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"EFEITO DO TRATAMENTO NEONATAL COM 17-β-ESTRADIOL SOBRE O PÊNIS DE RATO EM DIFERENTES IDADES: ASPECTOS ESTRUTURAIS DO ÓRGÃO E EXPRESSÃO DO RECEPTOR DE ANDRÓGENO PELAS CÉLULAS MUSCULARES LISAS E ENDOTELIAIS *IN VITRO*"

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientador: Prof. Dr. Hernandes Faustino de Carvalho

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Aos ratos,

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RESUMO

Os hormônios androgênicos (testosterona e diidrotestosterona) regulam a diferenciação e o crescimento das estruturas penianas via receptor de andrógenos (AR), tendo este função reguladora da transcrição de genes relacionados a aspectos do desenvolvimento de indivíduos do sexo masculino. A presença de receptores de estrógenos no pênis permite assumir que o 17-β-estradiol (E2) e moléculas similares tenham efeito direto sobre sua fisiologia. De forma geral, o estrógeno tem efeito anti-androgênico, atuando sobre o eixo hipotálamo-hipófise e, assim, reduzindo a produção de testosterona pelos testículos. O estrógeno é essencial para funcionamento reprodutivo em machos, no entanto, a exposição ao estrógeno ou xenobióticos durante períodos críticos do desenvolvimento pode ter conseqüências negativas para o trato reprodutivo e para a fertilidade, através de um mecanismo conhecido como *imprinting* estrogênico. Um dos efeitos do *imprinting* estrogênico causado por altas doses de estrógeno é o comprometimento do desenvolvimento peniano. Embora seja controverso na literatura, este efeito se daria pela regulação negativa da expressão do AR e reduzida resposta aos andrógenos. Sendo assim, para estudar o efeito do *imprinting* estrogênico no desenvolvimento do pênis foram administrados 25 µL de óleo de milho contendo E2 a 15 mg/kg (dose alta) (Putz, et al., 2001 a, b) a ratos Wistar, nos dias 1, 3 e 5 após o nascimento e observação dos efeitos nos períodos pré-púbere (28 dias), púbere (49 dias) e adulto (90 dias). Foi feito ainda isolamento de células musculares lisas (CML), cultivadas com e sem T, e endoteliais do órgão. Para cada situação, a expressão do AR foi verificada por Western blotting e a localização por imunocitoquímica. Para o órgão e as células CML, a expressão do RNAm do AR foi analisado por *Real-time* PCR. Nos animais adultos foram quantificados: colágeno solúvel, hidroxiprolina e glicosaminoglicano (GAG). O tratamento neonatal com E2 resultou na queda do peso corporal, má formação do pênis, menor quantidade de hidroxiprolina e maior quantidade de GAG. A expressão do AR aumentou em animais de 28 dias e reduziu aos 90 dias. Nessas idades a marcação do AR foi menos intensa nos animais estrogenizados em todos os compartimentos penianos. Nas CML, a expressão do AR exibiu padrão diferente quando cultivadas com ou sem T. Nas células endoteliais a expressão não varia com a idade, porém diminui naquelas isoladas de animal tratado. A exposição neonatal ao E2 causa má formação do pênis o que pode estar relacionado à alteração da expressão do AR no órgão, nas CML e endoteliais presentes no mesmo.

ABSTRACT

The androgens, testosterone and dihydrotestosterone, regulate the differentiation and growth of penile structures through the androgen receptor (AR), which regulates the transcription of genes associates with several aspects of the development of male individuals. In contrast to the prostate, the AR expression in the penis of the rat falls with age according to the androgen levels reached in the adult. The presence of estrogen receptor in the penis allows the assumption that 17-β- estradiol (E2) and similar molecules have direct effect on its physiology. It is known that estrogen has an anti-androgenic effect acting on the hypothalamic-pituitary axis reducing the production of testosterone by the testes. The estrogen is essential for reproductive function in males, but the exposure to estrogen or xenobiotics during critical periods of the development has negative consequences for the reproductive tract and fertility, through a mechanism known as estrogenic imprinting. One of the effects of estrogenic imprinting caused by high doses of estrogen is defective penile development. Although controversial in the literature, this effect occurs by down regulation of androgens receptors and reduced response to androgens. To study the effect of estrogenic imprinting on penis development, Wistar rats received subcutaneous injections of 25 μL of corn oil containing E2 at a dose of 15 mg/kg body weight (Putz, et al., 2001 a, b) on days 1, 3, and 5 after birth and observation of the effects on 28, 49 or 90 days after birth (prepubertal, pubertal and adulthood stages, respectively). Smooth muscle cells (SMC) and endothelial cells were isolated from the organ. For each situation, AR expression was verified by Western blotting and the localization by immunocitochemstry. Androgen receptor mRNA expression was done for the penis and SMC by Real-time PCR. In adult animals soluble collagen, hydroxyproline and glycosaminoglycans (GAGs) were quantified. Neonatal treatment with E2 resulted in reduction of weight and abnormal development of the penis at all ages, reduction in hydroxyproline and increase in GAGs. The AR expression increased at 28 days, but not at 90 days and in these ages the staining intensity of AR was smaller in all penile compartments. In SMC, AR expression exhibited a different expression pattern when cultured with or without T. In endothelial cells, the AR expression increased on day 28, reducing in the other ages, but without difference in comparison to control, what leds us to believe that endothelial cells do not interfere in the reduction AR expression after sexual maturation. The neonatal treatment with E2 leds to abnormal penile development what may be related to an alteration of AR expression in the organ and in their SMC and endothelial cells.

LISTA DE ABREVIATURAS

ACTH	Hormônio Adrenocorticotrófico, do inglês Adrenocorticotrophic Hormone
AMP cíclico	Adenosina Monofosfato
AR	Receptor de Andrógeno, do inglês Androgen Receptor
ARE	Elemento Responsivo ao Andrógeno, do inglês Androgen Responsive Element
BSA	Bovine Serum Albumin
B2M	Beta-2-microglobulin
cGMP	Guanosina Monofosfato Cíclica, do inglês cyclic guanosine monophosphate
CREB	cAMP response element binding
CML	Célula Muscular Lisa
CpG	Regiõsos do DNA ricas em citosina e guanina
CS	Chondroitin sulphate
DBD	Domínio de Ligação ao DNA do inglês, DNA Binding Domain
DES	Dietilestilbestrol
DHT	Diidrotestosterona, do inglês Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene blue
DNA	Ácido desoxirribonucléico, do inglês desoxiribonucleic acid
DS	Dermatan sulphate
EBSS	Earle's Balanced Salt Solution
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Receptor de Estrógeno, do inglês Estrogen Receptor
E2	17-β-estradiol
FBS	Fetal Bovine Serum
FSH	Hormônio Folículo Estimulante, do inglês Follicle-stimulating Hormone
GAG	Glicosaminoglicano, do inglês glycosaminoglycan
GH	Hormônio do crescimento, do inglês Growth Hormone
GHRH	Hormônio liberador do hormônio do crescimento do inglês Growth Hormone Releasing Hormone
GnRH	Hormônio Liberador de Gonadotrofina, do inglês Gonadotropin Releasing Hormone
GTP	Guanosina Trifosfato

GusB	Beta-glucuronidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHT	Hipotalâmico-Hipofisário-Testicular
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HS	Heparam sulphate
HSP	Heat Shock Protein
HRP	Horseradish peroxidase
IgG	Immunoglobulin
KDa	Kilodalton
LBD	Domínio de Ligação com o Ligante, do inglês Ligant Binding Domain
LH	Hormônio Luteinizante, do inglês Luteinizing Hormone
MHC	Myosin Heavy Chain
MPC	Magnetic Particule Concentrator
NEM	N-ethylmaleimide
NOS	Óxido nítrico sintase, do inglês nitric oxide sintase
PBS	Saline phosphate buffer
PCR	Polymerase chain reaction
PDE	Fosfodiesterase, do inglês phosphodiesterase
PGK1	Phosphoglycerate kinase 1
PMSF	Phenylmethanesulfonyl
PPiA	Peptidylprolyl isomerase A
RNA	Ácido ribonucléico, do inglês ribonucleic acid
SEM	Standard error of the mean
SHBG	Globulina ligada ao Hormônio Sexual, do inglês Sex Hormone Binding Globulin
SMC	Smooth Muscle Cell
SP1	Fator de transcrição envolvido na expressão gênica no início do desenvolvimento de um organismo
T	Testosterona ou testosterone
TBP	TATA binding protein
TBS-T	Tris buffered saline plus 0.2% tween 20
VWF	von Willebrand Factor

SUMÁRIO

1. Introdução

O desenvolvimento peniano é regulado pelos andrógenos, testosterona (T) e dihidrotestosterona (DHT), que ditam sua diferenciação e crescimento (George e Wilson, 1994). Os andrógenos exercem sua função via receptor de andrógeno (AR), uma proteína com 110 kDa, membro da família dos receptores nucleares. Uma vez ativado, o AR atua como fator de transcrição regulando a transcrição de diversos genes associados com vários aspectos do desenvolvimento de indivíduos do sexo masculino, inclusive do pênis.

Os estrógenos provavelmente participam desse processo, uma vez que os receptores de estrógeno (ERα e ERβ) estão amplamente distribuídos no pênis adulto e em desenvolvimento, como no corpo cavernoso, uretra, epitélio vascular e células neuronais, sugerindo papel do sistema estrógeno-ER não só no desenvolvimento do órgão, como também em suas funções (Crescioli, *et al*., 2003; Schultheiss, *et al*., 2003, Dietrich *et al.*, 2004).

Animais incapazes de expressar ER (Eddy, *et al*., 1996) ou a enzima aromatase (Fisher *et al.*, 1998; Honda *et al.*, 1998), responsável pela conversão da T em 17-β-estradiol (E2), são inférteis. Além disso, animais expostos, de forma inapropriada, a componentes estrogênicos durante períodos críticos do desenvolvimento exibem anormalidades reprodutivas, como cistos epididimais, retenção testicular, diminuição dos testículos, micropênis e hipospádias (Toppari, *et al.*, 1996). Essa exposição ao estrógeno em períodos críticos do desenvolvimento e a manifestação de seus efeitos na adolescência e na vida adulta denomina-se *imprinting* estrogênico (Prins, 2008).

Neste contexto, para estudar o desenvolvimento do pênis de animal estrogenizado e a expressão do AR, foram administrados 25 µL de óleo de milho contendo E2 numa dosagem de 15 mg/kg (dose alta) (Putz, et al., 2001 a, b) a ratos da linhagem Wistar, nos dias 1, 3 e 5 após o nascimento e observação dos efeitos nos períodos pré-púbere (28 dias), púbere (49 dias) e adulto (90 dias). Foi feita ainda análise *in vitro*, na qual, células musculares lisas (CML) e endoteliais, importantes no processo de ereção, foram isoladas do órgão. No cultivo de CML, foram usados meios de cultura com e sem T. Para cada situação, a expressão do AR foi verificada. Foi ainda, nos animais adultos, quantificado colágeno total solúvel, hidroxiprolina e glicosaminoglicano.

Os resultados deste trabalho mostraram redução do peso corporal, do pênis, da próstata e dos testículos nos animais tratados com E2, além de uma má formação do pênis caracterizada pela redução do ângulo reto na transição entre a glande e o corpo do órgão, redução do comprimento e diâmetro peniano e aumento da deposição de adipócitos na região do corpo cavernoso.

O corpo do pênis do animal adulto estrogenizado apresentou fibras colágenas mais compactas, evidenciada pela coloração com picrosirius e observação em microscopia de polarização; o tratamento neonatal com E2 parece levar a um arranjo diferenciado da matriz extracelular.

O tratamento neonatal com E2 levou ao aumento da expressão do AR aos 28 dias e redução aos 90 dias. As CML isoladas de animal estrogenizado e cultivadas com T suplementar não exibiram diferença quanto à expressão do AR, no entanto na ausência desse andrógeno a expressão aumentou aos 90 dias. Ainda, o tratamento neonatal levou a queda da expressão do AR aos 28 dias, na presença de T no meio de cultura. Entretanto, nas células endoteliais, a expressão do AR aumentou aos 28 dias, diminuindo em seguida, e assim permanecendo. Diferentemente do que ocorre nas CML, nas células endoteliais a expressão do AR não varia ao longo da idade.

Esse estudo determinou que a exposição neonatal ao E2 afeta o desenvolvimento peniano, o que pode ser devido à variação na expressão do AR no órgão, nas CML e endoteliais, alteração da localização celular do AR (citoplasmática, nuclear e citoplasmática, nuclear), variação dos níveis séricos de testosterona e/ou estradiol nos períodos pré-púbere, púbere e adulto.

Dessa forma, podemos afirmar que a exposição neonatal ao E2 pode alterar a expressão do AR no pênis e sua morfofisiologia de forma irreversível, podendo resultar em disfunção erétil. O processo pelo qual isso ocorre ainda é desconhecido. No entanto, é possível que o estrógeno afete o processo de diferenciação das estruturas penianas de forma a alterar o desenvolvimento, crescimento e função do órgão.

2. Revisão Bibliográfica

2.1 Estrutura e função do pênis

O pênis é um órgão do aparelho sexual masculino com função, na reprodução e na excreção da urina. Anatomicamente consiste de três partes: glande, corpo e raiz. A raiz corresponde à porção proximal do órgão e está inserida no interior do corpo do animal, na região púbica. Segue-se a ela o corpo, que corresponde a um prolongamento fálico e a glande, localizada distalmente. O pênis de roedores possui duas características únicas; a formação de um ângulo reto na região de transição entre o corpo do órgão e a glande e um osso cilíndrico que se estende da porção distal do corpo peniano ao topo da glande **(Figura 1)**.

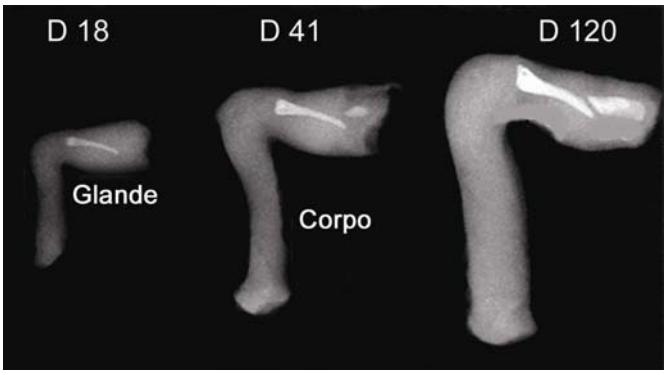

Figura 1. Radiografia do pênis de rato da linhagem Sprague-Dawley nas idades de 18, 41 e 120 dias. Observe o ângulo reto entre o corpo do órgão e a glande; e o osso na região da glande (Adaptado de Goyal, *et al*., 2004).

Histologicamente consiste de dois corpos cavernosos pareados, dorsalmente localizados, separados por um septo intercavernoso contendo vasos sanguíneos, nervos constituídos por fibras nervosas autonômicas (parasimpáticas e simpáticas) e somáticas, e de um corpo esponjoso localizado ventralmente. Tanto o corpo cavernoso como o esponjoso apresentam um parênquima musculovascular cuja unidade funcional é o sinusóide cavernoso. Estas são cavidades alveolares forradas por células musculares lisas (CML) e endotélio. Cada sinusóide se encontra irrigado por uma arteríola que é um ramo terminal das artérias helicina e é drenado por vênulas que confluem com as dos sinusóides vizinhos para formar as veias emissárias que atravessam a membrana albugínea. O corpo cavernoso constitui-se ainda do estroma intercavernoso e da túnica albugínea, além de poucos adipócitos. A túnica albugínea forma a cápsula do corpo do pênis e consiste de uma camada fibrosa externa e uma camada celular interna que envolve o corpo cavernoso. Já o estroma intercavernoso, provavelmente uma continuação da túnica albugínea, contém fibras colágenas e numerosos fibroblastos. O corpo esponjoso contém a uretra localizada centralmente, envolta por epitélio de transição e estroma peri-uretral formado por fibroblastos, fibras colágenas e vasos sanguíneos **(Figura 2)**.

O influxo sanguíneo ao corpo esponjoso é realizado pela artéria do bulbo, enquanto a helicina, o faz ao corpo cavernoso. Esta artéria origina os espaços cavernosos e as CML (Benson, 1994).

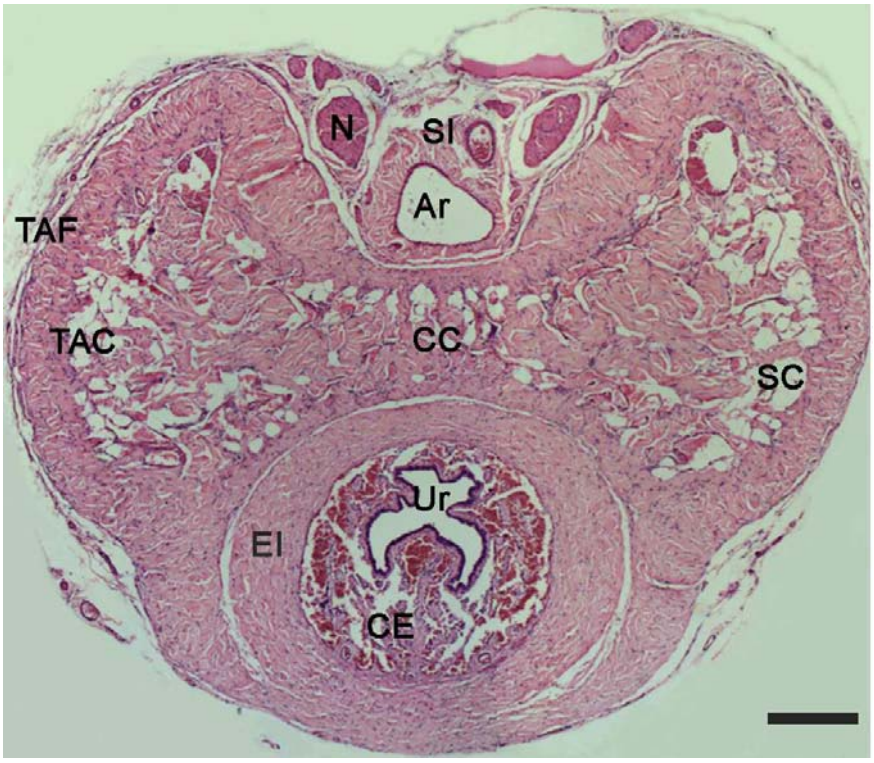

Figura 2. Corte transversal do corpo do pênis de rato Wistar com 90 dias, evidenciando os compartimentos penianos. CC (Corpo cavernoso), SC (Sinusóide cavernoso), TAC (Túnica albugínea celular) TAF (Túnica albugínea fibrosa), EI (Estroma intercavernoso), CE (Corpo esponjoso), Ur (Uretra), SI (Septo intercavernoso), Ar (artéria peniana), N (nervos). Coloração com Hematoxilina-eosina. Barra 400µm.

A ereção do pênis envolve uma interação complexa entre o sistema nervoso central e fatores locais. De maneira geral, é um evento neurovascular modulado por fatores psicológicos e hormonais. Com o estímulo sexual, as células endoteliais do pênis sintetizam e liberam o óxido nítrico que se difunde para as CML, ativando a enzima guanilato ciclase que catalisa a conversão de GTP (guanosina trifosfato) em cGMP (guanosina monofosfato cíclica). Isto leva à ativação dos canais iônicos de cálcio, diminuindo sua concentração citosólica, causando o relaxamento das CML do pênis, dilatação das artérias e arteríolas penianas, aumento do fluxo sanguíneo e preenchimento dos espaços cavernosos com sangue **(Figura 3)**. Devido ao fato do pênis ser envolto pela túnica albugínea, um tecido que não se distente facilmente, o aumento do fluxo sanguíneo aumenta não somente o volume peniano como também a pressão intrapeniana, causando compressão mecânica das veias emissárias, o que impede a drenagem do sangue, resultando na rigidez do pênis.

