MARINA TREVIZAN GUERRA

"ESTRUTURA DO TRATO GENITAL E FUNÇÃO REPRODUTIVA DA PROLE MASCULINA E FEMININA DE RATOS EXPOSTOS AO PROPIONATO DE TESTOSTERONA *IN UTERO* E DURANTE A LACTAÇÃO"

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Orientadora: Profa. Dra. Wilma De Grava Kempinas

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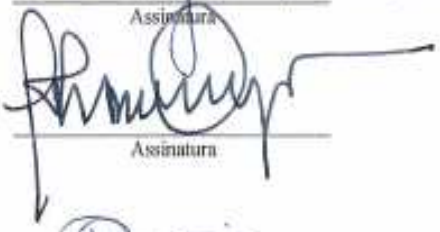
Prof. Dr. João Lauro Viana de Camargo 
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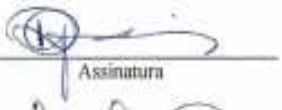
Profa. Dra. Daniela Cristina Ceccatto Gerardin 
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"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original."

Albert Einstein

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"O verdadeiro amor nunca se desgasta. Quanto mais se dá mais se tem."

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Sumário

Resumo

Resumo

Os desreguladores endócrinos são substâncias químicas que podem mimetizar ou antagonizar os hormônios endógenos, alterando o equilíbrio hormonal necessário para o desenvolvimento correto. Sabe-se que a exposição de organismos a agentes hormonalmente ativos durante períodos críticos do desenvolvimento pode levar a alterações permanentes detectadas somente na vida adulta. Em várias espécies animais estes compostos podem atingir o feto via placenta ou a prole, pelo leite materno. Embora pesquisas iniciais tenham focado em estrógenos e antiandrógenos ambientais, a presença de compostos com atividade androgênica tem sido descrita como contaminante em rios e animais destinados a alimentação. Vários estudos demonstraram que a exposição de animais a altos níveis de andrógenos durante o período perinatal pode causar alterações morfológicas, bioquímicas e funcionais permanentes em vários órgãos e sistemas. A exposição perinatal a andrógenos exógenos pode causar masculinização fisiológica e comportamental em fêmeas e levar a alterações reprodutivas em machos. O objetivo deste trabalho foi avaliar os possíveis efeitos da exposição a um agente androgênico sobre o sistema genital e a função reprodutiva de animais expostos durante períodos críticos de desenvolvimento. Para tanto, ratas prenhes foram alocadas em quatro grupos experimentais: controle, que recebeu somente óleo de milho (veículo), e tratados com propionato de testosterona nas doses de 0,05mg/kg, 0,1mg/kg, ou 0,2mg/kg. Os tratamentos foram realizados por via subcutânea, do dia gestacional 12 ao final da lactação. A prole feminina foi avaliada quanto ao número de células germinativas fetais, peso corpóreo e distância anogenital no dia pós-natal 1, número de mamilos, idade de instalação da puberdade, histologia uterina e ovariana, dosagens hormonais, comportamento sexual, fertilidade, níveis de receptores esteroides uterinos através de imunohistoquímica, assim como taxa de proliferação/morte celular uterina e testes uterotróficos. Nos filhotes do sexo masculino foram avaliados, na idade adulta e meia idade, a histologia testicular e epididimária, contagens, morfologia e motilidade espermáticas, dosagens hormonais, comportamento sexual e fertilidade. Os resultados demonstraram que em fêmeas, a exposição perinatal ao propionato de testosterona ocasionou alteração na intensidade de marcação dos receptores esteroides uterinos em todas as doses testadas, diminuição nos índices de proliferação e morte celular

no tecido uterino nas doses de 0,1 e 0,2mg/kg e aumentou a predisposição uterina a responder a estímulos estrogênicos na dose de 0,1mg/kg e 0,2mg/kg. Entretanto, estas alterações não foram capazes de prejudicar a diferenciação sexual feminina e a fisiologia normal do sistema genital feminino. Em relação aos filhotes do sexo masculino, a exposição perinatal a andrógenos provocou redução na produção e nas reservas espermáticas na idade adulta, sem, contudo, alterar a fertilidade destes animais após acasalamentos naturais. Podemos concluir que a exposição perinatal a andrógenos, nas doses utilizadas neste experimento, provocou o aparecimento de alterações tardias no sistema genital feminino como modificações nos receptores esteroides uterinos e anormalidade na resposta a estímulos estrogênicos e, no sistema genital masculino, alterações na produção espermática. Estas repercussões, entretanto, não foram capazes de alterar o desempenho geral de fertilidade em ambos os sexos.

