imunorreação de AR, aumentando essa marcação na próstata ventral de machos do grupo EE PRÉ/PUB. O EE como um DE age sobre a ação funcional dos receptores nucleares, o AR, alterando a (De Coster e van Labereke, 2012; Thayer et al., 2001). A ação dos andrógenos é essencial para o desenvolvimento da próstata e durante a vida adulta mantém a funcionalidade glandular (Cooke et al., 1991). Deste modo, a exposição ao EE durante períodos críticos do desenvolvimento pode alterar a atividade do AR, e este contribui para o aumento da proliferação das células epiteliais, alteração na interação epitélio-estroma, predispondo ao aumento do surgimento de lesões neoplásicas nas próstatas durante a senescência. A maioria dos trabalhos abordam os efeitos de estrógeno sintético sobre a glândula prostática de ratos (Prins et al., 2001; 2007; Timms et al., 2005), o

nosso resultados mostram que o gerbilo da Mongólia é um ótimo modelo para o estudo dos efeitos de estrógeno sintético sobre a próstata.

Períodos como o pré-natal e a puberdade são cruciais para o desenvolvimento prostático, diferente dos humanos, nos roedores o início da ramificação prostática inicia-se na fase fetal e continua no período neonatal (Cunha et al., 2004). Assim, a ação sobre o desenvolvimento prostático será efetiva sobre a glândula, caso os animais sejam submetidos à exposição aos DE na fase pré-natal ou na fase pós-natal (neonatal e puberdade). Os nossos resultados até o momento revelaram que a exposição ao EE durante o período pré-natal e a puberdade permitiu o surgimento de severos efeitos no epitélio e estroma da próstata masculina e a próstata feminina de gerbilos senis. A alteração dos hormônios esteroides (testosterona e estrógeno) devido à exposição precoce ao EE está relacionada com o aumento da predisposição ao surgimento de prostatite e neoplasia intraepitelial prostática na senescência. Entretanto, os efeitos sobre as próstatas apresentaram-se diferentes, pois se observou que a próstata ventral masculina foi mais sensível à ação da exposição do EE comparando-se com a próstata feminina. Pelo fato de que os machos apresentam baixos níveis de estrógeno endógeno (Xi et al., 2010), a ação de disruptores estrogênicos durante fases críticas do desenvolvimento exacerba o desenvolvimento de lesões pré-cancerígenas na próstata masculina, prejudicando a saúde da glândula durante a senescência.

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Tabelas e Figuras

Tabela 1. Descrições dos anticorpos utilizados nas técnicas de imunohistoquímica.

Figura 1. Esquema do delineamento, representando os grupos experimentais. Machos e fêmeas do grupo EE/PRÉ-PUB receberam 15 μ g/kg/dia de etinilestradiol (EE) do 18th ao 22th dias do

período embrionário (e) e durante 42th ao 49th dias pós natais. A prole foi sacrificada com 12 meses de vida. No grupo controle, a prole não foi exposta ao EE, nenhum período de vida.

Tabela 2. Dados biométricos dos machos e fêmeas senis dos grupos experimentais.

Valores expressos como média \pm desvio padrão (n=5).

Tabela 3. Dados morfométricos e sorológicos dos machos e fêmeas senis do grupos experimentais.

Valores expressos como média \pm desvio padrão (n=5). *Diferença significativa entre os grupos ($p \leq 0,05$).

Figura 2. Corte histológico da próstata ventral masculina e da próstata feminina de gerbilo senil corada com Hematoxilina e Eosina. **Grupo controle:** A-B (σ) e F-G (φ). Aspecto morfológico da próstata masculina e feminina, destaque para o epitélio simples prismático (Ep) que circundam os ácinos prostáticos com região luminal (Lu). Presença de camada de músculo liso (Mu) no estroma prostático (st) e vasos sanguíneos (vs). **Grupo EE/PRÉ-PUB:** C-E (σ). Presença de neoplasia intraepitelial prostática (***) com presença de células atípicas, com mucina no citoplasma (seta larga). H-I (φ). Atrofia acinar (ac) e epitélio prostático (Ep). Regiões com NIP e presença de pequenas vesículas no citoplasma das células secretoras epiteliais (ponta de seta), caracterizando a metaplasia mucinosa (seta tracejada).