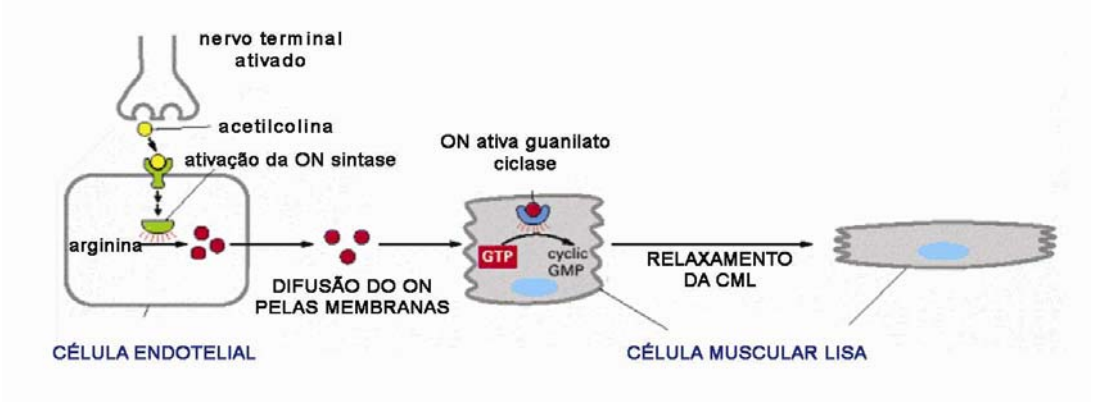

Figura 3. Mecanismo da ereção peniana. Com a estimulação sexual, a acetilcolina é liberada dos nervos terminais atingindo a parede vascular, ativando a enzima óxido nítrico sintase, o que leva à conversão da arginina em óxido nítrico, nas células endoteliais. O óxido nítrico se difunde dessas células até as células musculares lisas, onde ativa a guanilil ciclase, produzindo GMP cíclico, o que causa o relaxamento das CML (células musculares lisas), aumentando o fluxo sanguíneo e o preenchimento dos espaços cavernosos com sangue, levando à ereção do pênis (Adaptado de Alberts *et al*., 2002).

Os níveis celulares de cGMP refletem um balanço dinâmico entre sua produção e degradação pela fosfodiesterase-5, expressa no corpo cavernoso. A cessação da liberação do neurotransmissor leva à contração das CML, restaurando a saída venosa, de modo que o sangue é drenado e a flacidez retorna (Priviero *et al.*, 2007; Saad *et al.*, 2007).

2.2 Andrógenos

A diferenciação e o crescimento do pênis são completamente dependentes da estimulação androgênica (Sharpe, 1994; George e Wilson, 1994). Este estímulo inicia-se com a testosterona (T) circulante produzida nos testículos e intensifica-se com sua redução a diiidrotestosterona (DHT), pela enzima 5α-redutase presente em tecidos periféricos, inclusive no pênis. Altas concentrações desta enzima são encontradas nesse órgão no seu período máximo de crescimento (Wilson e Walker, 1969).

A T é produzida, na sua maior parte (cerca de 95%) pelos testículos. As glândulas adrenais contribuem com menos de 5% da produção dos esteróides sexuais (Coffey, 1992; Cheng *et al.,* 1993), sendo regulada pelo hormônio adrenocorticotrófico (ACTH). Apenas 2 a 3% da T encontram-se disponível na sua forma livre, sendo que o restante encontra-se ligado a proteínas séricas como a globulina de ligação ao hormônio sexual (SHBG), albumina e globulina de ligação com corticoesteróide (Vermeulen, 1973). Entre as três proteínas, a SHBG possui a maior afinidade pela T.

A T e a DHT exercem influência durante a embriogênese, a puberdade e a vida adulta. Durante o desenvolvimento fetal causam diferenciação da genitália masculina. Na puberdade contribuem para o desenvolvimento e manutenção das características secundárias. No adulto, respondem pela manutenção da massa muscular e óssea, distribuição da gordura corporal, eritropoiese, crescimento prostático e espermatogênese (Saad *et al*, 2007).

Além disso, os andrógenos desempenham papel fundamental na ereção peniana, uma vez que estudos feitos com animais castrados mostraram resposta erétil diminuída, porém reversível pelo tratamento com T ou DHT (Bradshaw *et al*., 1981**;** Clark *et al*., 1988) e queda na expressão do RNAm para a enzima óxido nítrico sintase (NOS), responsável pela síntese do óxido nítrico, em relação aos animais tratados com T (Mills *et al*., 1999). Ainda, a infusão de vasodilatador ou vaso constritor no pênis resultou em declínio da pressão cavernosa máxima nos animais tratados com andrógeno, mas não nos castrados (Mills *et al*., 1992). Foi também observado, que o tratamento de ratos com o anti-andrógeno flutamida bloqueia a manutenção da ereção peniana pela testosterona (Gray *et al*., 1980).

Os andrógenos atuam como modulador dos níveis de cGMP, exercendo papel na sua formação e degradação. No entanto, o mecanismo pelo qual isso ocorre ainda é desconhecido. Acredita-se que fatores epigenéticos e de transcrição tecido-específicos possam ter ação na mediação dos efeitos da testosterona nessa ação bivalente de produção e degradação do cGMP (Morelli *et al*., 2005; Saad *et al*., 2007).

Estudos feitos em ratos e coelhos concluíram que a privação androgênica além de afetar a função erétil, induz alterações estruturais no corpo cavernoso, como aumento de matriz extracelular e acúmulo de adipócitos abaixo da túnica albugínea (Traish *et al*., 2003). Andrógenos inibem a diferenciação de células progenitoras estromais em adipócitos e promovem sua diferenciação em CML (Bhasin et al, 2003), dessa forma, acredita-se que no corpo cavernoso, os andrógenos regulam a diferenciação de células progenitoras em CML e inibem a diferenciação em adipócitos, sendo assim, a castração deveria favorecer o acúmulo de adipócitos no corpo cavernoso alterando sua função (Traish, *et al.,* 2005).Ainda, a privação androgênica parece ter promovido a diferenciação de células progenitoras estromais em linhagens adipogênicas, que acumulam lipídeos, e comprometem a função erétil (Traish *et al*., 2005).

2.3 Regulação do eixo hipotalâmico-hipofisário-testicular (HHT)

O eixo hipotálamo-hipófise regula uma série de aspectos fisiológicos por meio de uma combinação de sistemas neurais e endócrinos. A secreção de gonadotrofinas (hormônios luteinizantes – LH e folículo estimulante – FSH) pela adeno-hipófise é regulada pelo hipotálamo, através do hormônio liberador de gonadotrofinas (GnRH). O LH é uma glicoproteína que estimula a ovogênese nas fêmeas e a espermatogênese nos machos. Neste último, a sua principal ação ocorre através do estimulo da produção de testosterona pelas células de Leydig (Klonoff e Karam, 1995). O hipotálamo, por meio da liberação do hormônio liberador do crescimento (GHRH), regula a liberação pela adeno-hipófise, do ACTH e do hormônio do crescimento (GH), respectivamente. O ACTH regula a produção de testosterona pelas adrenais, enquanto o GH estimula a síntese de proteínas, aumenta o número de mitoses e o crescimento de quase todas as células e tecidos.

A privação androgênica leva à regressão da próstata (Kerr e Searle, 1973; Isaacs 1994). Da mesma forma, compostos que bloqueiam a ação do AR, impedindo a ação da enzima 5α-redutase ou tendo efeito inibitório sobre o eixo HHT, têm ação anti-androgênica e induzem a regressão prostática. Por inibir o eixo hipotálamo-hipófise causando uma inibição na produção de testosterona pelos testículos, o estrógeno tem efeito anti-androgênico, causando então regressão prostática, dentre outros efeitos, quando aplicado a indivíduos adultos.

2.4 Receptor de andrógeno

Os andrógenos exercem suas funções via receptor de andrógenos (AR), que atua como fator de transcrição regulando a expressão de diversos genes associados com vários aspectos do desenvolvimento de indivíduos do sexo masculino.

O AR tem 110 kDa, é um membro da família dos receptores nucleares e possui diversos domínios funcionais. A região amino-terminal contém dois tipos de repetições, uma região de poli-glutamina e outra de poli-glicina. Cada tipo de repetição apresenta um número variável de aminoácidos e está associado com polimorfismos nas regiões correspondentes no gene. Na porção amino-terminal, há ainda uma outra região com nove prolinas repetitivas, mas com número bastante constante. Segue-se uma região de domínio de ligação ao DNA (DBD), que consiste de dois dedos de zinco; o primeiro dedo liga-se ao elemento responsivo ao andrógeno (ARE) e o segundo dedo estabiliza a ligação do complexo através de interações

hidrofóbicas com o primeiro e contribui para a especificidade da ligação do receptor com o DNA. A região em dobradiça (*hinge*), que se localiza na porção carboxi-terminal, apresenta a seqüência consenso de acetilação (KLKK) e o sinal de localização nuclear e é, portanto responsável pela translocação do receptor do citoplasma para o núcleo assim que este se liga ao hormônio. O domínio de ligação com o ligante (LBD) corresponde à região de ligação com o hormônio esteróide e, juntamente com a região amino-terminal, é o local de interação com cofatores transcricionais (coativadores e co-repressores) (Roy *et al*., 1999; Geldman, 2002) (**Figura 4**).

A região codificadora do gene do AR possui 8 éxons, intercalados com íntrons de tamanhos variáveis, de menos de 1 Kbp a mais de 26 Kbp **(Figura 4A)**. A região promotora não possui TATA box nem CAAT box (Lindzey *et al*., 1994). Entretanto, apresenta sítios de ligação para a proteína de ligação com o elemento de resposta ao AMP cíclico (CREB) (Mizokami *et al*., 1994; Lindzey *et al*., 1993) e para a SP1 (Lindzey *et al*., 1993).

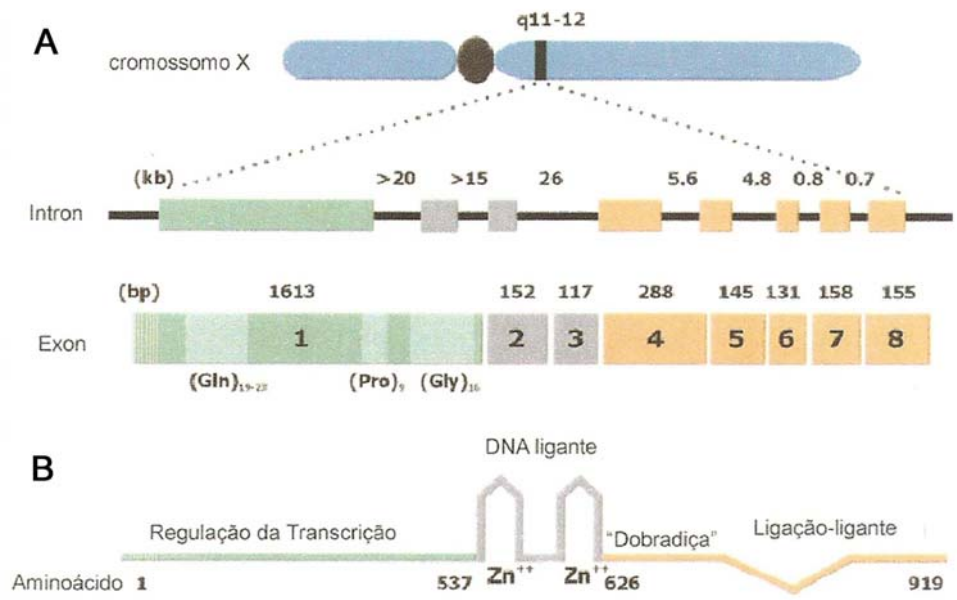

Figura 4. Principais regiões do gene e proteína do AR. A) O AR está localizado no cromossomo X, na região q11-12, apresenta íntrons e éxons com extensões variadas. B) Na região amino-terminal há 3 locais de repetição dos aminoácidos, representando o sítio de regulação da transcrição, seguindo uma região de ligação ao DNA, consistindo de dois dedos de zinco, uma região de dobradiça (já na porção carboxi-terminal) apresentando o sinal de localização nuclear, e finalmente, o local de ligação com o ligante, ou seja, os andrógenos (Modificado de Quigley et al., 1995).

O gene do AR humano apresenta pelo menos cinco regiões ricas em CpG, que se estende da posição –500 até a 1500 (Kinoshita *et al*., 2000; Sasaki *et al*., 2002), permitindo o silenciamento do gene por metilação do DNA em alguns tipos celulares tumorais prostáticos.

2.5 Regulação do receptor de andrógeno

A regulação da expressão e atividade do AR é um processo complexo dependente de vários elementos de regulação em níveis transcricionais, pós-transcricionais e pós-traducionais. A molécula inativa do AR localizada no citoplasma é ativada pela ligação com a T ou com a DHT, passa por uma série de modificações pós-traducionais, dissocia-se de uma proteína HSP (*Heat Shock Protein*), dimeriza-se e é translocada para o núcleo, onde, juntamente com uma série de co-ativadores ou co-repressores, liga-se aos AREs e ativa ou inativa genes (**Figura 5**).

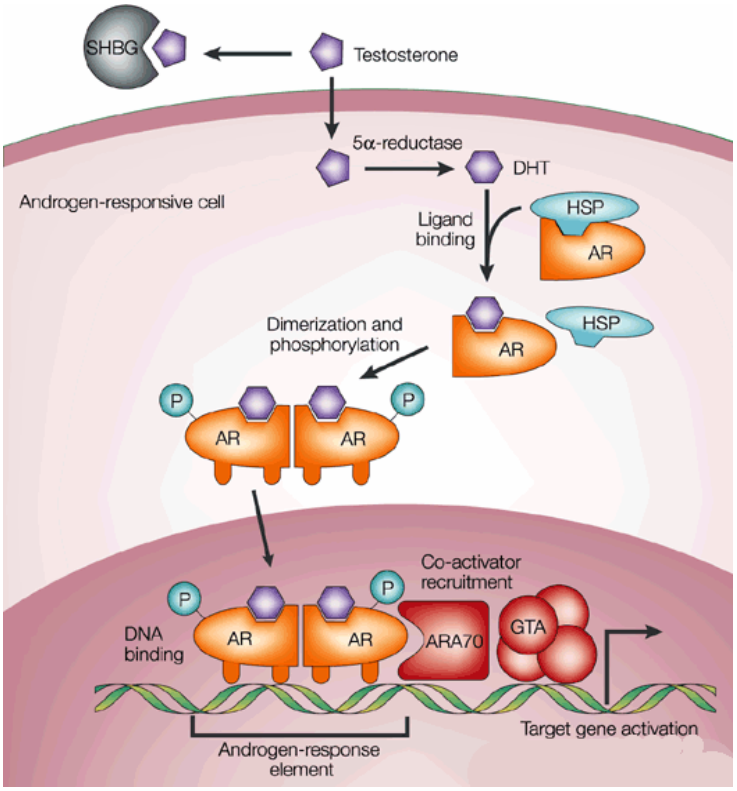

Figura 5. Ação do andrógeno. A T livre entra na célula e é convertida a DHT. A ligação com o AR o dissocia de uma HSP, deixando livre o sítio para a ligação da DHT. O complexo AR-andrógeno se dimeriza e é translocado para o núcleo, onde no DNA, se liga ao elemento responsivo ao andrógeno e em seguida, a coativadores, ativando genes alvo (Adaptado de Feldman & Feldman, 2001).

Hoje são conhecidas ações não genômicas do andrógeno, que envolveriam a ativação de várias vias de sinalização e diferentes efeitos sobre o comportamento celular (Heilein e Chang, 2002). As características principais destas ações são a sua velocidade de ocorrência, antecipando qualquer efeito de ativação e transcrição gênica (Castoria *et al*., 2003).

Estudos realizados com pênis de rato mostraram que o padrão de regulação da expressão do AR nesse órgão difere do que ocorre em outros órgãos como na próstata ventral (Quarmby *et al*., 1990; Krongrand *et al*., 1991). Do período pré-púbere ao púbere, por volta da quarta e sétima semana, respectivamente, a expressão do AR acompanha o aumento da concentração de testosterona (Rajfer *et al*., 1980; Takane *et al*., 1990), como na próstata ventral. Entretanto, no pênis, após a maturação sexual a expressão do AR cai (Rajfer *et al*., 1980), mesmo sob contínua presença de altas concentrações de andrógeno (Resko *et al*., 1968).

Os níveis de expressão do AR variam em células tumorais e linhagens estabelecidas de tumores prostáticos. No caso dos tumores, a expressão é bastante heterogênea. Dentre as linhagens, a LNCaP expressa o AR, enquanto a PC3 apresenta uma quantidade mínima e a DU145 não expressa AR (Nakayama *et al*., 2000). Os autores investigaram o padrão de metilação de duas regiões correspondentes a ilhas CpG na região promotora do gene do AR e demonstraram que a ausência de expressão na DU145 pode ser atribuída a um elevado índice de metilação nestas regiões. Além disto, o tratamento destas células com o inibidor de metilação azacitidina, restaura a expressão do AR. Os mesmos autores demonstraram uma grande heterogeneidade no padrão de metilação destas regiões em células provenientes de tumores prostáticos. Situação similar foi demonstrada para células tumorais prostáticas de ratos, para as quais foram identificados 19 dinucleotídeos CpG passíveis de metilação no gene do AR (Takahashi *et al*., 2002).

O mesmo mecanismo de hipermetilação que resulta no silenciamento do gene foi descrito para outros receptores nucleares, como o receptor de estrógeno (ER) e o receptor de progesterona (Sasaki *et al*., 2002; 2003).

2.6 Os disruptores estrogênicos

Disruptores endócrinos são agentes exógenos que interferem na síntese, secreção, transporte, conexão, ação ou eliminação dos hormônios naturais no organismo, responsáveis pela manutenção da homeostase, da reprodução, do desenvolvimento e/ou do comportamento (Kavlock et al., 1996).

Os disruptores endócrinos têm uma ampla série de configurações moleculares. A potência depende de sua ação sobre o órgão ou a célula alvo, além da finalidade específica. São exemplos de disruptores endócrinos: produtos de beleza e de higiene (cosméticos, filtros

solares, perfumes, sabonetes), fármacos (anticoncepcionais), selantes para dentes, solventes, surfactantes, agrotóxicos e os plásticos (PVC, poliestireno). Entre os disruptores endócrinos encontram-se os disruptores estrogênicos que se referem à substâncias químicas que exibem um grau de atividade similar ao estrógeno, esse termo pode ser aplicado a estrógenos naturais como fitoestrógenos e estrógenos sintéticos, como dietilestilbestrol (DES). O DES é um componente estrogênico sintético mais potente que o E2 e centenas ou milhares de vezes mais potente que outros componentes identificados como estrógeno exógeno **(Figura 6)**.

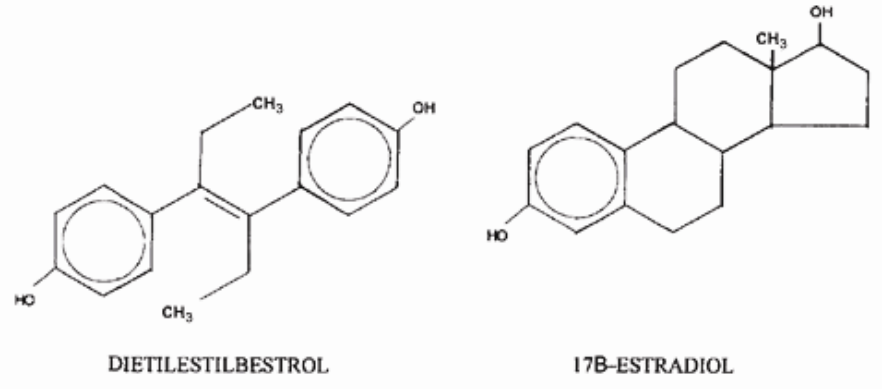

Figura 6. Estrutura química dos componentes estrogênicos dietilestilbestrol e 17β -estradiol.