Abstract

Abstract

Endocrine disruptors are chemicals that may mimic or antagonize endogenous hormones, altering the critical hormonal balance required for proper health and development. It is well known that exposure of organisms to some hormonally active agents during critical periods of development imprints permanent changes that can be detected later in adulthood. In many animal species these substances reach the fetus via placenta and/or the offspring via the mother's milk. Although first researches have focused on environmental estrogens and antiandrogens, androgenic activity has been described as contaminant in rivers and beef cattle. Previous studies demonstrated that exposure of experimental animals to high levels of androgens during perinatal age causes permanent morphological, biochemical, and functional alterations in several organs and systems. Perinatal exposure of female rodents to exogenous androgens results in both physiological and behavioral masculinization and causes reproductive disruption in male rodents. The aim of this study was to assess the possible effects of an androgenic compound on genital system and reproductive function of animails exposed during critical periods of development. Pregnant female rats were allocated into four experimental groups: control, which received corn oil (vehicle), and treated with testosterone propionate at doses of 0.05mg/kg, 0.1mg/kg and 0.2mg/kg. Treatments were performed subcutaneously, from gestational day 12 until the end of lactation. The female offspring was evaluated for fetal germ cell number, body weight and anogenital distance at post-natal day 1, number of nipples, puberty onset, histology of reproductive organs, hormonal levels, sexual behavior, fertility test, immunohistochemistry of steroid receptors, proliferation/apoptotic index, uterotrophic test and uterine stimulation with estrogens. On male offspring, at adult age and middle-age, histology of testis and epididymis, sperm counts, morphology and motility, hormonal levels, sexual behavior and fertility test were evaluated. Results demonstrated that, in females, the perinatal testosterone propionate exposure caused an alteration in the pattern of uterine steroidal receptors imunostaining in all tested doses, a decreased proliferation/apoptotic index in the uterine tissue at 0.1 and 0.2mg/kg and a greater predisposition from uterus in responding to estrogens stimulation at dose of 0.2mg/kg. However, these alterations were not capable of impairing female sexual differentiation and normal physiology of female genital tract. On

male offspring, the perinatal androgenization provoked a reduction in sperm production and reserves at adult age, without altering fertility after natural mating. We can conclude that perinatal exposure to androgens, at doses used in this experiment, provoked the appearance of late alterations in the female genital system as modifications in the uterine steroidal receptors and abnormalities in uterine response to estrogenic stimulation, and in male genital system, as spermatic production impairment. These repercussions, however, were not capable to alter the general fertility performance in both sexes.

Introdução

1. Introdução

1.1. O rato como modelo experimental

Um modelo constantemente utilizado na área de ciências médicas e biologia experimental para estudos *in vivo* é o rato. Este animal é constantemente usado em estudos de avalição de toxicidade de determinados produtos químicos, incluindo medicamentos, compostos químicos agrícolas, aditivos alimentares e uma diversidade de substâncias sintéticas ou naturais (Mutai, 2000).

O uso abrangente deste tipo animal deve-se a sua grande sensibilidade às substâncias tóxicas. Além disso, outras vantagens que os tornam um dos animais mais utilizados em pesquisa são o fácil manejo, o tamanho relativamente pequeno, suportam diversas rotas de administração de substâncias (gavage, dieta, aplicações intravenosas, subcutâneas e intraperitoneais) e se reproduzem constantemente durante toda a vida reprodutiva. Este último fato não representa somente uma vantagem na manutenção e obtenção destes animais, mas também a facilidade para avaliação de compostos químicos sobre o desempenho reprodutivo (Mutai, 2000).