Figura 6. Corte histológico da próstata ventral masculina e próstata feminina de gerbilo senil corada pela técnica de reticulina de Gömori. **Grupo controle:** A (σ) e C (φ). Fibras reticulares (seta branca) e colágenas (*), componentes do estroma prostático. **Grupo EE/PRÉ-PUB:** B (σ) e D (φ). Ruptura das fibras reticulares (seta larga preenchida) na região subepitelial, principalmente em locais com presença de adenocarcinoma (**). Desorganização das fibras reticulares localizadas na região subepitelial (seta larga).

Figura 4. Corte histológico de próstata ventral masculina e feminina de gerbilo senil submetidas à técnicas imunohistoquímica. *Imunohistoquímica para AR.* **Grupo controle:** A (σ) e B (φ)

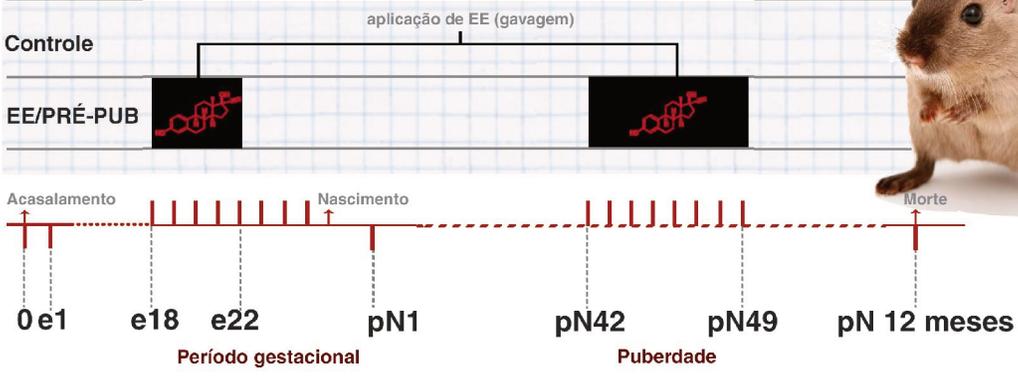
Imunoreatividade positiva nas células do epitélio prostático (setas). **Grupo EE/PRÉ-PUB:** C (♂). Observou-se diminuição da imunoreatividade do AR (seta tracejada). E (♀) Não se observou alterações nesta imunoreatividade (seta larga), comparada ao controle. *Imunohistoquímica para ER α .* **Grupo controle:** E (♂) e F (♀). Presença de imunoreatividade de ER α nas células estromais (setas largas) e nas células epiteliais (seta). Epitélio (Ep) e estroma (St) prostático. **Grupo EE/PRÉ-PUB:** G (♂). Aumento dessa imunoreatividade nas células epiteliais (seta larga preenchida). H (♀). Não se observou esse aumento nas células estromais (seta) e epiteliais (seta larga). *Imunohistoquímica para ER β .* **Grupo controle:** I (♂) e J (♀). Imunoreatividade positiva nas células epiteliais (setas tracejadas). **Grupo EE/PRÉ-PUB:** L (♂) e M (♀). Evidência de imunoreatividade de ER β no citoplasma epitelial (ponta de seta).

Figura 5. Corte histológico de próstata ventral masculina e feminina de gerbil senil submetidas à técnicas imunohistoquímica. *Imunohistoquímica para PCNA.* **Grupo controle:** A (♂) e B (♀). Imunoreatividade positiva nas células epiteliais (setas). Epitélio (Ep). **Grupo EE/PRÉ-PUB:** Aumento desta imunoreatividade (seta larga), principalmente em locais com NIP (seta larga preenchida). *Imunohistoquímica para p63.* **Grupo controle:** E (♂) e F (♀). Imunomarcagem de p63 nas células basais (setas tracejadas) do epitélio (Ep). **Grupo EE/PRÉ-PUB:** G (♂). Observou-se diminuição da imunoreatividade de p63 em regiões com NIP (*). H (♀). A imunomarcagem de p63 na camada de células basais (seta tracejada). *Imunohistoquímica para α -actina de músculo liso.* I (♂) e J (♀). Presença desta imunoreatividade na camada muscular prostática (setas). **Grupo EE/PRÉ-PUB:** L (♂). Ausência da imunoreatividade de α -actina em locais com presença de adenocarcinoma (***) e inflamação (IF). M (♀). Aumento desta imunoreatividade, principalmente em regiões com NIP.