2.7 *Imprinting* estrogênico

Parte dos andrógenos circulantes é convertida a estrógenos em vários tecidos periféricos, através da enzima aromatase (Simpson *et al.*, 1999). Receptores para estrógenos estão amplamente distribuídos no trato reprodutivo de várias espécies (Couse e Korach, 1999), inclusive em ratos (Hess *et al*., 1997). Camundongos incapazes de expressar genes para receptor de estrógeno, ERα ou ERβ (Eddy *et al*., 1996; Couse e Korach, 1999) ou a enzima aromatase (Fisher *et al*.; Honda *et al*., 1998) são inférteis. A aromatase é abundantemente expressa em diferentes compartimentos penianos de ratos no período neonatal. Porém, a expressão cai com a idade, o que evidencia sua influência nos estágios iniciais do desenvolvimento peniano (Jesmin *et al*., 2004).

A exposição ao estrógeno em períodos críticos do desenvolvimento e a manifestação de seus efeitos na adolescência e na vida adulta denomina-se *imprinting* estrogênico. Efeitos opostos sobre a próstata, quando diferentes dosagens são empregadas caracterizam a ação não normotônica do estrógeno nesse período, ou seja, dosagens baixas resultam em estímulo do

crescimento prostático, enquanto dosagens mais elevadas resultam em redução do crescimento (Putz *et al*., 2001a).

Dentre os efeitos do *imprinting* estrogênico, encontra-se também uma reduzida resposta a andrógenos na próstata (Rajfer e Coffey, 1979; Naslund e Coffey, 1986). Este efeito é bem caracterizado pelo fato de que a expressão do AR atinge apenas 43% dos níveis no controle, mesmo se os animais são tratados com andrógenos.

Anormalidades reprodutivas foram observadas em indivíduos do sexo masculino nascidos de mulheres que receberam DES durante a gravidez (Shawn, 2000, Titus-Ernstoff, 2008). Ainda, animais de laboratório expostos a componentes estrogênicos durante o período crítico de desenvolvimento exibiram alterações no aparelho reprodutor, incluindo cistos epididimais, retenção dos testículos, testículos pequenos, micropênis, hipospádias (Toppari *et al*., 1996) e câncer testicular (Colborn *et al*., 1993; Toppari *et al*., 1996).

A exposição de ratos machos neonatais ou adultos ao estrógeno leva à infertilidade (Goyal *et al.*, 2001; 2003). Estudo feito com ratos tratados no período neonatal com DES mostrou que a perda da fertilidade parece ser permanente e relacionada com a substituição do espaço cavernoso por células adiposas. Foi observada também a má formação do pênis, caracterizada pela redução do osso peniano e do ângulo reto na transição entre a glande e o corpo do órgão, além de menores comprimento e diâmetro. No entanto, não foi observada variação quanto à expressão do AR entre esses animais (Goyal *et al*., 2004b), como demonstrado por imunocitoquímica.

Em outros trabalhos, a exposição neonatal ao estrógeno causou redução da expressão do AR no restante do trato reprodutivo masculino, como epidídimo, ducto deferente, vesícula seminal (McKinnell *et al*.; Williams *et al.*, 2001) e próstata (Prins e Birch, 1995; Williams *et al*., 2001; Woodham *et al*., 2003).

Além dos efeitos descritos acima, o *imprinting* estrogênico leva à feminilização por ação direta no hipotálamo, além de atuar antecipando a puberdade em ratos (Putz *et al.*, 2001b). Altas dosagens de estrógeno neonatal induzem também uma quase completa eliminação da expressão do AR na próstata, como determinado por imunocitoquímica (Putz *et al.*, 2001a). Foram observados ainda rearranjos das junções comunicantes (Habermann *et al.*, 2001) e alterações na expressão dos receptores para ácido retinóico na próstata (Prins *et al.*, 2002). A redução da expressão do AR deve-se provavelmente à destinação do produto protéico ao sistema de degradação por proteassomos (Woodham *et al.*, 2003). Embora sejam conhecidos alguns efeitos do *imprinting* estrogênico, o mecanismo molecular envolvido permanece desconhecido.

3. Objetivos

3.1 Objetivo geral

Os objetivos gerais deste estudo foram estudar os efeitos da administração de alta dosagem de estradiol (E2) no desenvolvimento peniano e na expressão do receptor de andrógeno (AR) no pênis de rato, nos períodos pré-púbere, púbere e adulto e em células musculares lisas e endoteliais isoladas do órgão nestas diferentes idades.

3.2 Objetivos específicos

1) Verificar possível variação no peso dos animais e no peso relativo do pênis, próstata e testículo.
2) Analisar morfologicamente os diferentes compartimentos penianos em cortes histológicos corados com hematoxilina-eosina.
3) Analisar a expressão do AR nos diferentes compartimentos penianos, por imunocitoquímica.
4) Analisar a localização do AR (nuclear, citoplasmática e nuclear-citoplasmática) nas células musculares lisas e endoteliais isoladas do órgão.
5) Analisar a expressão do AR no pênis e nas células isoladas, por *Western blotting*.
6) Verificar possíveis alterações no conteúdo e organização de colágenos fibrilares no pênis do animal adulto.
7) Padronizar o controle interno adequado para quantificação do RNAm do AR no pênis e nas células musculares lisas nas condições experimentais por *Real- time PCR*.

4.1. Effects of neonatal 17-beta-estradiol treatment on the rat penis at different ages: structural aspects of the organ and androgen receptor expression by smooth muscle cells and endothelial cells in vitro

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ABSTRACT

The androgens, testosterone and dihydrotestosterone, regulate the differentiation and growth of penile structures through the androgen receptor (AR), which regulates the transcription of genes associates with several aspects of the development of male individuals. In contrast to the prostate, the AR expression in the penis of the rat falls with age according to the androgen levels reached in the adult. The presence of estrogen receptor in the penis allows the assumption that 17-β- estradiol (E2) and similar molecules have direct effect on its physiology. It is known that estrogen has an anti-androgenic effect acting on the hypothalamic-pituitary axis reducing the production of testosterone by the testes. The estrogen is essential for reproductive function in males, but the exposure to estrogen or xenobiotics during critical periods of the development has negative consequences for the reproductive tract and fertility, through a mechanism known as estrogenic imprinting. One of the effects of estrogenic imprinting caused by high doses of estrogen is defective penile development. Although controversial in the literature, this effect occurs by down regulation of androgens receptors and reduced response to androgens. To study the effect of estrogenic imprinting on penis development, Wistar rats received subcutaneous injections of 25 μL of corn oil containing E2 at a dose of 15 mg/kg body weight (Putz, et al., 2001 a, b) on days 1, 3, and 5 after birth and observation of the effects on 28, 49 or 90 days after birth (prepubertal, pubertal and adulthood stages, respectively). Smooth muscle cells (SMC) and endothelial cells were isolated from the organ. For each situation, AR expression was verified by Western blotting and the localization by immunocitochemstry. Androgen receptor mRNA expression was done for the penis and SMC by Real-time PCR. In adult animals soluble collagen, hydroxyproline and glycosaminoglycans (GAGs) were quantified. Neonatal treatment with E2 resulted in reduction of weight and abnormal development of the penis at all ages, reduction in hydroxyproline and increase in GAGs. The AR expression increased at 28 days, but not at 90 days and in these ages the staining intensity of AR was smaller in all penile compartments. In SMC, AR expression exhibited a different expression pattern when cultured with or without T. In endothelial cells, the AR expression increased on day 28, reducing in the other ages, but without difference in comparison to control, what leds us to believe that endothelial cells do not interfere in the reduction AR expression after sexual maturation. The neonatal treatment with E2 leds to abnormal penile development what may be related to an alteration of AR expression in the organ and in their SMC and endothelial cells.

1. Introduction

Testosterone (T) and/or dihydrotestosterone (DHT) are required for differentiation, growth, and maintenance of both structure and function of male reproductive organs, including the penis (Sharpe, 1994; George and Wilson, 1994). Androgens play a basic role in penile erection. Castrated animals show reduced erectile response which is restored by the treatment with T or DHT (Bradshaw et al., 1981**;** Clark et al., 1988).

Androgens exert its functions through AR, which act as a transcription factor, regulating the transcription of diverse genes related to male development and function. Moreover, estrogen action appears to be essential for reproductive functioning in males and exposure to estrogen during critical periods of development results in abnormal development of the penis, epididymal cysts, cryptorchidism, smaller testes, microphallus, and hypospadias (Toppari et al., 1996), with consequent reproductive abnormalities.

The estrogen receptors (ER) are broadly and abundantly expressed in various penile compartments and cell types (erectile tissues, urethral epithelium, vascular and neuronal cells), indicating the complexity and significance of the estrogen-ER system in penile physiology (Crescioli et al., 2003). Receptors for estrogen, ERα and ERβ, are distributed in the male reproductive tract of various species (Couse et al., 1999). Mice lacking expression of ER (Couse et al., 1999; Eddy et al., 1996) or aromatase enzyme (Fisher et al., 1998; Honda et al., 1998) are infertile.

Studies have shown that estrogen results in the replacement of cavernous spaces by adipocytes in the corpora cavernosa (Goyal et al., 2004a) and that androgen deprivation appears to affect erectile function, by inducing structural alterations in the cavernous body (Traish et al., 2003), increase of extracellular matrix and accumulation of adipocytes below of the tunic layer of the cavernous body. Nevertheless the androgen deprivation seems to promote the differentiation of mesenchymal stem cells into adipogenics lineages that eventually affect erectile function (Traish et al., 2005).

The erection of the penis involves a complex interaction between the central nervous system and local factors. With sexual stimulation, the endothelials cells of the penis synthesize and liberate nitric oxide (NO) that diffuses out to SMC, activating the enzyme guanylate cyclase that catalyzes the conversion of GTP (guanosine triphosphate) into cGMP (cyclic guanosine monophosphate), leading to the relaxation of SMC and increasing the blood flow and filling of the cavernous spaces with blood. The cellular cGMP level reflects a dynamic balance between its production and degradation by cyclic nucleotide

phosphodiesterases (PDEs) expressed in the cavernous body. Testosterone plays role in balancing the cGMP levels (Goyal et al., 2004b), and both cGMP formation and degradation are affected by T. The ceasing of the release of the neurotransmitter takes to the contraction of the smooth muscle cells (SMC), restoring the venous flux, so that the blood is drained (Priviero et al., 2007; Saad et al., 2007).

Moreover, the extracellular matrix whose main components are collagens, elastic fibers and hyaluronan are essential for the organization and function of the erectile tissue (Pinheiro et al., 2000) Being thus, alterations of anyone of these penile components by estrogenic action, for example, can cause an abnormal morphology of the penis. Therefore it is necessary to understand the erection dysfunction reported in animals that received xenobiotics during critical periods of the development.

The abnormal organization of the extracellular matrix in the corpus cavernosum has been implicated with a number of erectile dysfunctions. According to Wespes et al (1991), the percentage of collagen fibers replacing the SMC is increased in impotent patients with corporeal veno-occlusive dysfunction and arterial lesions.

Considering the potential hazardous effects of estrogenic compounds in animal and human health, our goal is to understand the mechanisms through which estrogens affect critical developmental processes in the male reproductive tract with impact in erectile dysfunction. The objectives of this study were to determine effects of neonatal 17-β-estradiol (E2) exposure on penile development, on AR expression in the rat penis at different ages and in the isolated SMC and endothelial cells.

2. Materials and methods

2.1 Animals and treatments

Ten adult male and female Wistar rats were purchased from the Multidisciplinary Center for Biological Investigation (CEMIB-UNICAMP) and were bred. Pregnancy was accompanied for the determination of day of birth. The pups were sexed according of the urogenital-distance. Offspring size was adjusted to eight male pups per litter. Pups received subcutaneous injections of 25 µL of corn oil containing E2 (Sigma, St. Louis, MO, USA) at a dose of 15 mg/kg body weight on days 1, 3, and 5 after birth (Putz et al., 2001a). Control animals received corn oil only. Pups were observed weekly until sacrificed on 28, 49 or 90 days after birth (prepubertal, pubertal and adulthood stages, respectively). Six penises were collected for *in vivo* analysis and SMC and endothelial cells isolation. Animals received water

and food *ad libitum*. The University's Committee for Ethics in Animal Experimentation Ethic approved all animal procedures (Protocol nr. 1337-1).

2.2 Collection of the organs

The animals were weighted and the penis, ventral prostate and testis were collected. The penises were either fixed in 4% paraformaldehyde for morphological analysis; snap frozen in liquid nitrogen and stored at -70°C for posterior extraction of total proteins or RNA; or placed in EBSS medium containing 5% of penicillin/streptomycin solution (Nutricell, Campinas SP, Brazil) for cell isolation cultures.

2.3 Testosterone and estradiol measurements

Five animals of each age and group were anesthesized with xylazin (50-175 µL/kg) and ketamine (80-280 µL/kg). A blood sample was collected from the heart of each animal in tubes (Serum Activator of Coagule and Gel) (Vacuette) and centrifuged at 25500 g. The serum was frozen at -70°C until assayed. Testosterone and E2 were measured using a COAT-A-COUNT testosterone radioimmunoassay (Siemens Medical Solutions Diagnostics) according to the manufacturer's protocol. The sensitivity of the assay was 3.34 pg/mL and 3.65 ng/dL, respectively. All samples were quantified in a single assay. The variation coefficient was 4.89% for testosterone and 2.35%, for estradiol.

2.4 Smooth muscle cell culture

Primary cultures of 28, 49 and 90-day-old of Wistar rat penis SMC were established by organ explant cultures. SMC were prepared using the protocol described by Gerdes et al. (1996). The penis was minced into 1-2 mm^3 fragments and placed in phenol red free Dulbecco's Modified Eagle's Medium (DMEM) (Nutricell), 10% fetal bovine serum (FBS) (Nutricell), supplemented with 5 µg/mL insulin (Novo Nordisk, São Paulo SP, Brazil) in 24-well culture plates. In half of the plates, testosterone cypionate (0.5 µg/mL) (Novaquímica – Sigma Pharma, Hortolândia SP, Brazil) was added to the medium. The medium was replaced every 48 h and the cell migration out of the explants was followed with a phase contrast microscope.

After reaching confluence, SMC were replated using trypsin/EDTA (Nutricell) and cultured in 25 cm^2 culture flasks (Sarstedt, Mannheim, Germany) at 37 °C in a humidified atmosphere with 5% CO_2.

Immunocitochemstry for myosin heavy chain (MHC) was done to characterize the purity of cultures. Usually, 95% of the cells stained positively.

2.5 Endothelial cell culture

After collection, the penis of Wistar rats on 28, 49 and 90 days after birth were minced into 1-2mm^3 fragments and incubated overnight at 37 °C with agitation in HAM-F12 medium (Nutricell) containing collagenase type I (GIBCO, Auckland, New Zealand) (1mg/mL). After complete dissolution of the tissue, usually after 12 h, the cellular suspension was centrifuged at 515 g for 10 min and 3 mL of trypsin/EDTA (0.25%) (Nutricell) were added to the pellet. After 30 min incubation at 37 °C, 3 times of the initial volume of medium HAM F-12 (Nutricell), 10% of FBS (Nutricell), 0.5% L-Glutamine, 0.3% sodium bicarbonate and 1% penicillin (1.000 U/mL) and streptomycin (250 μg/mL) (Nutricell) were added and centrifuged for 10 min at 515g. The cell suspension was cultured in 75 cm^2 culture flasks (Sarstedt) at 37 °C in a humidified atmosphere with 5% CO_2. After reaching confluence, endothelial cells were obtained by immunomagnetic separation, using Dynabeads (Dynal AS, Oslo, Norway).

In brief, beads linked with anti-CD31 antibody were incubated with the cells at 4 ºC under constant rotation. After 30 minutes, the immunomagnetic separation was done with a Magnetic Particule Concentrator (MPC1) (Dynal) according to the instructions of manufacturer. After 3-4 stages of immunomagnetic separation, the endothelial cells were cultured in 25 cm^2 culture flasks, covered with collagen, at 37° C in a humidified atmosphere with 5% CO_2.

Endothelial cell specific von Willebrand factor (VWF) was used to characterize the purity of cultures. Usually, 95% of the cells stained positively.

2.6 Hydroxyproline quantification

Fragments of the penis from five animals each, control or E2 treated, , were homogenized, delipidated in acetone and chloroform, dried, weighed and submitted to hydrolysis in 6N HCl (1 mL/10 mg tissue) for 18 h at 120 °C. The hydrolyzed was then

treated with a chloramine-T solution for 20 min at 20 °C; perchloric/aldehyde acid was then added, and the mixture was left in a water bath at 60°C for 15 min according to the method described by Stegemann and Stalder (1967). Absorbance was read at 550 nm in a Hewlett-Packard (Palo Alto, CA, USA) 845A spectrophotometer. Different concentrations of hydroxyproline (Sigma, St. Louis, MO, USA) were used for the standard curve.

2.7 Soluble collagen quantification

Fragments of the penis, control or E2 treated, from five animals each, were homogenized in phosphate buffered saline (PBS) pH 7.4 and 0.5M acetic acid added (50 µL/1 mg tissue). After 24h, the samples were centrifuged at 15600g for 20 min. Absorbance was read at 550 nm in a Hewlett-Packard (Palo Alto, CA, USA) 845A spectrophotometer. Different concentrations of collagen (Sigma, St. Louis, MO, USA) were used for the standard curve.

2.8 Glycosaminoglycan quantification

Fragments of the penis, control or E2 treated, from five animals each, were homogenized, delipidated in acetone, dried, and weighed. The samples were digested in 10 mL/g dry tissue in a solution of 0.05 M TRIS-acetate (pH 8) containing maxatase (4 mg/mL) for 24 h at 60°C. After digestion, the samples were precipitated with trichloroacetic acid for 10 min and centrifuged at 3000 rpm for 15 min. Glycosaminoglycans (GAGs) were isolated by precipitation with two volumes of cold methanol overnight at 4°C. The precipitates containing GAGs were air-dried, resuspended in water, and quantified by the dimethylmethylene blue (DMMB) method (Farndale et al., 1986). Contaminating nucleic acid was eliminated by digestion with DNase/RNase (10 mg/mL) purified from *Flavobacterium heparinum*. The extracted GAGs were identified by electrophoresis in a 0.05 M 1,3-diaminopropane buffer system at pH 9.0 as described previously (Dietrich and Dietrich, 1976). The GAGs present in the gel were fixed with 0.1% cetylpyridinium chloride plus 0.3 M NaCl for 1 h and washed with 0.1% cetylpyridinium chloride every 15 min for 2h. The individual bands were visualized by staining with 0.1% toluidine blue in 50% ethanol and 1% acetic acid (Augusto et al., 2008).

2.9 Androgen receptor immunocytochemistry

2.9.1 Penis

The penis had been fixed in paraformaldehyde 4% in PBS pH 7.2, for 48 hours. The tissue was dehydrated with increasing concentrations of ethanol, cleared in xylene and embedded in Paraplast and sectioned (5 µm) using a rotating microtome (Zeiss). Sections were heated for 5 min in 10mM citrate buffer, pH 6.0 in a microwave at maximal potency, cooled for 10 min, treated for 10 min with 3% hydrogen peroxide to quench endogenous peroxidases, and incubated for 1h 1% BSA in TBS-T. Androgen receptor protein was immunolocalized using the N-20 anti-AR antibody (Santa Cruz Biotechnology, Santa Cruz CA, USA) diluted 1:100 in solution of 1% bovine serum albumin (BSA) (Sigma) in tris buffered saline plus 0.2% Tween 20 (TBS-T). While one section on each slide served as a control and received on only 1% BSA, the other section on the same slide received primary antibody and then slides were incubated for 2h at room temperature in a humidified chamber. The primary antibody was reacted with a species-specific biotinylated secondary antibody, and the biotin was detected with an avidin-biotin peroxidase Super kit ABC (Easy Path) using n,n-diaminobenzidine as a chromogen. Developed sections were dry in air and coverslipped using Entellan (Merck). For AR quantification, the intensity of AR staining was visually verified, using optical Zeiss microscope, in 3 sections of 3 different animals of each age and treatment. The intensities were determined as strong, moderate, weak and negative (no staining).

2.9.2 Smooth muscle cell and endothelial cell

The SMC and endothelial cells were plated on glass coverlips in 24-well plates, and maintained in serum containing medium until semi-confluence. Coverlips were washed three times in PBS pH7.4 and fixed for 15 min in 4% paraformoldehyde.