O rato é o animal experimental mais popular em estudos sobre a fisiologia da reprodução. As vantagens em se utilizar ratos e não outros animais nestes tipos de avaliações são a presença de ciclos reprodutivos mais curtos e estágios como instalação da puberdade, ciclo estral, duração da gestação e lactação muito menores quando comparados com animais de maior porte também utilizados com fins de pesquisa. Além disso, apresentam sinais físicos externos considerados indicativos do desenvolvimento sexual e de fácil monitoramento (Mutai, 2000; Ojeda e Skinner, 2006).

1.2. Diferenciação sexual

1.2.1. Diferenciação sexual do cérebro

A diferenciação sexual no sistema nervoso central (SNC) de mamíferos é dependente de níveis circulantes de testosterona durante estágios críticos de desenvolvimento (MacLuscky e Naftolin, 1981) e este processo tem sido bem estudado em ratos (Diaz et al., 1995).

Diferentemente dos ovários, onde a secreção basal de estrógenos não é detectada até o quinto dia após o nascimento (Weniger, 1993), a testosterona secretada pelos testículos em desenvolvimento durante o final da gestação e o ínicio da vida neonatal possui fundamental importância na determinação do fenótipo sexual masculino (Jost et al., 1973; Cruz e Pereira, 2012). A conversão de andrógenos em estrógeno no SNC é um passo fundamental para que a diferenciação sexual aconteça (Naftolin et al., 1975; McEwen et al., 1977).

O andrógeno produzido pelos testículos fetais/neonatais (Quadros et al., 2002) apresenta dois picos de produção importantes: o primeiro entre os dias gestacionais 18-19 e o segundo nas primeiras horas após o nascimento (Corbier et al., 1978; Weisz and Ward, 1980; Huhtaniemi, 1994). A aromatase (enzima responsável pela conversão de testosterona em estradiol) encontra-se presente no cérebro nos estágios iniciais do desenvolvimento e alguns estudos em ratos demonstram uma maior atividade desta enzima durante o período perinatal em animais do sexo masculino (Reddy et al., 1974).

Desta forma então, o estradiol é o responsável pela masculinização cerebral (Sakuma, 2009), causando dimorfismo sexual no rato, estabelecendo o comportamento sexual típico masculino (montas, intromissão e ejaculações) e o estado endocrinológico sexo-específico na idade adulta do animal (MacLusky e Naftolin, 1981; Hoepfner e Ward, 1988; Adkins-Regan, 1988; Rhees et al., 1997; Bakker, 2003; Sakuma, 2009; Piffer et al., 2009).

A administração de testosterona para uma fêmea neonatal pode alterar os padrões hormonais e inibir a manifestação de lordose na idade adulta, indicando uma defeminização cerebral (Diaz et al., 1995). Este processo também pode ser induzido por tratamento neonatal com estrógenos (Maeda, 2000).

1.2.1.1. Mecanismo de proteção contra estrógenos de origem materna

O feto é protegido da grande quantidade de estrógeno secretado pelos ovários materno devido à presença de uma proteína feto-específica de ligação ao estrógeno denominada α-fetoproteína. Esta proteína é sintetizada pelo fígado durante o período perinatal em ambos os sexos (MacLusky e Naftolin, 1981, Toran-Allerand, 1984) e liga-se

somente ao estrógeno circulante. Desta forma, andrógenos secretados pelos testículos fetais chegam livremente ao cérebro, onde serão localmente convertidos em estrógenos. Alguns estrógenos artificiais como o dietilestilbestrol, que não apresentam uma boa ligação a α-fetoproteína, podem afetar o desenvolvimento morfológico e sexual cerebral, oasionando mudanças fisiológicas e comportamentais no indivíduo adulto.