Tabela 1.

<i>Anticorpos</i>	<i>Descrição</i>	<i>Diluição</i>
PCNA	Camundongo monoclonal IgG _{2a} , PC-10: sc-56, Santa Cruz Biotechnology CA, EUA.	1:100
p63	Camundongo monoclonal IgG _{2a} , 4A4: sc-8431, Santa Cruz Biotechnology CA, EUA.	1:100
AR	Coelho policlonal IgG, N-20: sc-816, Santa Cruz Biotechnology CA, EUA.	1:100
ER α	Coelho poli clonal IgG, H-184: sc-7207, Santa Cruz Biotechnology CA, EUA	1:50
ER β	Coelho policlonal IgG, H-150: sc-8974, Santa Cruz Biotechnology CA, EUA	1:50

Grupos experimentais | prole ♀/♂



Abreviações:
e = dias embrionários
pN = dias pós-natais
EE = Etilil Estradiol

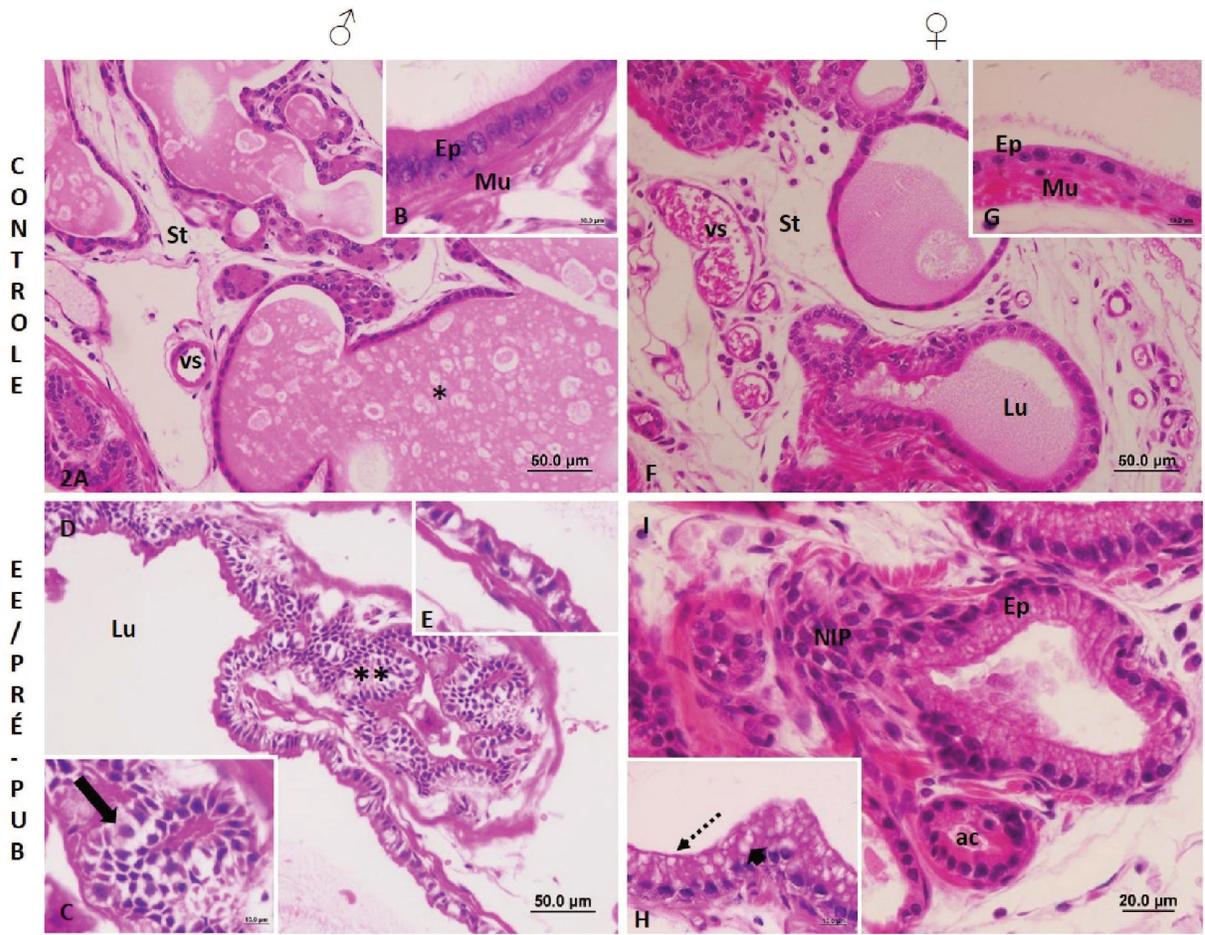
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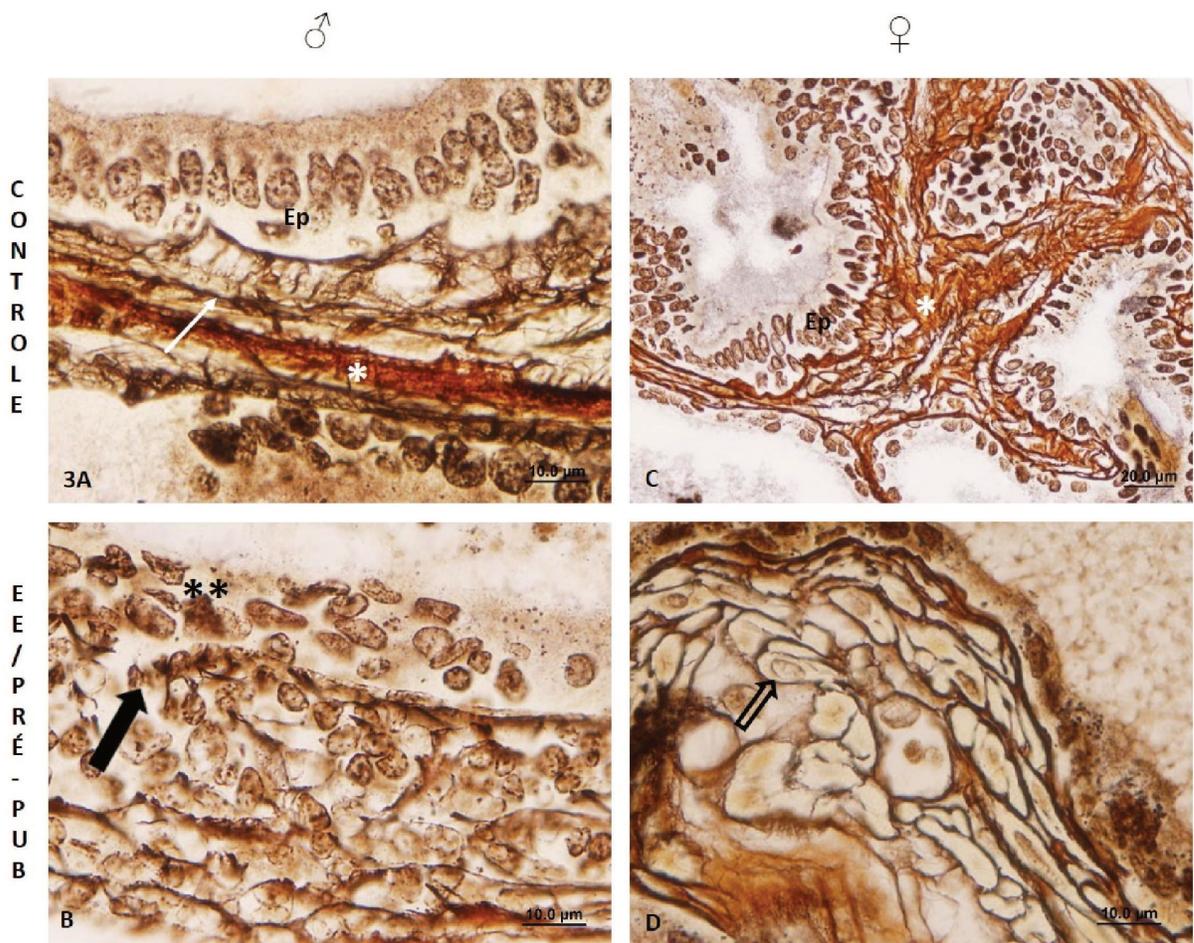
Tabela 2.