The cells were permeabilized by exposure to TBS-T three times at room, followed by 15 min of incubation with 3% hydrogen peroxide to remove endogenous peroxidases, then washed three times with TBS-T and blocked in TBS-T with 3% BSA for 1h at room temperature. The same anti-AR antibody was diluted in solution of 1% BSA in TBS-T, and applied to the cells for 2h at room temperature. Coverlips were washed three times with TBS-T and incubated with the same horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG

in 1:200 dilution in TBS-T for 1h at room temperature. The cells were then washed in TBS-T and counterstained with hematoxylin. For AR quantification, AR localization was visually verified in 100 cells from 3 differents coverlips from each age and treatment, in cytoplasmic, nuclear/cytoplasmic or nuclear. Observations were done using optical Zeiss microscope.

3.0 Western blotting analysis for androgen receptor

For extraction of proteins, the penis had been homogenized in 1mL (for each 250mg of tissue) of protein buffer extraction (150mM of NaCl, 1% Triton X100, 1mM of EDTA, 10mM of Tris-HCl pH 7.4, 1mM of EGTA, 1mM of HEPES, 100mM of PMSF) and 10 µL/mL of coquetel inhibiting of proteases (BIORAD). For the cells, SMC and endothelial cells were cultured until 90% confluence. Then cells were scraped using trypsin/EDTA and centrifuged for 10 min at 515g. The cell pellet was diluted in protein buffer extraction (1 mM aminohexanoic acid, 5 mM benzamidine, 1 mM PMSF, 5 mM NEM, 10 mM EDTA, 600 mM Tris and 0.1% Triton X-100). Total proteins concentration was measured using Bio-Rad Bradford protein assay.

Western blotting was performed after electrophoresis with 75µg protein of each lysate on 10% polyacrilamide gel under reducing conditions and electric transfer for nitrocellulose membranes (Hybond - ECL, Amersham Biosciences). Membranes were blocked for 1h with 5% non fat dry in TBS-T, at room temperature with agitation. Anti-AR polyclonal antibody (N-20; cat. Sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluited 1:500 in 1% non fat dry in TBS-T and incubated for 2h at room temperature with agitation. After 3 TBS-T washes, HRP conjugated goat anti-rabbit IgG (cat. 61-8100; Zymed Laboratories, South San Francisco, CA, USA) and anti-actin HRP conjugated goat polyclonal IgG (I-16; cat. Sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added 1:2500 and 1:2000 dilution, respectively in TBS-T for 1h at room temperature with agitation. After another set of washes, detection of bound antigen was achieved by chemiluminescence (Santa Cruz Biotechnology). Band intensity was determined by densitometry using Scion Image Software (Scion Corporation).

3.1 RNA extraction, transcription and Real Time PCR

Total RNA was extracted, from the penis and SMC, using TRizol reagent (Invitrogen Life Technologies, São Paulo, SP, Brazil) according to manufacturer. The quantification and

integrity of RNA was assayed by spectrophotometry using Ultraspec 2100 Pro (Amersham Biosciences, São Paulo, SP, Brazil). For cDNA synthesis, 5µg of total RNA samples was reverse transcribed with 20U SuperScript III (Invitrogen Life Technologies) and Oligo(dt)$_{12-18}$ Primer (Invitrogen Life Technologies) according to the supplier's instruction. The cDNA was also quantified by spectrophotometry.

Ten internals controls genes (B2M, TBP7, TBP, GusB, PPiA, 18S, PGK1, HPRT, β-actin and GAPDH) were tested to verify which one best fitted to experimental conditions of the penis and SMC. The analyzes were done using the geNorm (Primer design Ltd.) and NormFinder tools. B2M was the internal control that showed less variation of expression on the experimental conditions and was tested with AR gene for the validation assay. The primers and probe sequences for AR were designed using Primer Express (Applied Biosystems Software). AR: forward, 5' ATG ACT CTG GGA GCT CGT AAG C 3'; reverse: 5' TGG AGT TTT CTC CTT CTT CTT GTA G 3', all synthesized by Applied Biosystems. The internal control gene was analyzed with TaqMan Gene Expression Inventoried Assay (Rn00560865_m1 - Applied Biosystems).

The two-steps multiplex reaction was then used to evaluate relative expression levels of AR mRNA, in 7300 Real Time PCR System (Applied Biosystems), by the Comparative C_T Method. The PCR reactions were set up in a volume of 20 µl. The reaction components were 20 ng of cDNA, 2x TaqMan Master Mix (Applied Biosystems); for the internals controls reactions, the inventoried assay was used 1x and for AR, 600 nM of each primer and 300 nM of the probe.

3.2 Statistical Analysis

Data were expressed as the mean ± standard deviation (SD) of measurements for all experiments. Differences between groups were determined by two way analysis of variance (ANOVA). When the differences were determined, paired comparison of mean values was assessed by Tukey's test (MINITAB 14 software). The differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1 Animals and organs weight

The mean body weight of treated animals, was significantly reduced ($p<5$) in comparison with that of controls on days 49 (198.34 ± 14.32 vs. 179.48 ± 16.17 g) and 90 (315.65 ± 42.50 vs. 269.17 ± 27.50 g) **(Figure 1)**. The mean relative weight of the penis, prostate and testes in treated animals, were significantly smaller than in the controls ($p<5$) **(Figure 2)**.

3.2. Testosterone and estradiol measurements

The mean serum testosterone level in control animals increased from a trace levels on day 28 to about 0.82 ng/mL on day 49 and to 4.48 ng/mL on day 90. In treated animals testosterone serum level also increased from 0.54 ng/mL at day 49 to about 3.37 ng/mL on day 90, with no detectable variation on day 28 the level does not changed. The mean plasma estradiol level reduced at days 49 and 90 in controls and treated animals in comparison with that at day 28 and, at this age, the estradiol levels were markedly different between the two groups (0.09 ng/mL in control to 1.58 ng/mL in treated animals) **(Figure 3).**

3.3. Morphology

The microscopic organization of the penis body in animals at 28 days after birth showed cavernosus sinusoids irregular in diameter and mainly concentrated under the cellular layer of tunica albuginea in treated rats **(Fig. 4, A and D)**. In the corpus cavernosus, in both groups, amount substantial adypocites, characterized by round, empty spaces in the hematoxylin and eosin-stained sections, were seen mainly under the cellular tunica albuginea; in treated animals these cells were more numerous and appeared spread throughout the corpus cavernosus **(Fig. 4, B and E)**. Moreover, in treated rats there was an increase of the cavernosus sinusoids in corpus spongiosus and a decrease in the stromal tissue, reducing the sustentation of urethra **(Fig. 4, C and F)**. The stromal tissue was apparently less abundant in collagen fibers than that at 28-day control group.

At day 49, the corpus cavernosus penis in control rats contained wider cavernous sinusoids in comparison with that at day 28 **(Figs. 4 and 5; A, B)**. In treated 49-day rats,

cavernous sinusoids had been reduced, tunica albuginea appeared larger and the stromal tissue appears more fibrous in comparison with that in control animals **(Figs. 5 A, B, D and E)**. Moreover adypocites was abundant in the corpus cavernosus in treated group than in controls **(Figs. 5 B and E)**. The corpus spongiosus reduced in treated group **(Figs. 5 C and F)**.

The morphology of the corpus cavernosus in rats at 90 days was essentially similar to that of the pubertal rats, except for an apparent increase of the cavernous sinusoids and density of fibers in the intercavernous stroma **(Figs. 5 and 6; A, B)**. In adulthood, the corpus cavernosus of treated animals was dominated by wide sinusoids, the boundaries of which were distinct and demarcated by thin fibers; also the tunica albuginea was reduced in thickness and contained fewer fibers and cells, in comparison to controls **(Fig. 6 B and E)**.

3.4 Analyses of soluble collagen, hydroxyproline and GAG

Sections of the adult penis stained with picrossirius showed an arrangement of the collagen fibers apparently more compacted, in the penis estradiol treated animal, in comparison to control; the neonatal E2 treatment appears to affect the organization of the extracellular matrix **(Figura 7 and 8)**.

Analysis of the penis morphology led the observation of the disappearance of the Littré glands, present in the corpus spongiosus, in the adult animals treated neonatally with estradiol **(Figure 9)**.

The E2 treatment reduces the amount of soluble collagen and hydroxyproline **(Figure 10)** and increase the GAG in the adult penis **(Figure 11)**.

3.5. Androgen receptor localization analyses

3.5.1 Penis

Immunocytochemical detection of AR was analyzed in three morphological components of the body penis, corpus cavernosus, corpus spongiosus and intercavernosus septum of each age and treatment. The intensity of staining at 28-day varied from weak to moderate and from negative to weak in corpus cavernosus **(Figure 12 A and C)** and spongiosus, in control and treated rats, respectively. In the intercavernosus septum there was no staining, in both, control and treated animals. In urethral epithelial cells there were a citoplasmic staining that varied from moderate, in control, to weak in treated group **(Figure 12 B and D)**. In pubertal rats (49d), the intensity of staining was strong in the corpus

cavernosus in control and moderate in treated **(Figure 13 A and C)**. In the corpus spongiosus the staining varied from weak to strong in control animals. In treated group, the staining varied from moderate to strong in the corpus cavernosus and it was moderate in the corpus spongiosus. In the intercavernosus septum the intensity of staining was weak, in both, control and treated rats. In adult rats (90d), the intensity of staining varied from moderate to strong in the corpus cavernosus and spongiosus in control rats, negative to moderate in the corpus cavernosus and from weak to moderate, in treated animals **(Figure 14 A and C)**. In the intercavernosus septum the intensity of staining varied from negative to moderate and it was negative in treated animals **(Table1)**.

3.5.2 Smooth muscle cell and endothelial cell

SMC specific myosin heavy chain (MHC) and von Willebrand factor (VWF) to endothelial cell were used to characterize the purity of cultures **(Figure 15)**.

Immunocytochemistry of AR in the cells is described under the staining in the cytoplasm (C) and nucleus (N). The cytoplasmic staining of AR in SMC cultured without or with testosterone decreased with age, while the nuclear staining increased **(Figure 16)**. In the cells isolated from treated animals the cytoplasmic and nuclear staining of AR in SMC cultured without or with testosterone, was the opposite from those isolated from control animals **(Figure 16)**. The staining of AR in endothelial cells, in controls, was predominantly cytoplasmic at 28 and 90 days. In those cells isolated from treated animals, the cytoplasmic staining was higher at 28 and 90 day, in the otherwise the nuclear staining does not varied between the ages **(Figure 17)**.

3.6. Androgen receptor protein expression analyses

It was apparent that AR content in penis, SMC and endothelial cells are affected by the neonatal treatment with estradiol. In the penis, quantification of the bands **(Figure 18)** showed that E2 treatment caused significant variation in AR protein at 28 and 90 days after birth in comparison to controls. Expression of AR was highest at 49-day and it reduced from prepubertal to adults rats in treated animals. In the SMC cultured in free testosterone medium, the AR expression reduces with age and the treatment does not caused significant variation in AR protein **(Figure 19)**. On the other hand, SMC cultured with testosterone supplementation, showed that E2 treatment caused significant variation in AR protein only at 28 days after birth

in comparison to control. Expression of AR reduced with age **(Figure 20)**. The AR content in endothelial cells varied also at day-28 in comparison to control. Expression of AR was the same between controls **(Figure 21)**.

3.7. Androgen receptor mRNA expression analyses

The AR mRNA expression does not varied with age, but the neonatal treatment with E2 leds to an increase of mRNA expression at 28 days animals **(Figure 22)**. In SMC cultured with no testosterone, AR mRNA expression was the same in cells isolated from control and treated animals **(Figure 23)**, but the testosterone supplementation leds to an increase of AR mRNA in those cells isolated from E2 treated animals **(Figure 24)**.

4. Discussion

The treatment with E2 resulted in body weight reduction in the animals at 49 and 90 days after birth, but not on day 28. Moreover, the weight of penis, prostate and testis were also reduced in all ages and there was an increased deposition of adipocytes in the penis corpus cavernosus, indicating that the E2 treatment affects important steps in the development of male reproductive tract. These alterations are permanent and led to infertility (Goyal et al. 2004b).

According to Goyal et al. (2004b) this adipogenesis was associated with the reduction of testosterone levels and increase of ER expression without, however, alteration on patterns of AR expression that was largely negative in penile compartments of immature, mature, and adult rats, in contrast to the observations of this study. Reasons for differences between the two studies may be attributed to differences in the lineages rats (Wistar vs. Sprague-Dawley), estrogenic compound (17β-estradiol vs. DES) and/or different methodologies (immunocytochemical vs. biochemical analyzes).

In adult penis, the amount of hydroxyproline reduced while the quantity of glycosaminoglycan increased in estradiol treated animals, in comparison to controls. Knowing that SMC contributes with the collagen synthesis (Moreland et al., 1995) and that, apparently, SMCs are substituted by adipocytes in treated animals (Cooke and Naaz, 2004), this explain the reduction of hydroxyproline amount in this group. The increase in fat cells deposition results in reduction of penis total area what could explain the appearance more compacted of collagen fibers in treated group.

In this work we found that the treatment with E2 led to an increase of AR expression at prepubertal rats and a reduced expression at adulthood, without alteration on testosterone levels. The estradiol levels were higher in the prepubertal and pubertal periods, but not at adulthood, maybe there was a residual of E2 in the 28-day animals treated.

It is shown in literature that the AR expression reaches the maximum level at the pubertal period and falls after sexual maturation, even with high levels of testosterone (Takane et al., 1990). This was associated with reduction in AR mRNA expression (Takane et al. and Gonzalez-Cadavid et al., 1991), what is in contrast with this work, in which there was no difference in AR mRNA expression in control groups. In the otherwise, in treated groups, AR mRNA presented low expression after 28 days of age. In this period, AR mRNA expression increased in treated group in comparison to control. Estradiol may act up-regulating AR expression on prepubertal and pubertal period and down-regulating in adult animals, suggesting that the balance between androgens and estrogen is critical in penile growth and differentiation. Exactly how estrogens and androgens and their respective receptors interact is unclear. However, we believe it may be that some of the penile effects currently attributed to androgens, such as androgen receptor down-regulation (Takane et al., 1990), may be mediated by estrogens, at the level of their mRNA or protein.

Differently to the organ, we found that in the SMC, cultured without T, the AR expression reached maximum peak in the cells isolated from 28-day-old animals and was the same in endothelial cells. In the cells obtained from estrogenized penis, the expression decreased with age in endothelial cells, increased at day-90 in the SMC not cultured with testosterone and decreased in those cultured with testosterone, even with the increase in AR mRNA expression in SMC, isolated from E2 treated animals and cultured with T. In SMC and in endothelial cells isolated from treated animals the cytoplasmic AR localization increased, while the nuclear localization decreased, what led us to suppose that the activation of AR is time and testosterone-dependent and that E2 treatment contribute to retain cytoplasmic AR localization.

When varying doses of estrogen were administered to SMC of the human fetal corpus cavernosus in vitro, they exerted a dose-dependent anti-proliferative effect, which was opposite to testosterone (Crescioli et al., 2003). This observation in human cells is consistent with in vivo data obtained for rats by Goyal et al. (2004a) and Yucel et al. (2003), demonstrating an inhibitory effect of DES on rat penile growth and differentiation.

Because of the different pattern of AR expression in vivo and in vitro, it is certain that testosterone regulates the expression differently. It is probable that other components act

together inhibiting testosterone action on AR expression in adulthood, but in vivo, testosterone does not interfere with the pattern of AR expression in prepubertal, pubertal and adults periods in culture, but in SMC isolated from treated animals, testosterone does not influence AR expression in adulthood.

In the prostate and seminal vesicle the maintenance of the differentiated state and of the secretory activity depends on the androgenic stimulation (Quigley et al., 1995). In the penis, after sexual maturation, the AR is also responsible for the maintenance of the differentiated state; however in independence of androgenics stimulations, other factors or endocrines mechanisms could be responsible for this pattern of AR expression regulation in the penis.

The influence of estrogen on penile development and function is mostly unclear. Much of what is known about the effects of estrogen on penile events is largely based on in utero exposure to environmental estrogens or mega pharmacological doses of estradiol (Toppari et al., 1996). The fact that estrogen receptors and aromatase are localized in diverse subpopulation of penile cells, epithelia, stroma, glandular cells, suggests that its function in the penis is likely to be cell specific and complex (Jesmin et al., 2002, 2004; Crescioli et al., 2003; Goyal et al., 2004a,b).

5. Conclusion

Neonatal E2 treatment with estradiol led to an abnormal development of the penis characterized by reduction in length and weight and morphological changes as reduction in the cavernosus sinusoids and accumulation of adipocytes in the corpus cavernosus. The treatment seems to increase the extracellular matrix in the penis, which appears to be more dense and fibrous. These results may be related to alterations in ARmRNA expression, AR protein expression and AR localization not only in penis, but also in SMC and endothelial cells presents in the organ, decreasing the erectile process.

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Figure 1

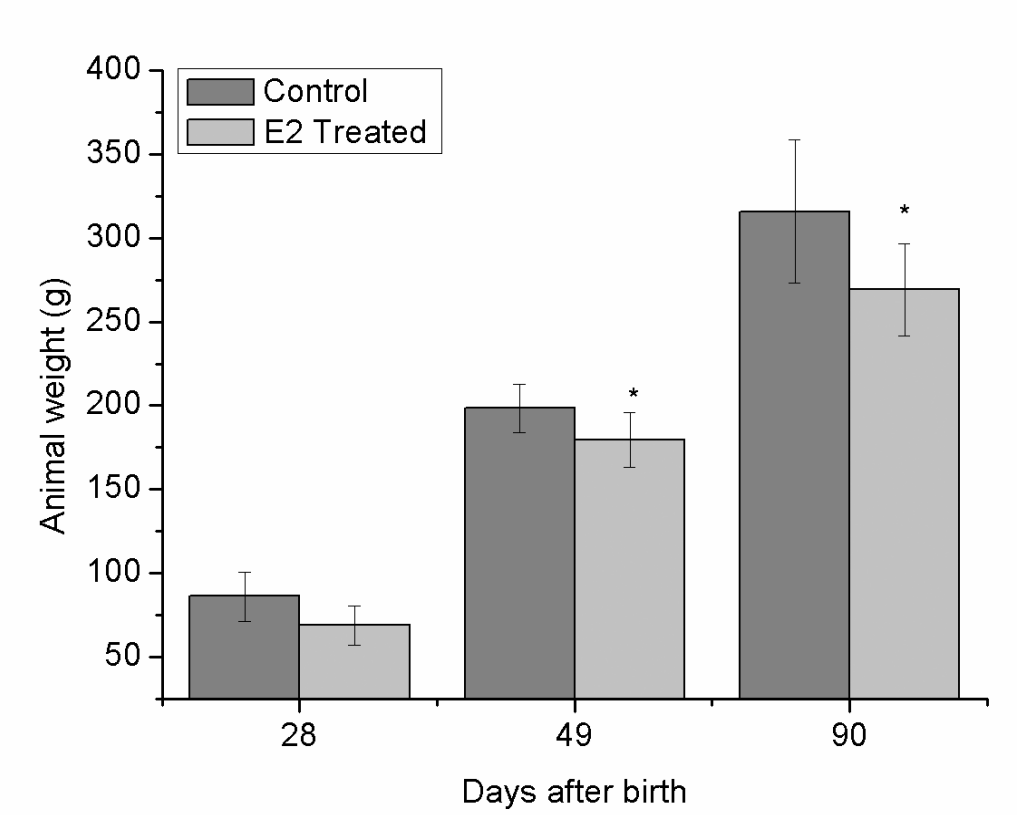

Figure 1. Body weight variation in prepubertal (28 days), pubertal (49 days) and adult (90 days) Wistar rats and the effect of early postnatal estrogenization. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups ($P \leq 0.05$), n=10.