1.2.2. Diferenciação sexual dos órgãos reprodutores

A gônada indiferenciada é derivada de células da crista genital e, antes da determinação sexual e da diferenciação gonadal em testículo ou ovário, fetos fêmeas e machos possuem o sistema urogenital idênticos (George e Wilson, 1994; Drews, 2000), com tecidos reprodutivos alvo expressando receptor de andrógeno (AR) em ambos os sexos (Bentvelsen et al., 1995).

A crista gonadal desenvolve-se no 13° dia gestacional (dia gestaciona - DG 13), quando possui somente células germinativas primordiais (Hebel e Stromberg, 1986). Estas células desenvolvem-se em ovogônias em fêmeas e em espermatogônias em machos, e finalmente formam ovários ou testículos (Huckins e Clermont, 1968), respectivamente. A cronologia do desenvolvimento da gônada indiferenciada é assimétrica, sendo que a gônada masculina desenvolve-se dias antes das primeiras modificações na gônada feminina (Jost et al., 1973).

O genoma masculino é programado para inibir o desenvolvimento dos ductos femininos (ductos de Muller) (Jost et al., 1972; Ford, 1982). O gene SRY no cromossomo Y é considerado um determinante testicular tanto em humanos quanto roedores (George e Wilson, 1994); quando este gene é expresso nas células somáticas de sustentação das gônadas indiferenciadas, o desenvolvimento é desencadeado (Schoenwolf et al., 2009).

Sob a influência de proteínas SRY, as células somáticas de sustentação diferenciam-se em células de Sertoli, que se associam às células intersticiais da gônada e organizam-se para formar os cordões testiculares, confinando as células germinativas. As células de Sertoli iniciam a produção do hormônio anti-Mulleriano entre os DG 13-14, concomitantemente ao processo de diferenciação dos cordões sexuais (Hirobe et al., 1992).

A produção deste hormônio induz a degeneração do ducto de Muller em torno do DG 17 (Josso et al., 1979; Hebel e Stromberg, 1986).

Após a diferenciação funcional dos testículos fetais, as células de Leydig secretam testosterona (Dyche, 1979; Habert e Picon, 1984; George e Wilson, 1994) e o andrógeno testicular liga e ativa os AR, levando a um processo de masculinização, incluindo estabilização e diferenciação do ducto de Wolff e aumento da distância anogenital (Huhtaniemi, 1994; Swan et al., 2005; Edwards et al., 2006). Os ductos de Wolf então se diferenciam em ductos eferentes, epidídimo, ductos deferentes e glândulas vesiculares (Robaire e Hermo, 1988; Welsh et al., 2006). Com esta diferenciação, a indução da enzima 5α-redutase permite a conversão da testosterona em di-hidrotestosterona, que por sua vez induz a diferenciação do seio urogenital em próstata, diferenciação da uretra masculina e falo e a formação do escroto (Wilson e Siiteri, 1973).

Em fêmeas, os ovários não produzem testosterona (Welsh et al., 2008) e as células somáticas de sustentação não possuem o cromossomo Y. Desta forma, o ducto de Wolff degenera-se em torno do DG 17 (Hebel e Stromberg, 1986; Huhtaniemi, 1994; George e Wilson, 1994; Drews, 2000) e as células de sustentação se diferenciam em células foliculares, que envolvem as células germinativas. Com a ausência de andrógenos e do hormônio anti-mulleriano, os ductos genitais masculinos e a estruturas sexuais acessórias não se desenvolvem e os ductos mulerianos fetais persistem, dando origem aos ovidutos, útero, cérvix e a parte anterior da vagina (Schoenwolf et al., 2009).

1.3. Hormônios gonadais

As gônadas sintetizam e secretam hormônios sexuais esteroides como andrógenos, estrógenos e progesterona. Estes hormônios gonadais possuem uma variedade de ações, incluindo a estimulação da produção de gametas, desenvolvimento dos órgãos reprodutivos e expressão de características sexuais secundárias e comportamento sexual. Além disso, as gônadas produzem uma variedade de hormônios não esteroidais que também influenciam as funções gonadais (Ohkura et al., 2000).