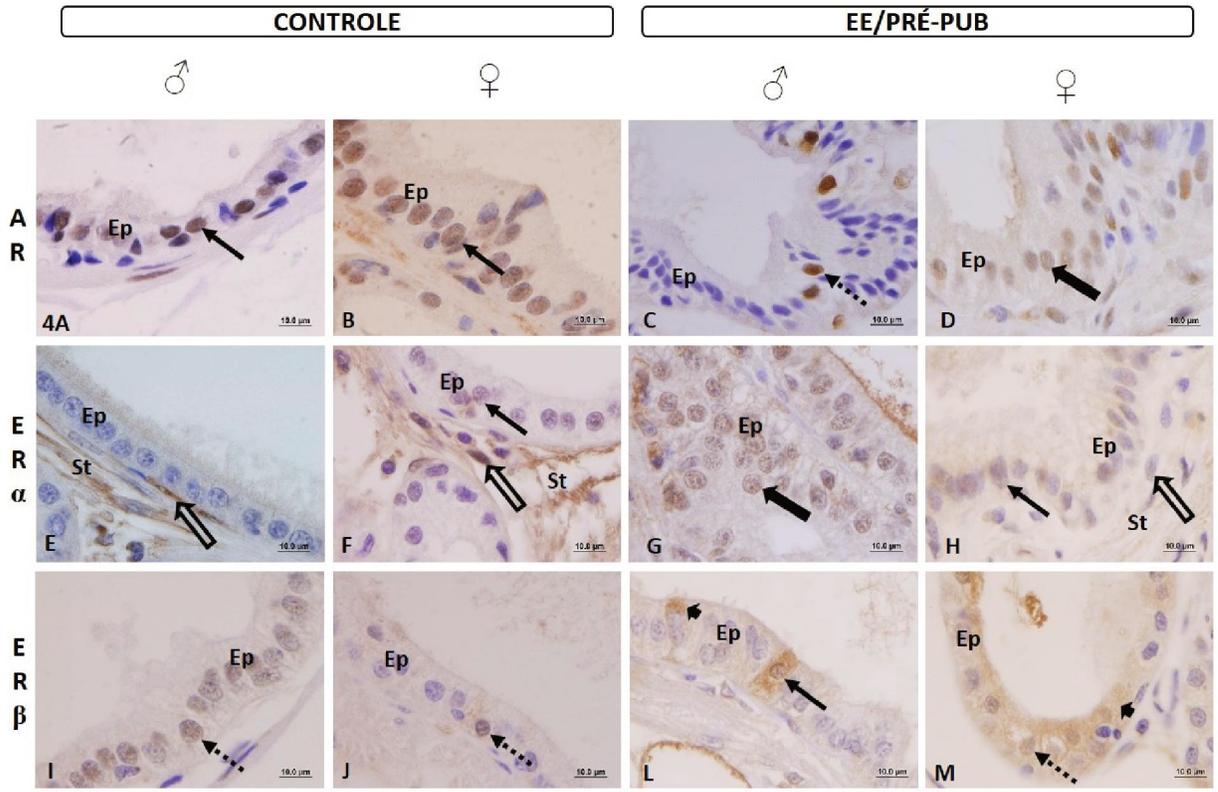
<i>Parâmetros</i>	<i>Grupos Experimentais</i>	
<i>Macho</i>	<i>Controle</i>	<i>EE/PRÉ-PUB</i>
Peso corpóreo (g)	86,66 ± 6,11	71,33 ± 1,15
Complexo prostático (g)	1,05 ± 0,22	0,74 ± 0,05
Próstata ventral (g)	0,02 ± 0,002	0,016 ± 0,008
Peso relativo da próstata ventral (x10 ⁻³)	0,25 ± 0,07	0,27 ± 0,09
Testículos (g)	1,24 ± 0,19	1,06 ± 0,06
Adrenal (g)	0,05 ± 0,01	0,044 ± 0,01
DAG (mm)	12,48 ± 1,49	12,47 ± 0,69
<i>Fêmea</i>	<i>Controle</i>	<i>EE/PRÉ-PUB</i>
Peso corpóreo (g)	68,60 ± 7,12	61,20 ± 8,32
Próstata + uretra (g)	0,04 ± 0,01	0,03 ± 0,003
Peso relativo da próstata + uretra (x10 ⁻³)	0,43 ± 0,22	0,47 ± 0,05
Ovários (g)	0,037 ± 0,016	0,05 ± 0,01
Adrenal (g)	0,04 ± 0,01	0,04 ± 0,01
DAG (mm)	3,93 ± 0,49	4,30 ± 0,70