Figure 2

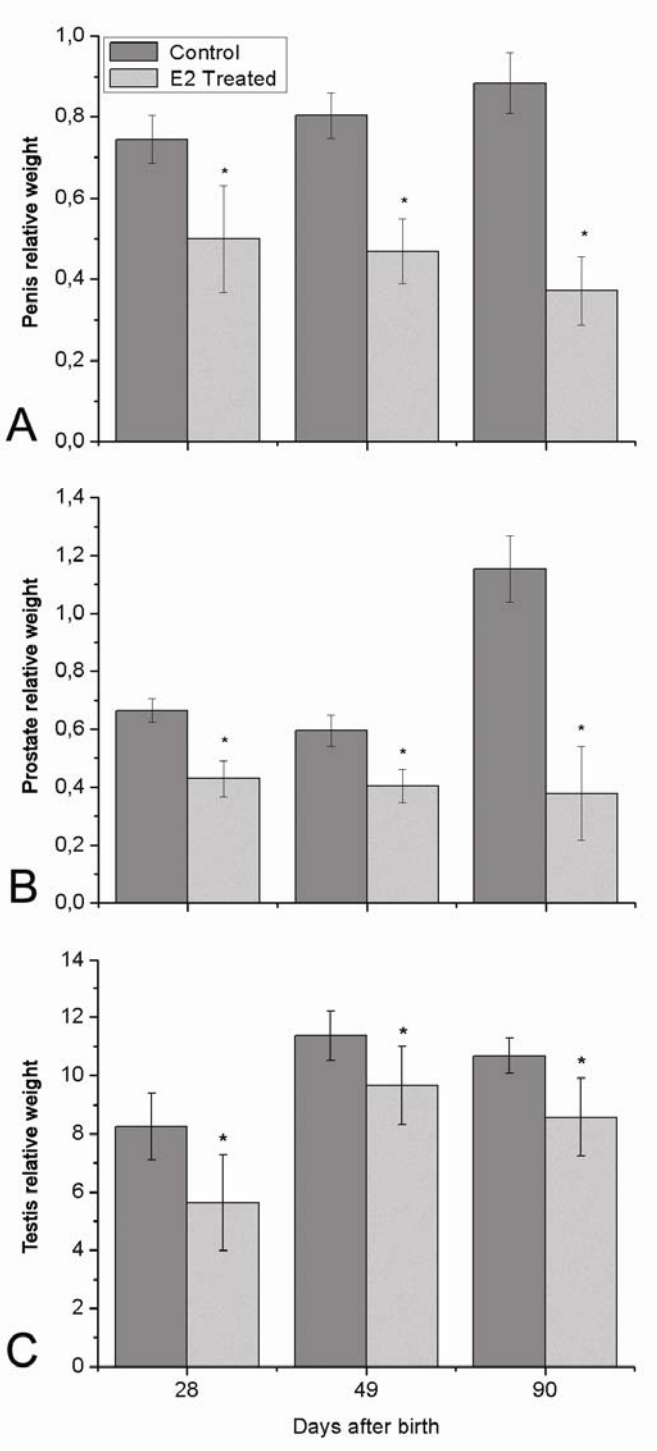

Figure 2. Penis (A), prostate (B) and testis (C) relative weight in prepubertal (28 days), pubertal (49 days) and adult (90 days) Wistar rats and the effect of early postnatal estrogenization. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups ($P \leq 0.05$), n=10.

Figure 3

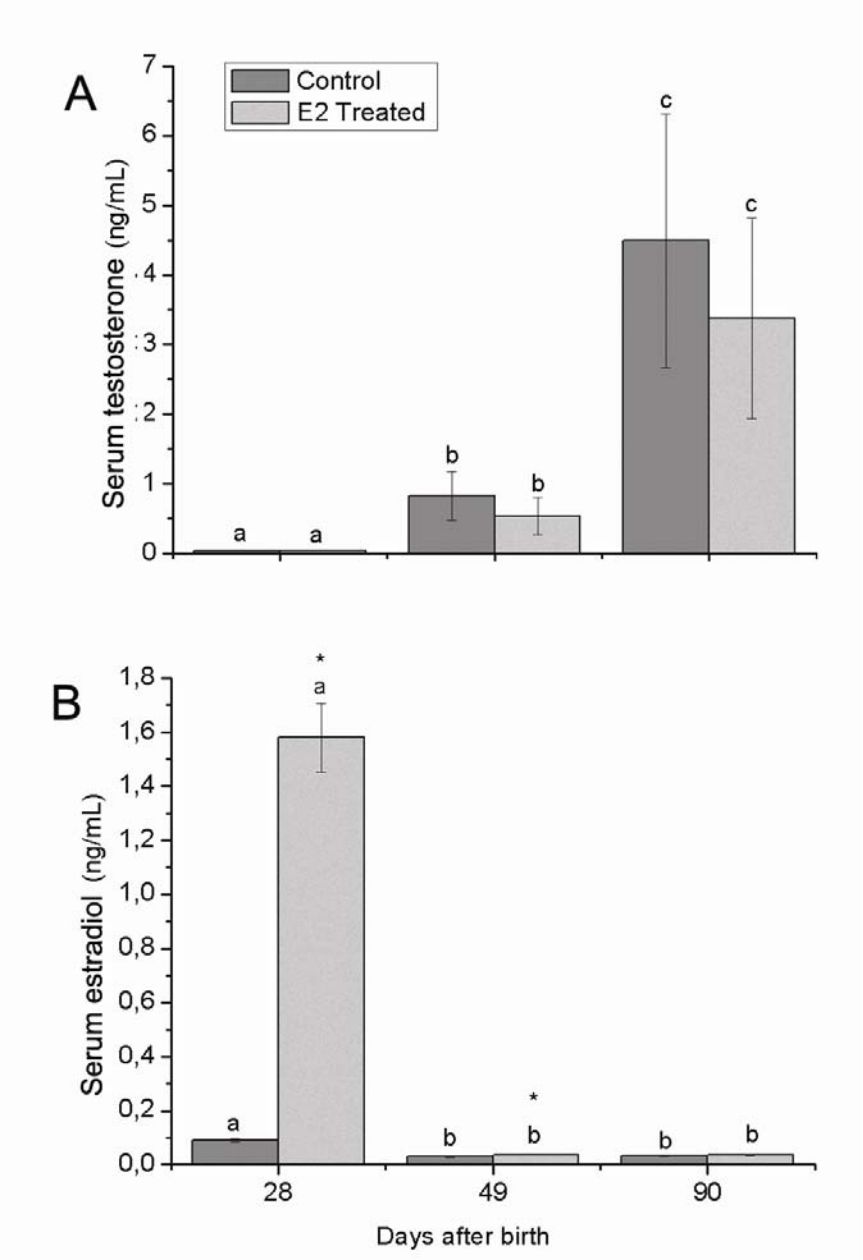

Figure 3. Effect of exposure of neonatal male rats to estradiol on plasma testosterone (A) and estradiol (B) at 28, 49 and 90 days of age. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups and the letters indicates differences between the estrogenized and control groups, separately ($P \leq 0.05$), n=5.

Figure 4

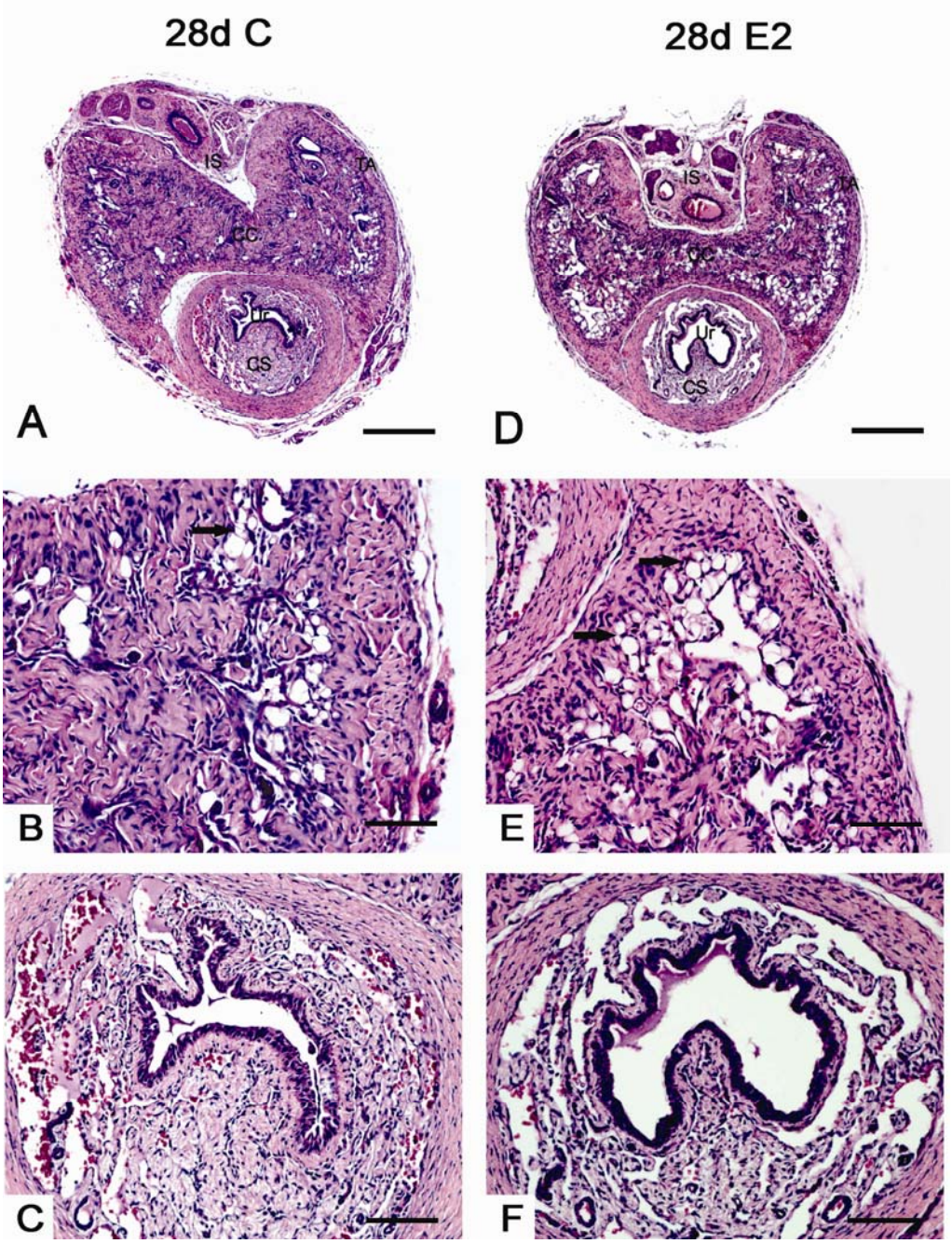

Figure 4. Micrographs from the penis body in control rats **(A–C)** and in rats treated with estradiol neonatally **(D-F)** at 28 days of age. **A**, **D**) Note spatial arrangement of different parts of the body penis between control and treated rats: paired corpora cavernosa (CC), corpus spongiosus (CS), urethra (Ur), intercavernosus septum (IS), tunica albuginea (TA); cavernosus sinusoids irregular in diameter and mainly concentrated under the tunica cellular albuginea in treated rats. **B, E)** Note much higher accumulation of adypocites in the corpora cavernosa penis of the treated rat (arrows). **C, F)** Increase of the cavernosus sinusoids in corpus spongiosus and a decrease in the stromal tissue. Hematoxylin and eosin staining. Bars: A, D = 400 µm, B, C, E, F= 200 µm.

Figure 5

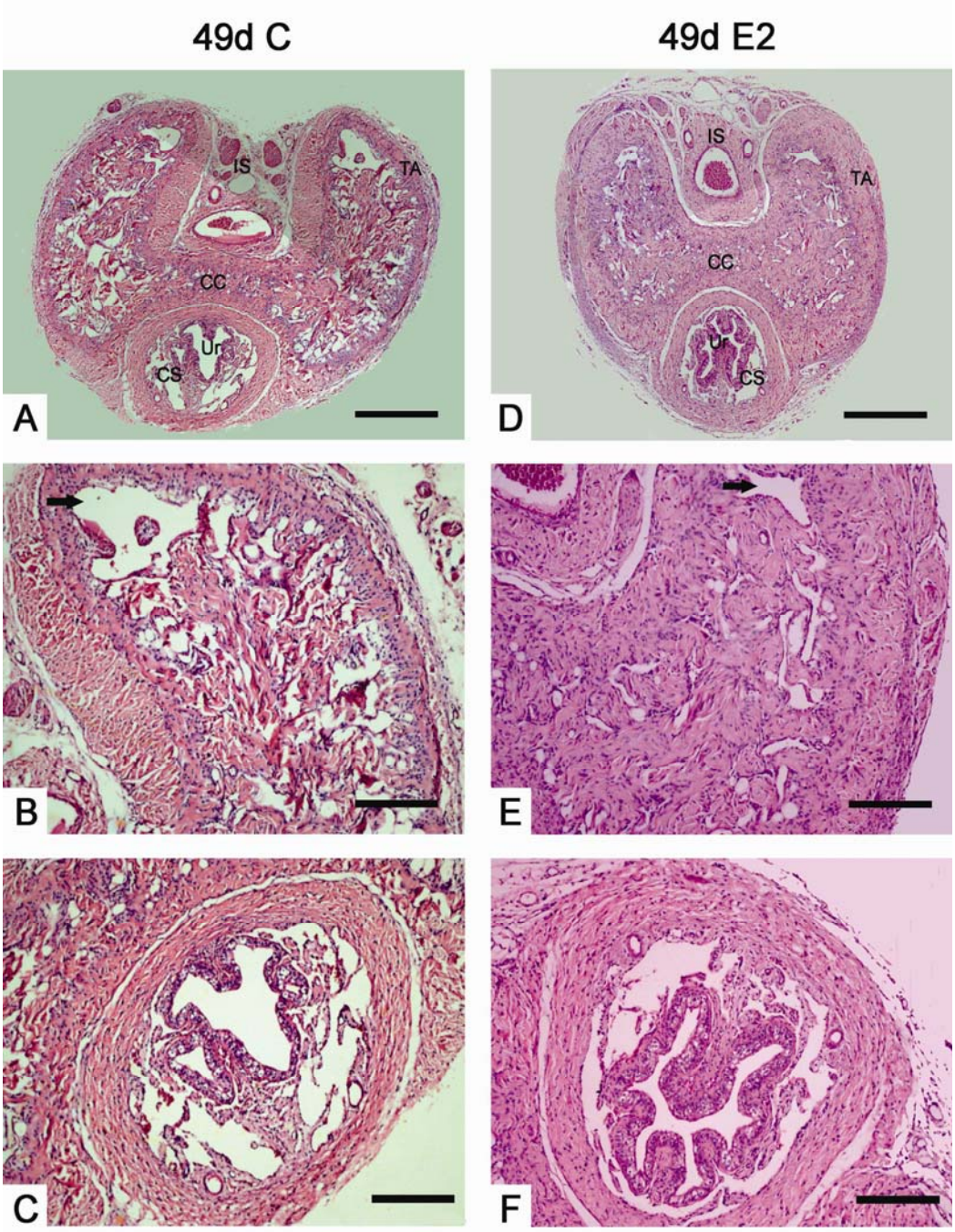

Figure 5. Micrographs from the penis body in control rats **(A-C)** and in rats treated with estradiol neonatally **(D-F)** at 49 days of age. **A**, **D)** Although spatial arrangement of different parts of the body penis between control and treated rats: paired corpora cavernosa (CC), corpus spongiosus (CS), urethra (Ur), intercavernosus septum (IS), tunica albuginea (TA); tunica albuginea appeared larger in treated group; **B, E)** Note cavernous sinusoids reduced and accumulation of empty-appearing adipose cells in corpus cavernosus of the treated rats (arrows). **C, F)** Corpus spongiosus showing reduction in treated group. Hematoxylin and eosin staining. Bars: A, D= 400 µm; B, C, E, F= 200 µm.

Figure 6

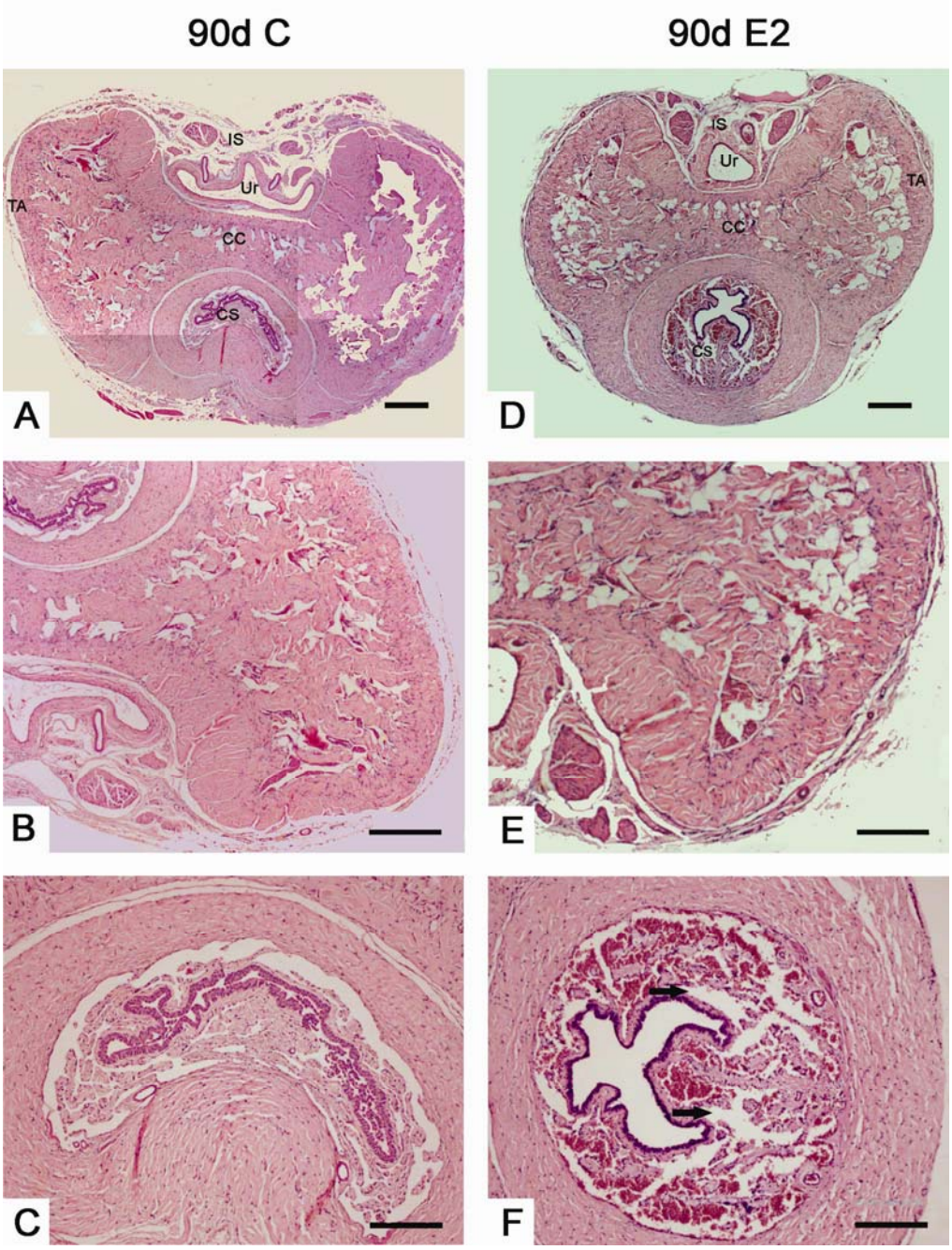

Figure 6. Micrographs from the penis body in control rats **(A-C)** and in rats treated with estradiol neonatally (**E**–**H**) at 90 days of age. **A**, **D)** Although spatial arrangement of different parts of the body penis between control and treated rats: paired corpora cavernosa (CC), corpus spongiosus (CS), urethra (Ur), intercavernosus septum (IC), tunica albuginea (TA); **B, E)** Note cavernosus space more developed in control than in treated rats and abundant intracavernosus stroma. Note accumulation of empty-appearing adipose cells in corpus cavernosus of the treated rats. **C, F)** Corpus spongiosus showing wide cavernosus sinusoids. Hematoxylin and eosin staining (arrows). Bars= A, D= 400 µm; B, C, E, F= 200 µm.