1.3.1. Esteroides gonadais

Os hormônios esteroides gonadais possuem uma estrutura comum de quatro anéis de carbono chamados núcleos esteroides (Gore-Langton e Armstrong, 1994). Eles são sintetizados a partir do colesterol (lipoproteínas de alta densidade - high-density lipotproteins - HDL) encontrado no plasma (Ohkura et al., 2000; Rang et al., 2004). Desta forma, estes hormônios são lipoprotéicos e possuem a habilidade de atravessar as membranas celulares, ligarem-se a receptores intracelulares e difundem-se até o núcleo para gerar uma resposta celular específica (O'Malley, 1984; Rang et al., 2004).

A síntese de esteroides gonadais está sob controle de gonadotrofinas produzidas pela hipófise em ambos os sexos. No sangue, os esteroides podem estar livres ou ligados a proteínas tais como albuminas e globulinas (Rosner, 1990) e esta ligação a proteínas plasmáticas pode resultar na retenção de esteroides em concentrações elevadas no sangue por um período maior (Ohkura et al., 2000).

1.3.1.1. Andrógenos

Os hormônios androgênicos estimulam características sexuais secundárias (Norris, 1996) e a maturação dos órgãos sexuais masculinos (Rang et al., 2004). Estes hormônios promovem o desenvolvimento e função dos órgãos sexuais acessórios e são fundamentais para a espermatogênese; também estimulam a expressão do comportamento sexual masculino e anabolismo de proteínas (Meisel e Sachs, 1994). Além disso, os andrógenos exerem um *feedback* negativo na secreção de gonadotrofinas.

A testosterona é o principal andrógeno natural, sintetizado em grande parte pelas células intersticiais do testículo (céluas de Leydig) e, em menores quantidades, pelo ovário e pelo córtex da adrenal (Baird, 1984; Hall, 1994; Rang et al., 2004). A ação da testosterona em órgãos-alvo, como a genitália externa, é expressa após a conversão em 5α-dihidrotestosterona (DHT) pela enzima 5α-redutase (Miller, 1988).

1.3.1.2. Estrógenos

Os hormônios estrogênicos são sintetizados nos ovários pelas células foliculares e corpo lúteo, pela placenta e, em menor quantidade, pelo córtex adrenal e pelos testículos.

Existem três estrógenos naturais produzidos pelo organismo: a estrona, 17β-estradiol e estriol. Dentre eles, o estrógeno mais potente é o 17β-estradiol, secretado pelos ovários (Ohkura et al., 2000).

No ovário, os estrógenos são sintetizados a partir de andrógenos por uma reação enzimática denominada aromatização (Miller, 1988). LH e FSH (hormônio luteinizante e hormônio folículo-estimulante) estimulam a síntese de andrógenos nas células foliculares da teca e a conversão em estrógeno pelas células da granulosa, respectivamente. Os estrógenos são responsáveis pelo desenvolvimento e a função de órgãos reprodutivos femininos acessórios, crescimento e desenvolvimento folicular e desenvolvimento das glândulas mamárias. A queratinização das células epidermais da vagina também está sob influencia de estrógenos (Norris, 1996, Range et al., 2004).

Estrógenos induzem o cérebro, na idade adulta, a produzir o comportamento sexual feminino, evidenciado pela lordose (Pfaff et al., 1994) e exercem controle sobre a liberação de GnRH (hormônio liberador de gonadotrofinas) e gonadotrofinas durante os ciclos ovulatórios (Karsch, 1984).

1.4. Puberdade

A puberdade é caracterizada por rápidas mudanças fisiológicas como crescimento acentuado e maturação das gônadas e do cérebro. Este processo implica na transição de um período não reprodutivo para um reprodutivo e o principal hormônio envolvido nesta regulação é o GnRH no hipotálamo, que estimula a liberação de LH e FSH pela hipófise (Terasawa e Fernandez, 2001).