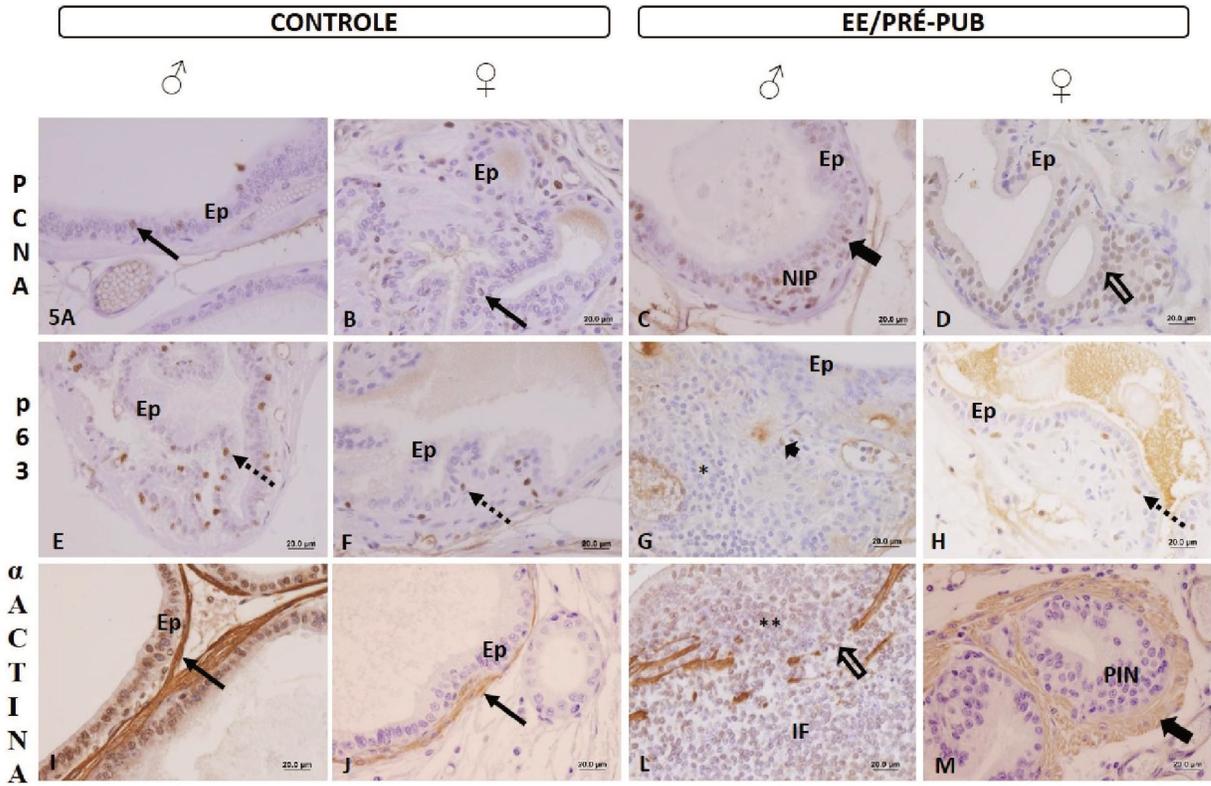
Tabela 3.