Figure 7

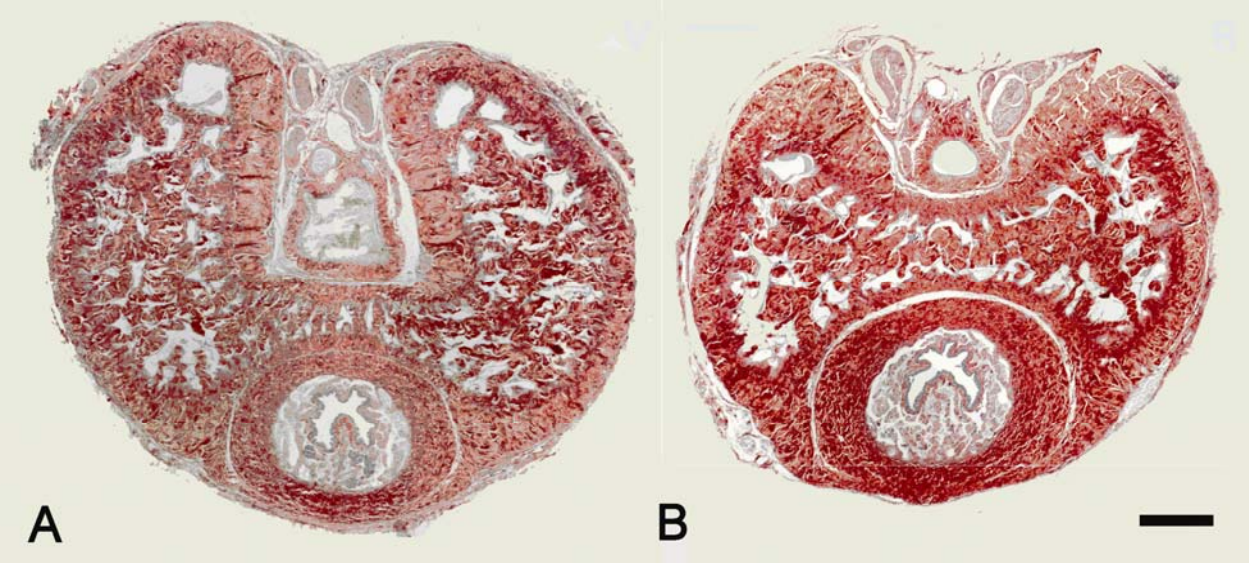

Figure 7. Micrographs from the body penis of adult rat in control (A) and in estradiol treated (B) rat. Observe the intense picrossirius staining in the treated animal penis, showing a more compacted arrangement of the collagens fibers. Bar= 400µm.

Figure 8

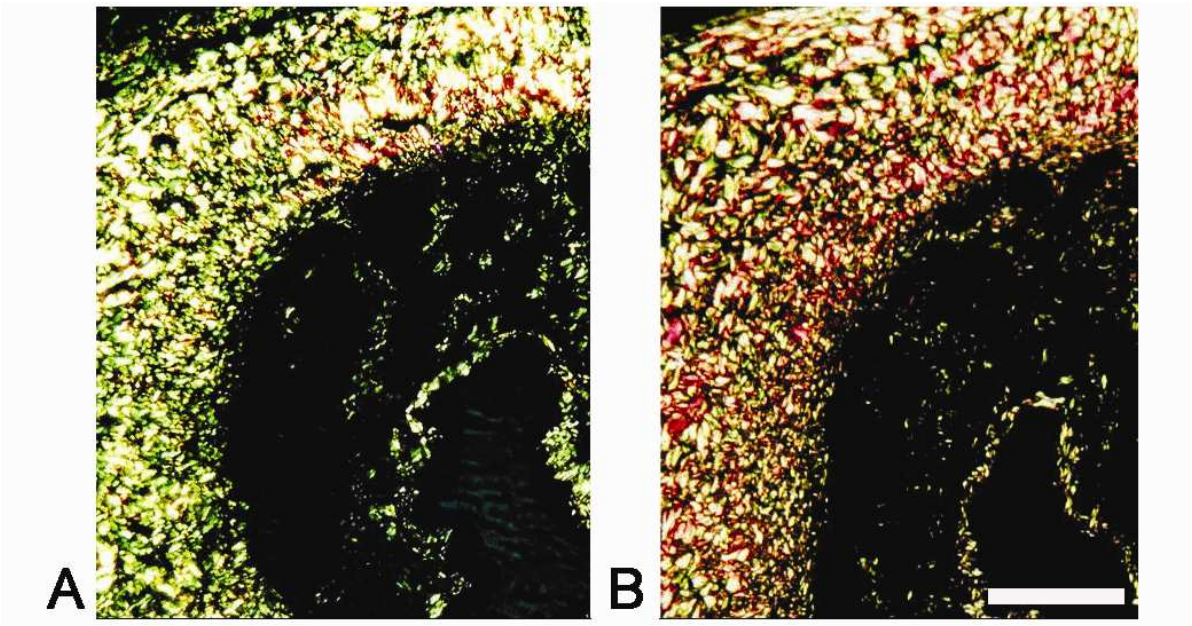

Figure 8. Stromal tissue of control animals penis at 90 days in control (A) and E2 treated (B). Note the colors of birefringence interference predominantly green in the control, and pink in the animal treated, what showing that the collagen fibers are more compacted. Picrossirius staining. Bar= 200µm.

Figure 9

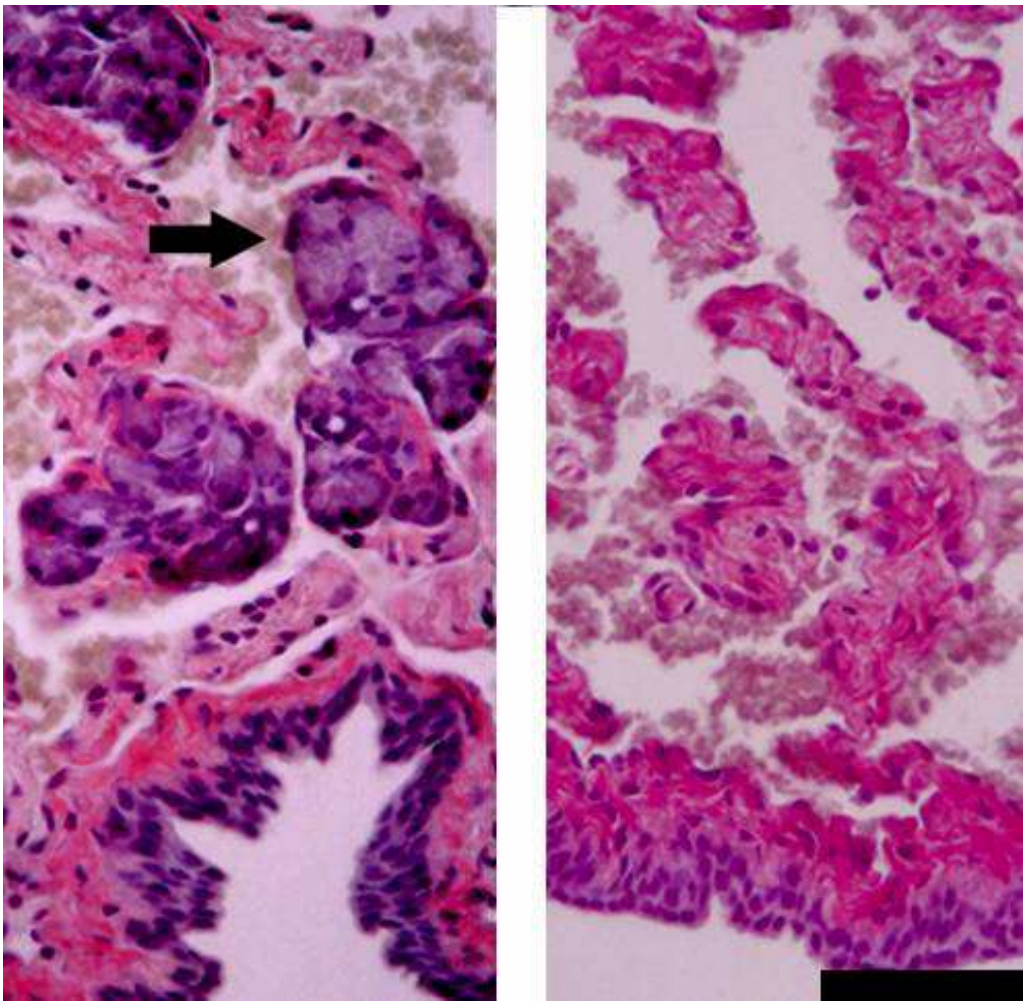

Figure 9. Corpus spongiosus of control animals penis at 90 days (A) and E2 treated (B). Note, in the control, the presence of the Littré glands (arrows), and their absence in the treated animal. Hematoxylin and eosin staining. Bar= 50µm.

Figure 10

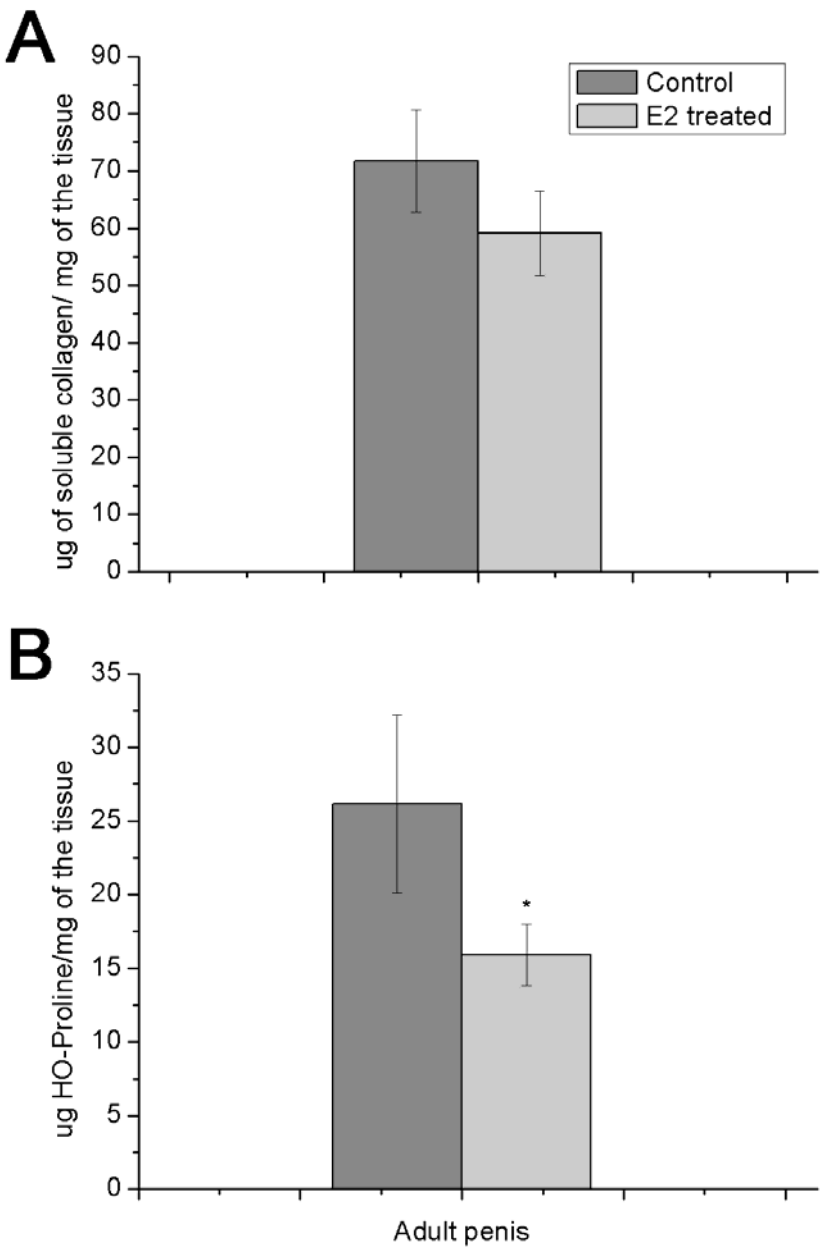

Figure 10. Soluble collagen (A) and hydroxyproline (B) quantification in the penis of animals at 90 days, control and E2 treated. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups ($P \leq 0.05$), n=6.

Figure 11

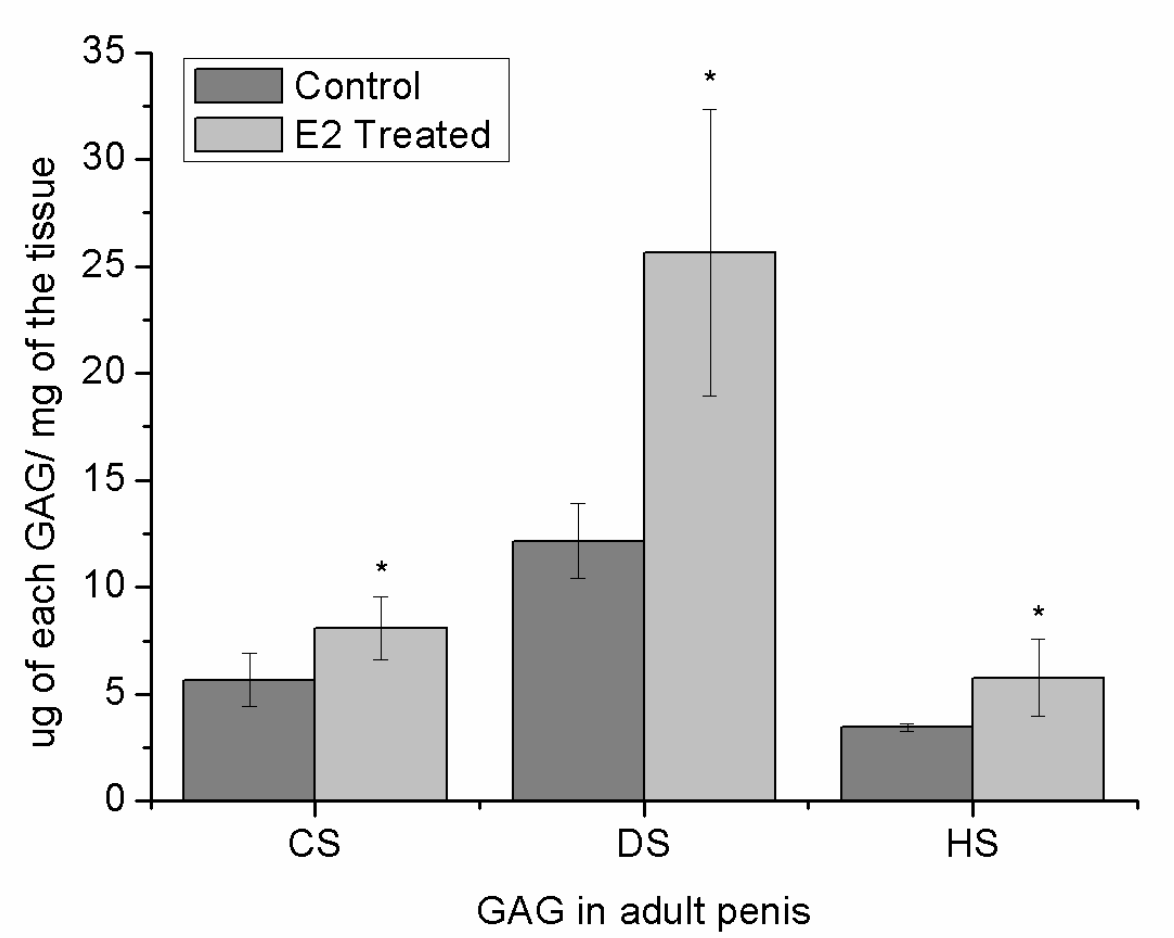

Figure 11. GAG quantification in the penis of animals at 90 days, control and E2 treated. CS: chondroitin sulphate, DS: dermatan sulphate, HS: heparam sulphate. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups ($P \leq 0.05$), n=5.

Figure 12

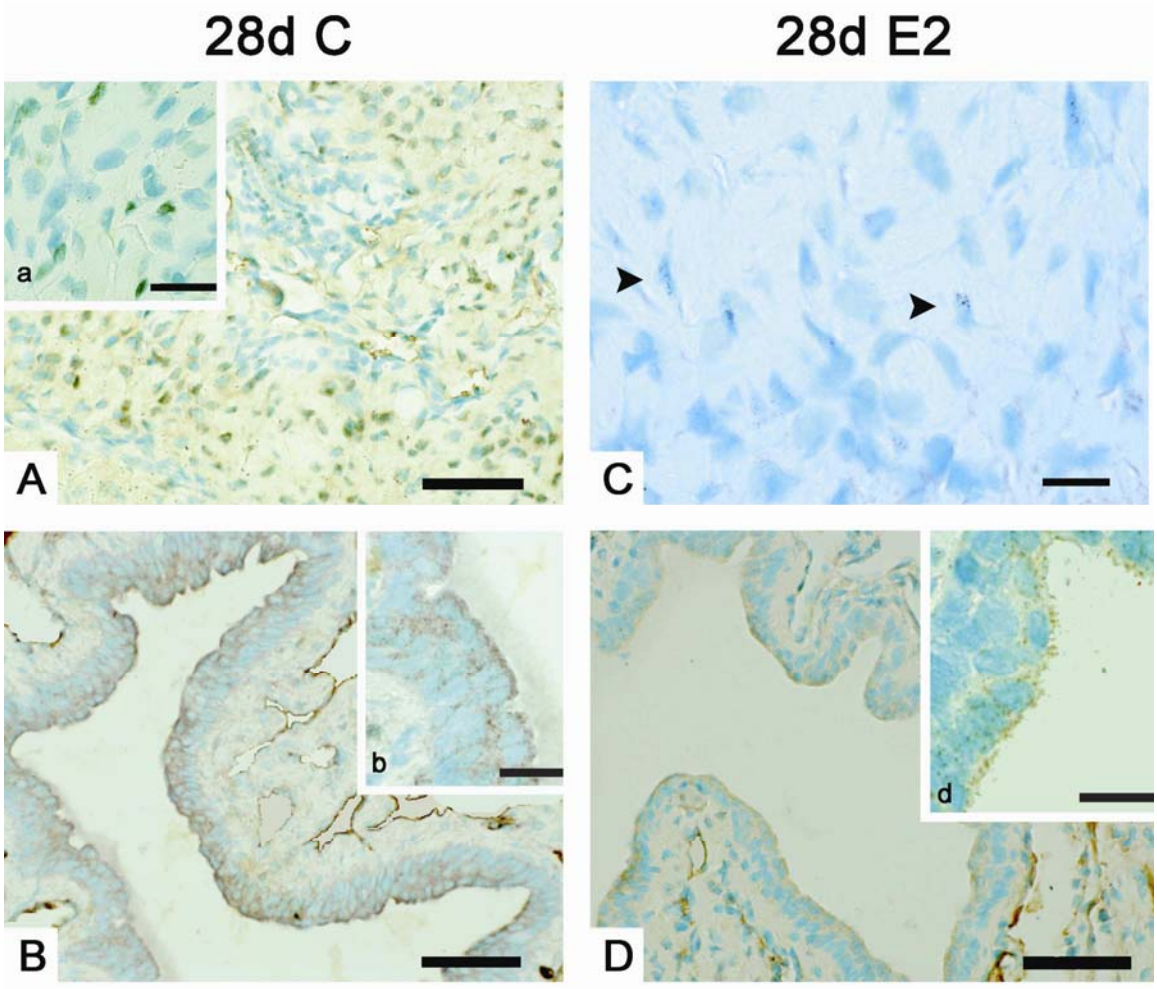

Figure 12. Immunolocalization of androgen receptor in different compartments of the penis at 28 days of age in control (A, B) and rats treated neonatally with estradiol (C, D). A, C) Nuclear staining less intense of stromal cell in corpus cavernosus in treated animals. B, D) Citoplasmic staining on urethral epithelial cells in both, control and treated animals. Note that estradiol treatment affected the staining intensity of androgen receptor-positive cells. Bars=100 µm (A, B, D), 50µm (C, b, d) and 20 µm (a).