1.4.1. Puberdade em machos

De acordo com Amann e Schandacher (1983) a puberdade é caracterizada quando um macho produz, pela primeira vez, uma quantidade de espermatozoides suficiente para fertilizar uma fêmea. O período puberal está associado ao rápido crescimento testicular, mudanças no padrão de secreção de LH, aumento gradual na concentração de testosterona sanguínea e início da espermatogênese. A puberdade não é um sinônimo de maturidade sexual, que ocorre meses mais tarde (Amann, 1970; Robb et al., 1978).

Previamente à instalação da puberdade, os níveis circulantes de testosterona em machos induzem um *feedback* negativo no eixo hipotalâmico-hipofisário (Negro-Vilar et al., 1973). Entetanto, com a maturação animal, este eixo torna-se progressivamente menos sensível a este controle. Neste período, a liberação pulsátil de GnRH aumenta, resultando no aumento de FSH e LH circulantes (Chowen-Breed e Steiner, 1989). Desta forma, a maturação testicular e a instalação da puberdade ocorrem secundariamente às mudanças de secreção de gonadotrofinas pela hipófise. Após a puberdade, os níveis gonadotrópicos são estabilizados (Marty et al., 2003).

Um sinal externo da instalação da puberdade em machos é a separação prepucial – quando o prepúcio separa-se da glande do pênis (Korenbrott et al., 1977). Inicialmente, o pênis do rato assemelha-se com o clitóris feminino e a glande dificilmente é exposta antes dos 30 dias após o nascimento (Maeda et al., 2000). Existe uma complexa relação entre a instalação da puberdade e o peso corpóreo do animal (Cameron, 1991).

1.4.2. Puberdade em fêmeas

Durante a puberdade em fêmeas, a secreção de esteroides sexuais estrogênicos é estimulada por um aumento na secreção dos hormônios hipotalâmicos e hipofisários. A instalação da puberdade em ratas resulta de uma cascata de eventos que estabelecem um padrão pulsátil de LH após a quarta semana pós-natal (aproximadamente aos 30 dias de idade) e levam à maturação ovariana (Andrews e Ojeda, 1981).

Em fêmeas, entre os DPN (dia pós-natal) 12-28 (Ojeda e Urbanski, 1994), a α-fetoproteína começa a ser eliminada pelo metabolismo hepático e como consequência, os níveis circulantes de estrógenos aumentam suficientemente para induzir o pico de LH. Este pico de LH, por sua vez, resulta na ovulação (Rang et al., 2004). Os níveis sanguíneos de estradiol aumentados levam a instalação da puberdade com ocorrência da abertura vaginal e do primeiro estro (US EPA, 1996; Guyton e Hall, 2002[a]). Antes deste período, o sistema genital feminino é considerado inativo (Westwood, 2008).

A abertura vaginal ocorre geralmente quando o animal atinge cerca de 100g de peso corpóreo (Maeda et al., 2000), o que ocorre em torno dos 35-41 dias após o nascimento, e o primeiro estro ocorre em torno dos 37-42 dias de idade (Guerra et al., 2011). Cerca de uma

semana após a abertura vaginal, o animal começa a demostrar ciclos estrais regulares (Ohkura et al., 2000).

1.5. Sistema genital feminino

O desenvolvimento do trato genital feminino (figura 1) consiste em quatro estágios: pré-natal, juvenil, adulto e envelhecimento. O estágio pré-natal compreende 20-22 dias a partir do dia da fertilização (dia pós-coito 1 até o nascimento) (Witschi, 1962). O período juvenil é dividido em neonatal (do nascimento até em torno do DPN 5), infantil (DPN 10 a 14) e imaturo (DPN 20-24) e acaba com a puberdade (Branham et al., 1985ª). A vida adulta compreende o período de idade mínima e máxima para acasalamentos (60-80 a 360-450 dias de idade) (Inglis, 1980) e o período de envelhecimento pode ter início aos 360 dias de idade e é caracterizada por estro constante, repetidas pseudogestações e finalmente anestro (Del Vecchio, 1992).