<i>Parâmetros</i>	<i>Grupos Experimentais</i>	
<i>Sorologia</i>	<i>Controle</i>	<i>EE/PRÉ-PUB</i>
Testosterona (ng/ml)	♂ 2,16 ± 0,65 ♀ 0,81 ± 0,35	1,21 ± 0,53* 1,46 ± 0,56
Estradiol (pg/ml)	♂ 12,15 ± 7,20 ♀ 8,30 ± 2,50	16,00 ± 5,00 24,00 ± 9,14*
<i>Morfometria</i>	<i>Controle</i>	<i>EE/PRÉ-PUB</i>
Altura epitelial (µm)	♂ 16,27 ± 3,80 ♀ 11,65 ± 3,50	9,34 ± 2,55* 14,23 ± 4,62*
Espessura da camada muscular (µm)	♂ 8,74 ± 3,00 ♀ 6,22 ± 3,13	5,34 ± 1,43* 7,14 ± 1,51









Conclusão

O etinilestradiol (EE) exposto durante as fases pré-natal e puberal alterou significativamente a estrutura glandular da próstata ventral masculina e a próstata feminina durante a senescência. Essa exposição promoveu também mudanças nos níveis dos hormônios esteroides, como a testosterona e o estradiol nos animais senis. Como o EE apresenta ação agonista de estrógeno esse fato confirma mais uma vez a sensibilidade da próstata à exposição aos estrógenos sintéticos quando comparada à próstata feminina. Enquanto que nas fêmeas, a exposição ao EE, principalmente no período puberal promoveu ação anabolizante sobre a próstata feminina, isso mostra que as próstatas de ambos os sexos responderam de formas diferentes à exposição ao estrógeno sintético. Com base nisso, a puberdade foi considerada uma fase tão crítica na exposição ao EE quanto o período pré-natal sobre os efeitos na próstata senil. A exposição à estrógenos sintéticos como o EE mesmo nas fases iniciais do desenvolvimento prostático prejudicou o aspecto estrutural e ultraestrutural da próstata e, aumentou a predisposição ao surgimento de lesões prostáticas invasivas, comprometendo a saúde da glândula prostática na senescência.

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Anexos

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação de Mestrado/tese de Doutorado intitulada **Efeitos da exposição à baixa dose de etinilestradiol durante as fases pré-natal e puberal sobre a próstata masculina e feminina de gerbilos senis.**

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

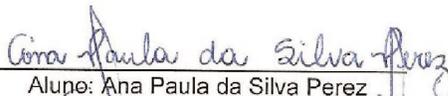
Tem autorização da(s) seguinte(s) Comissão(ões):

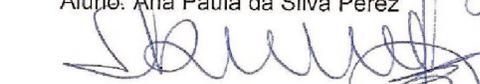
() CIBio – Comissão Interna de Biossegurança , projeto No. _____, Instituição: _____

(X) CEUA – Comissão de Ética no Uso de Animais , projeto No. 020/09, Instituição: Instituto de Biociências, Letras e Ciências Exatas IBILCE/UNESP.

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

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

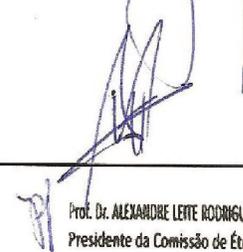

Aluno: Ana Paula da Silva Perez


Orientador: Dr. Sebastião Roberto Taboga

Para uso da Comissão ou Comitê pertinente:

Deferido () Indeferido

Carimbo e assinatura


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

Para uso da Comissão ou Comitê pertinente:

() Deferido () Indeferido

Carimbo e assinatura

COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL IBILCE

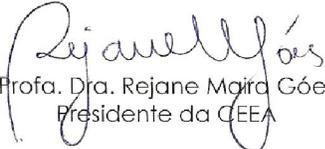
CERTIFICADO

Certificamos que o projeto de pesquisa intitulado "**Avaliação da exposição estrogênica intra-uterina e puberal como fator predisponente de lesões prostáticas em machos e fêmeas de gerbilos senis**" (protocolo nº. 020/09 CEEA), sob responsabilidade do Prof. Dr. Sebastião Roberto Taboga, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética em Experimentação Animal, em reunião de 10/12/2009.

CERTIFICATE

UNESP / IBILCE Ethical Committee for Animal Research (CEEA) hereby certify that the scientific investigation entitled "**Evaluation of estrogenic exposure in utero and pubertal development as a predisposing factor of prostatic lesions in male and female gerbils senile**" (protocol nº. 020/09 CEEA), on Sebastião Roberto Taboga responsibility, is in accordance with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and it was approved by the Committee of this Institute, on december 10th, 2009.

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


Profa. Dra. Rejane Maira Góes
Presidente da CEEA