Figure 13

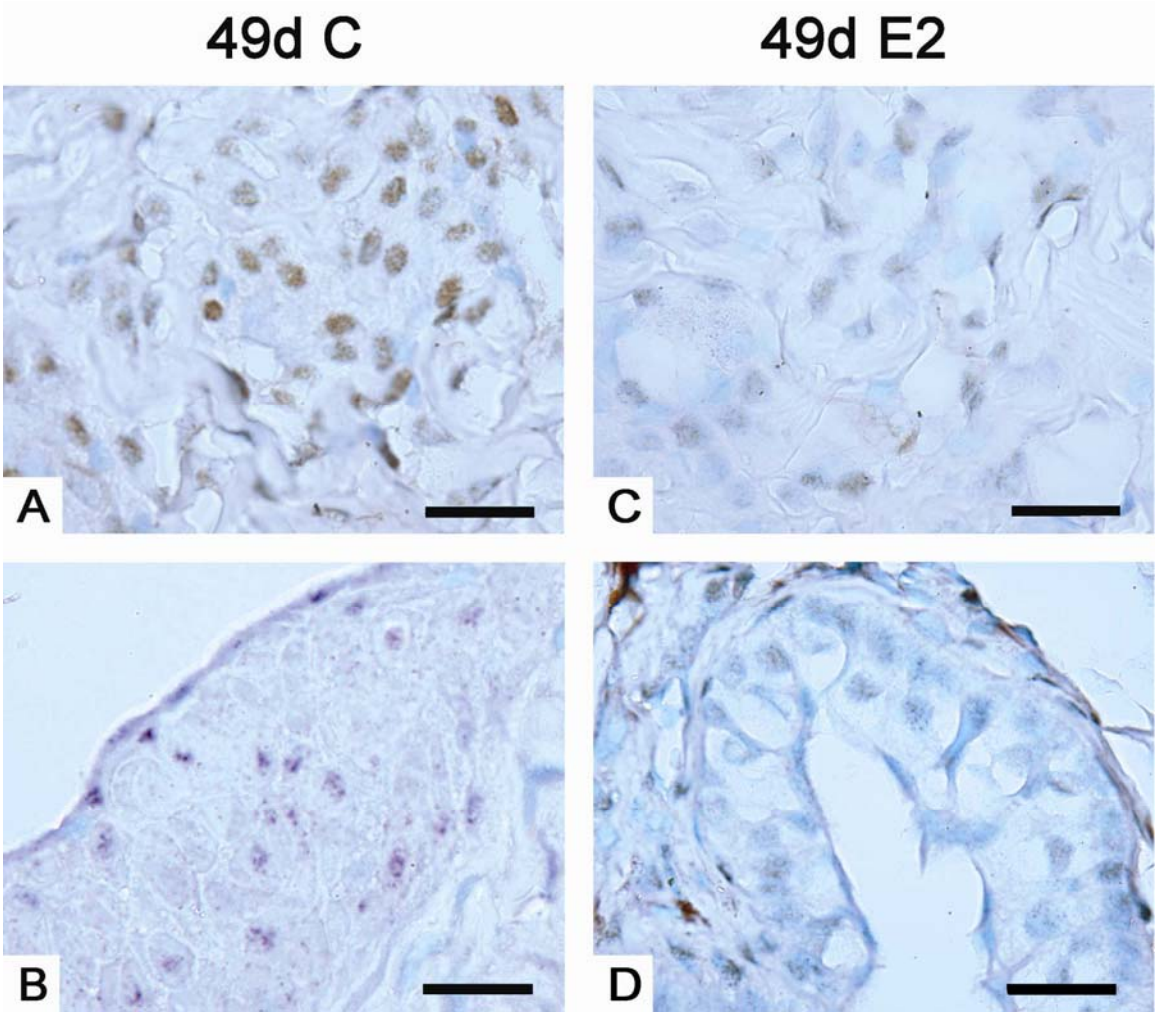

Figure 13. Immunolocalization of androgen receptor in different compartments of the penis at 49 days of age in control (A-C) and rats treated neonatally with estradiol (D-F). A, C) As at 28-day, the nuclear staining of stromal cell in corpus cavernosus was weak in treated animals. B, D) Intense staining in urethral epithelium in treated rats. Bars=50 µm (A, B, C, D).

Figure 14

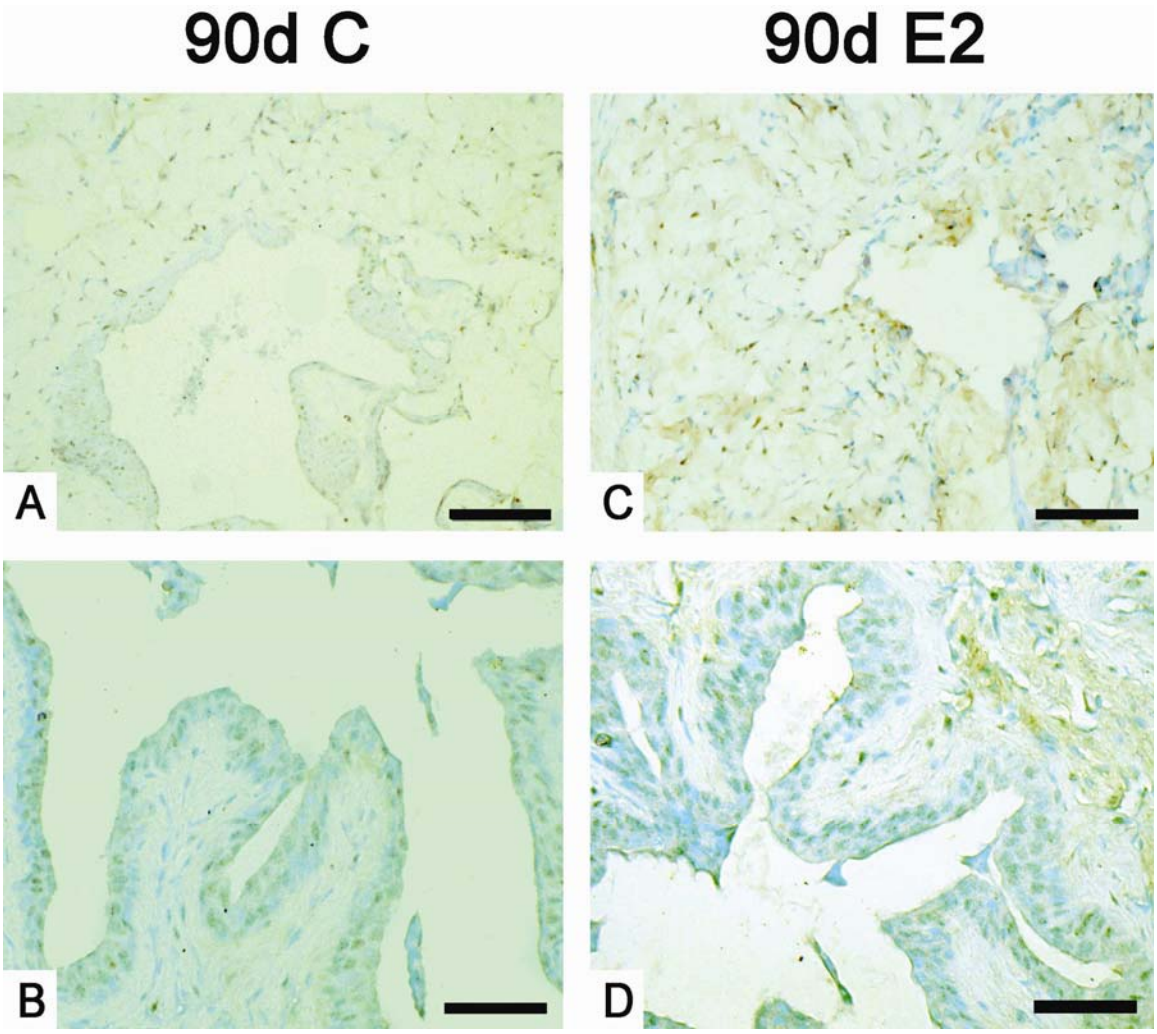

Figure 14. Immunolocalization of androgen receptor in different compartments of the penis at 90 days of age in control (A, B) and rats treated neonatally with estradiol (C, D). A, C) The nuclear staining of stromal cell in corpus cavernosus was, also, weak in treated animals. B, D) Intense staining in urethral epithelium in control rats in comparison with that in treated rats. Bars=100 µm (A,C) and 50µm (B,D).

Figure 15

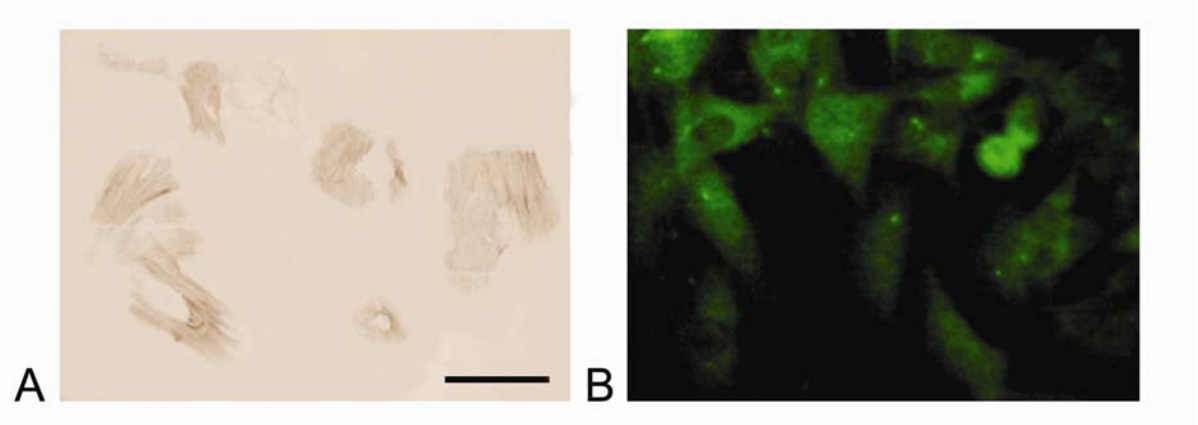

Figure 15. SMC specific myosin heavy chain (A) and von Willebrand factor (B) to endothelial cell were used to characterize the purity of cultures. Bar= 200µm.

Figure 16

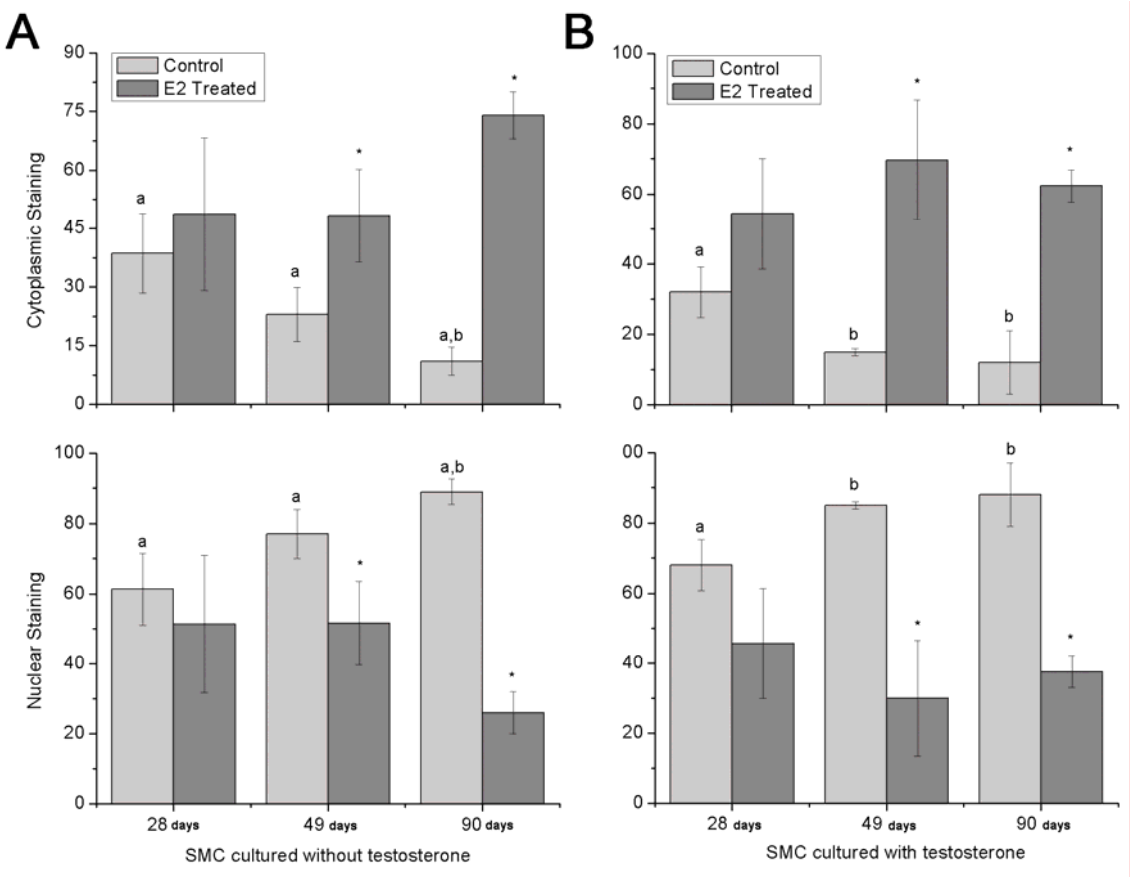

Figure 16. Androgen receptor localization in SMC isolated from the penis at 28, 49 and 90 days after birth, cultured without (A) and with (B) testosterone. The staining was cytoplasmic and nuclear. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups ($P \leq 0.05$), n=3.

Figure 17

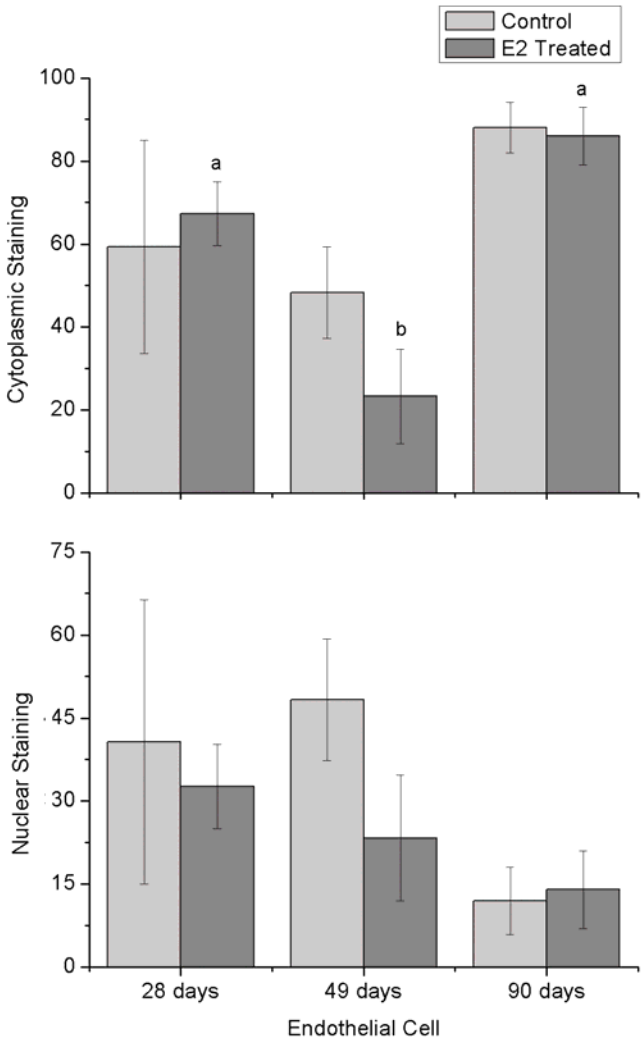

Figure 17. Androgen receptor localization in endothelial cells isolated from the penis at 28, 49 and 90 days after birth. The staining was cytoplasmic and nuclear. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups ($P \leq 0.05$), n=3.

Figure 18

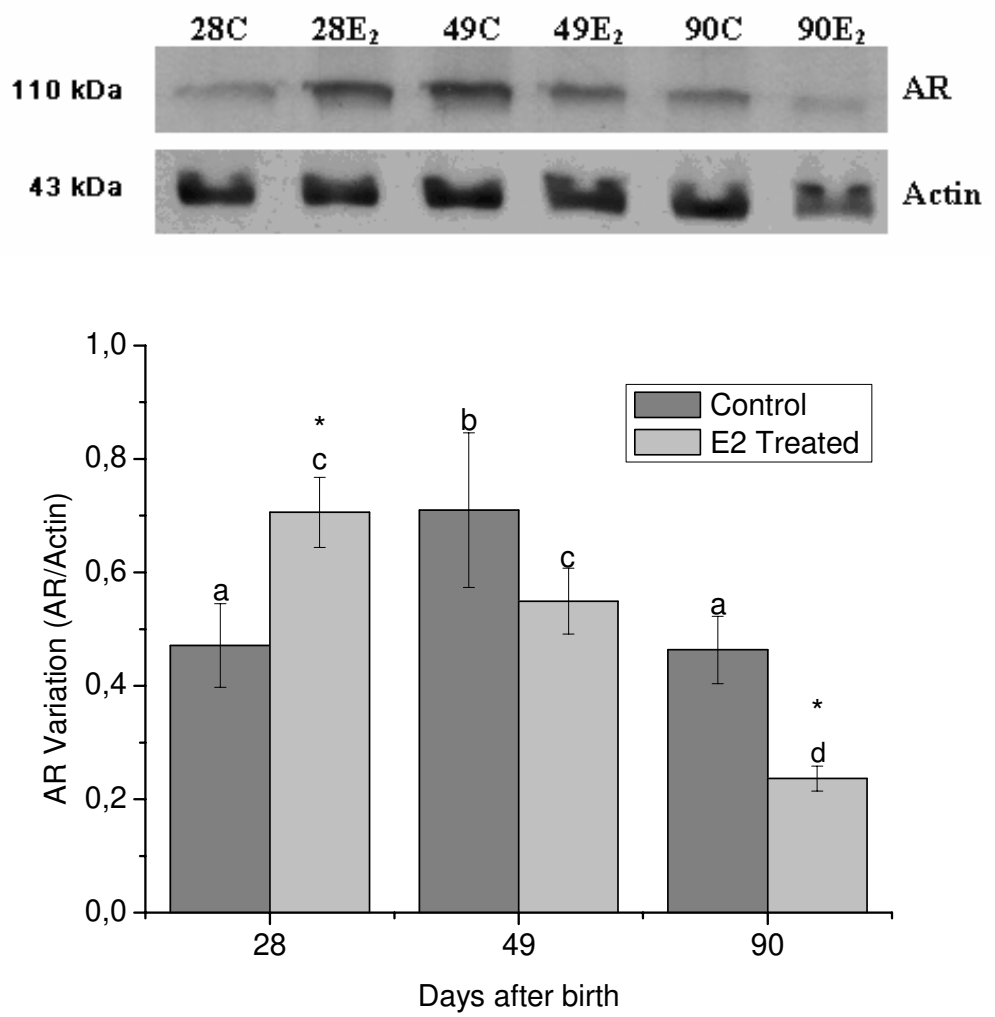

Figure 18. Representative Western blotting showing AR expression in the penis. **A)** Protein levels of AR at 28, 49 and 90 days after birth following neonatal treatment with estradiol. **B)** Actin was used as loading control. **C)** Variation of band density AR for penis. Only at 28 and 90 days after birth the expression of AR was significantly different of the control group. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups and the letters indicates differences between the estrogenized and control groups, separately ($P \leq 0.05$), n=3.

Figure 19

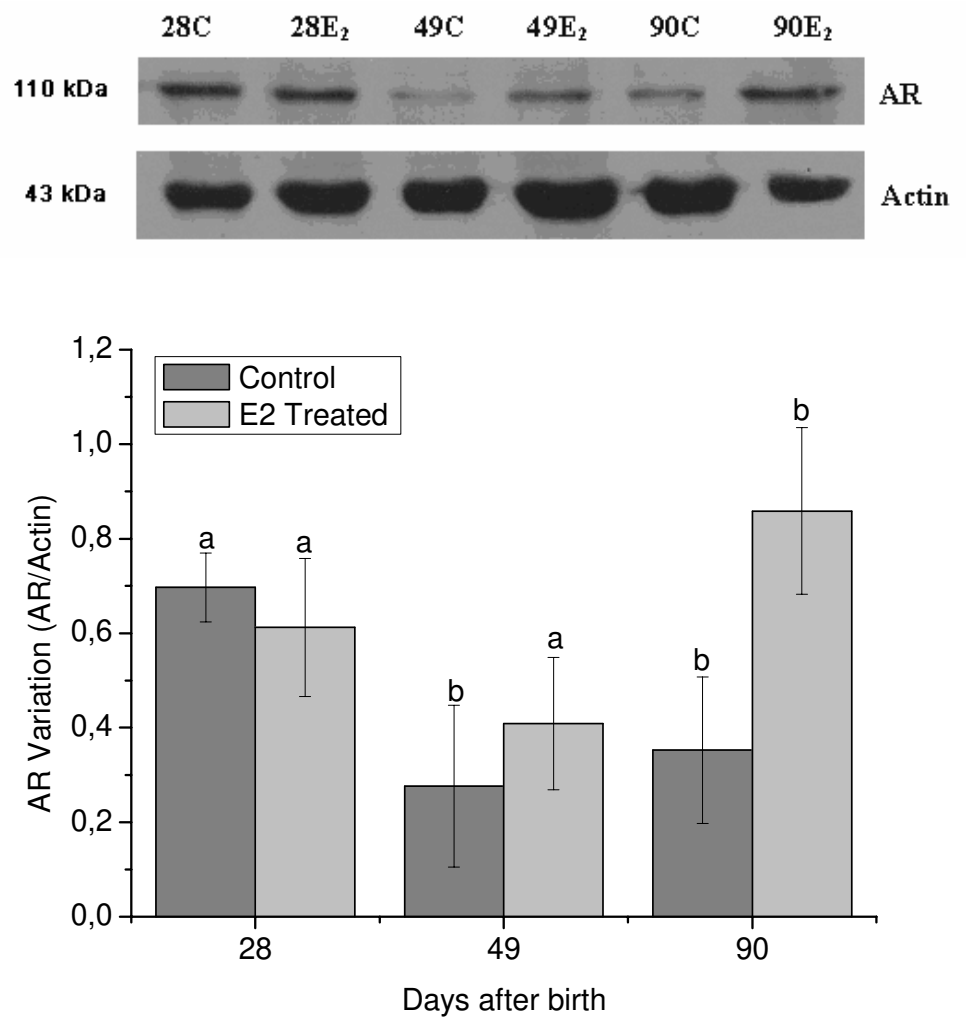

Figure 19. Representative Western blotting showing AR expression in SMC treated without testosterone supplementation in the medium. **A)** Protein levels of AR at 28, 49 and 90 days after birth following neonatal treatment with estradiol. **B)** Actin was used as loading control. **C)** Variation of band density AR for SMC. Only at 28 days after birth the expression of AR was significantly different of the control group. Data are expressed as mean ± SD. There were no statistical differences between the estrogenized and control groups. The letters indicates differences between the estrogenized and control groups, separately ($P \leq 0.05$). n=3.

Figure 20

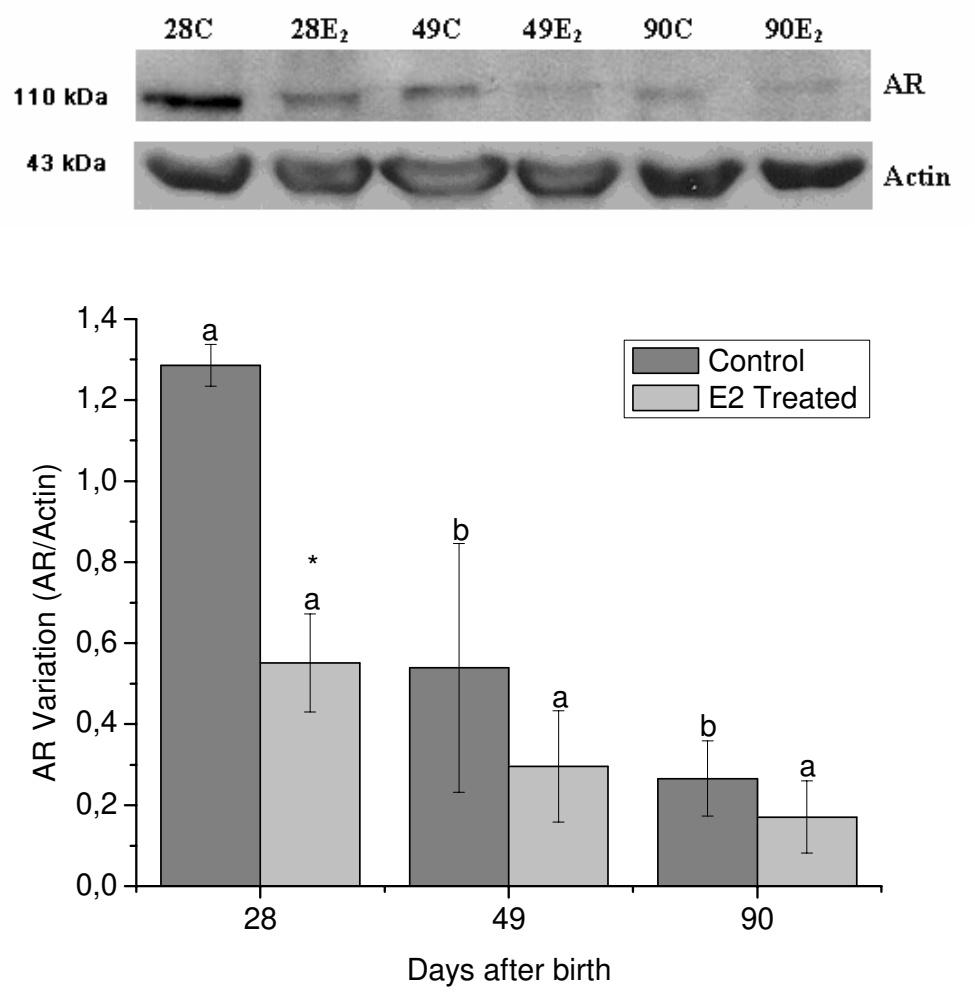

Figure 20. Representative Western blotting showing AR expression in SMC cultured with testosterone supplementation in the medium. **A)** Protein levels of AR at 28, 49 and 90 days after birth following neonatal treatment with estradiol. **B)** Actin was used as loading control. **C)** Variation of band density AR for SMC. Only at 90 days after birth the expression of AR was significantly different of the control group. Data are expressed as mean ± SD. The asterisks indicate statistical differences between the estrogenized and control groups and the letters indicates differences between the estrogenized and control groups, separately ($P \leq 0.05$), n=3.

Figure 21

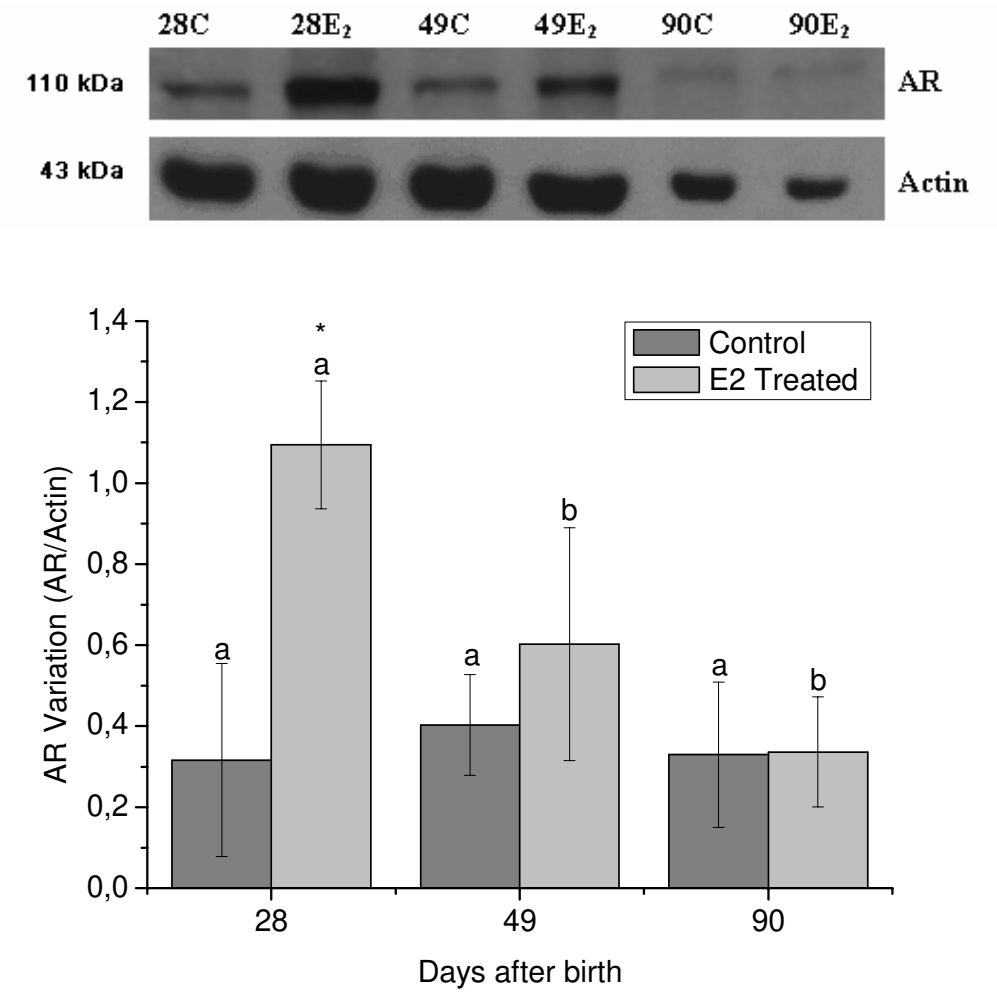

Figure 21. Representative Western blotting showing AR expression in endothelial cells. **A)** Protein levels of AR at 28, 49 and 90 days after birth following neonatal treatment with estradiol. **B)** Actin was used as loading control. **C)** Variation of band density AR for endothelial cells. Only at 28 days after birth the expression of AR was significantly different of the control group. Data are expressed as mean ± SD. Bars without a common superscript and with * are significantly different. The letters indicates differences between the estrogenized and control groups, separately *(P*≤0.05); n=3.

Figure 22

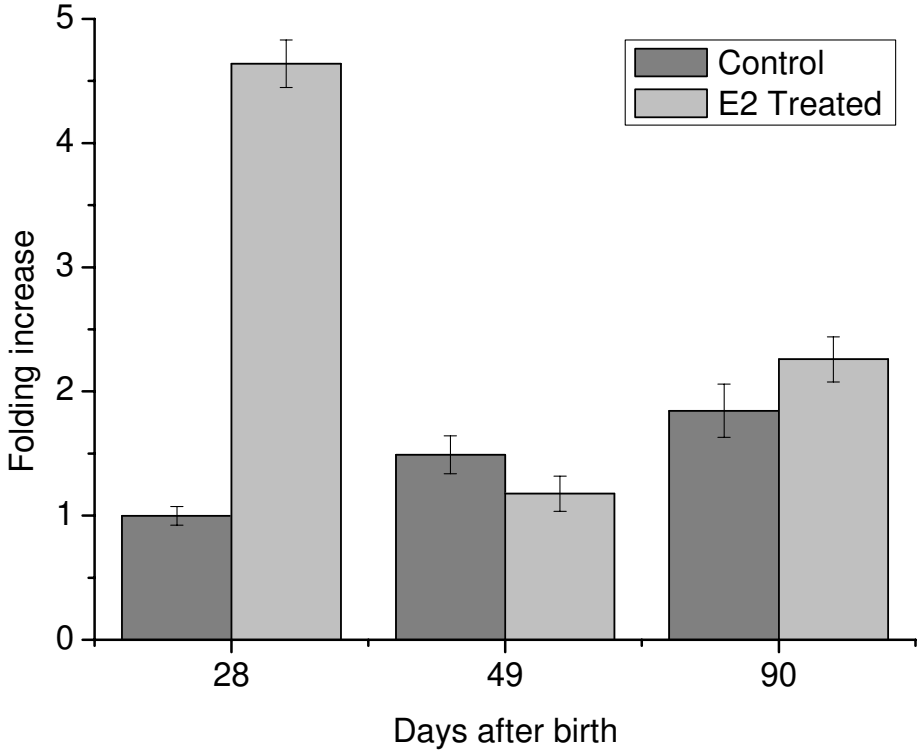

Figure 22. AR mRNA expression in the penis in SMC cultured without testosterone supplementation in the medium. The AR mRNA expression increased on 28 days animals, treated neonatally with E2. On the others groups there were no difference in AR mRNA expression between control and treated animals.

Figure 23

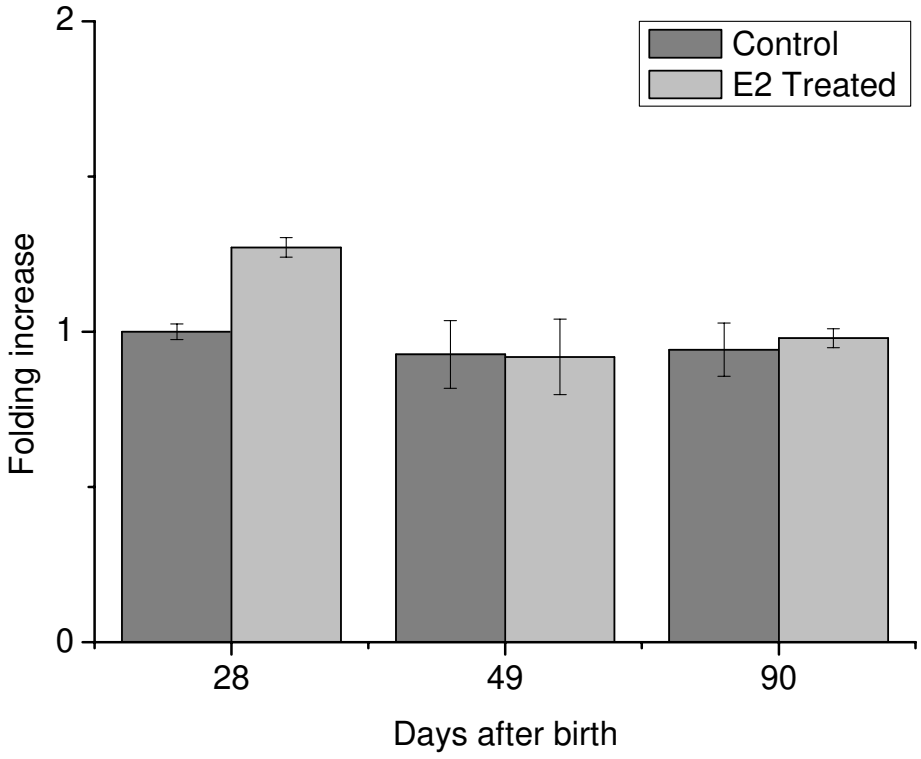

Figure 23. AR mRNA expression in SMC cultured with testosterone supplementation in the medium. There were no difference in AR mRNA expression in treated groups in comparison to those in control.

Figure 24

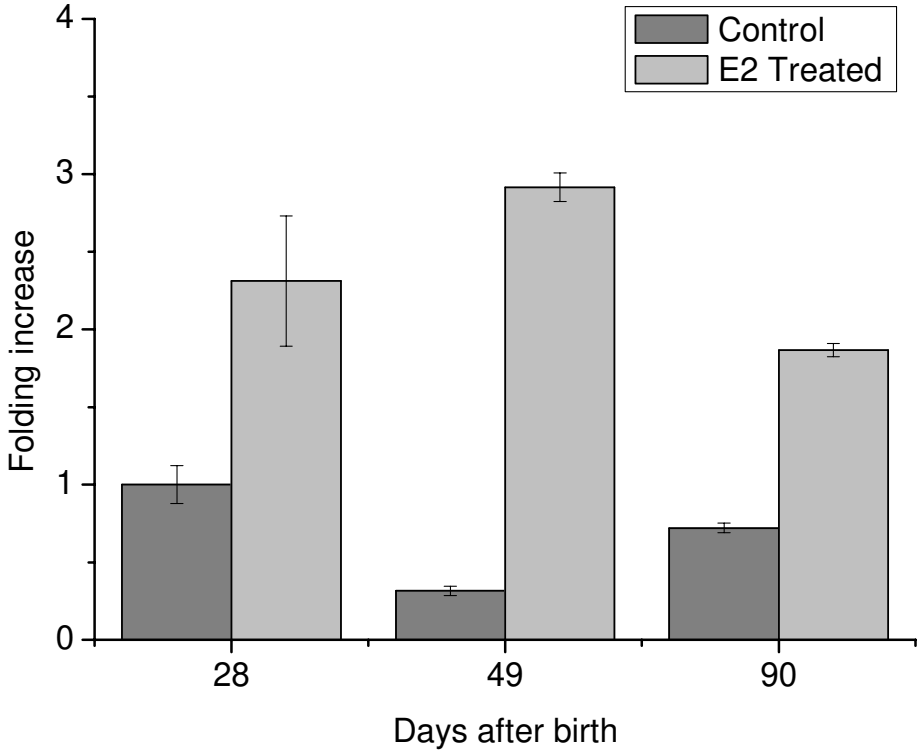

Figure 24. AR mRNA expression in the penis. The AR mRNA expression increased in all ages in treated animals in comparison to control.

Table 1. Comparison of immunostaining intensity of AR in body penis compartments. The staining intensity was scored as negative (-), weak (+), moderate (++), or strong (+++) and that for AR in penis of 49 days after birth animal was used as a benchmark for strong staining.

Penile compartment	Control			E2 Treated		
	28	49	90	28	49	90
Corpus Cavernosus						
Tunica albuginea cellular	+/++	+++	++/+++	+	+++	-
Tunica albuginea fibrous	+	+++	++	-	+++	++
Cavernosus Stroma	++	+++	++/+++	-/+	++	++
Corpus Spongiosus						
Urethral Epithelium	++	+	++	+	++	+/++
Peri-urethral Stroma	+	+++	+++	-/+	++	++
Intercavernosus Septum						
Endothelium	-	+	++	-	+	-
Nerves (peri-neurals cells)	-	+	-	-	+	-

Conclusões gerais

- A exposição neonatal ao E2 pode afetar o desenvolvimento peniano o que pode ser em parte devida à alteração na expressão do AR no pênis, nas CML e endoteliais isoladas do órgão.

- É possível que a exposição neonatal ao E2 afete o processo de diferenciação das estruturas penianas - adipogênese - de forma a alterar o desenvolvimento, crescimento e função do órgão de forma irreversível.

- Em animais adultos (90dias) o tratamento com E2 leva ao aumento do conteúdo dos glicosaminoglicanos: heparam sulfato, condroitin sulfato e dermatam sulfato, levando à maior compactação do colágeno no órgão.

- Outros fatores, não apenas a testosterona, regulam a expressão do AR, uma vez que este apresentou padrão de expressão diferente nas CML (cultivadas com e sem testosterona) em relação ao órgão.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha Tese de Mestrado intitulada "Efeito do tratamento neonatal com 17-β-estradiol sobre o pênis de rato em diferentes idades: aspectos estruturais do órgão e expressão do receptor de andrógeno pelas células musculares lisas e endoteliais *in vitro*"

( ) não se enquadra no Artigo 1º, § 3º da Informação CCPG 01/2008, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo nº _____), intitulado _____

(x) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº 1337-1).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo nº _____).

_____

Aluno(a): Lilian Caroline Vaz Cardoso

_____

Orientador(a): Hernandes Faustino de Carvalho

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Ana Maria Aparecida Guaraldo

Nome:

Função:

Profa. Dra. ANA MARIA A. GUARALDO
Presidente
Comissão de Ética na Experimentação Animal
CEEA/IB - UNICAMP