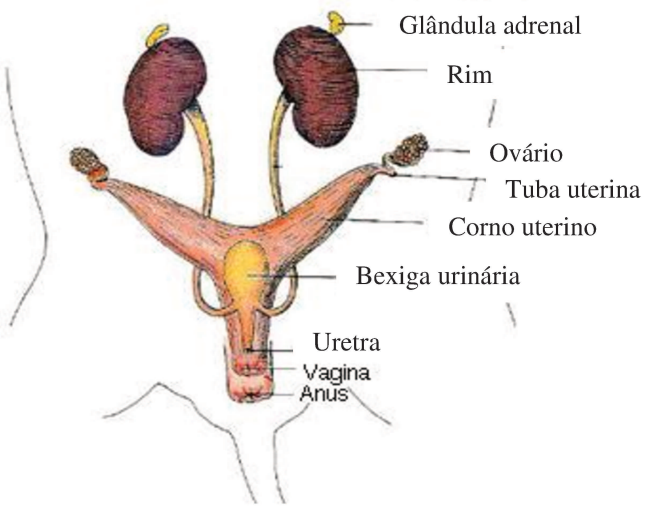

Figura 1. Órgãos sexuais femininos de ratas Wistar.

1.5.1. Ovários

Os ovários estão localizados caudalmente aos rins. Eles são envolvidos pela bursa ovariana, conectados aos cornos uterinos pelos ovidutos e cobertos por um epitélio simples cuboidal ou colunar, que na região do hilo funde-se com o epitélio escamoso da bursa. Próximo à região do hilo, numerosas fibras elásticas e musculares lisas aparecem (Komárek et al., 2000).

O ovário de mamíferos é formado por duas populações celulares: as células somáticas e as células germinativas. Em roedores, as células primordiais indiferenciadas sexualmente têm origem extra-gonadal por volta do dia embrionário 7.0-7.5 (Saitou et al., 2002) e começam a migrar para a gonada indiferenciada no dia embrionário 9.0-9.5 (Ginsburg et al., 1990). Nas gônadas femininas (XX), as células germinativas formam ninhos de ovócitos e entram em meiose I, que é interrompida em prófase I no dia embrionário 17.5 (Peters, 1970; Kurilo, 1981; McLaren, 2001; Moore e Persaud, 2008). Após o nascimento, os ovócitos entram em uma pausa de divisão em diacinese, onde permanecem até o momento da puberdade (Biggers, 1975).

O ciclo ovulatório em mamíferos inclui múltiplos eventos relacionados envolvendo a foliculogênese, ovulação e preparação do sistema genital para a fertilização e implantação levando a gestação (Boué et al., 1975).

A ovulação resulta de interações entre regiões hipotalâmicas, hipófise e ovário. O hipotálamo libera GnRH e como resposta, a hipófise é estimulada e os níveis de FSH e LH aumentam subitamente. Este surto ovulatório das gonadotrofinas ocasiona o desenvolvimento folicular e estimula o ovócito primário do folículo maduro a terminar a primeira divisão meiótica, além de fornecem estímulos para a cascata de eventos ovarianos que resulta na ovocitaçao do ovócito secundário em metáfase da segunda divisão meiótica (Boué et al., 1975; Schoenwolf et al., 2009).

A ovulação de um ovócito fertilizável requer o crescimento, maturação e diferenciação da célula germinativa, das células da granulosa e das células endócrinas da teca e a formação de corpo lúteo. Todos estes eventos são susceptíveis a efeitos tóxicos, assim como a mitose da ovogônia durante o período intrauterino,as divisões das células da

granulosa durante o crescimento folicular, a meiose da ovogônia para gerar ovócitos e a diferenciação das células da granulosa e da teca (Komárek et al., 2000).

Cerca de cinco a seis folículos por ovário desenvolvem-se em folículos pré-ovulatórios e liberam o ovócito a ser fertilizado. Após a ovulação, as células foliculares se diferenciam em corpo lúteo e iniciam a secreção de progesterona. Nem todos os folículos que são estimulados a crescer chegam a ovular; a maioria dos folículos degenera por um processo denominado atresia e diferenciam-se posteriormente em interstício (Komárek et al., 2000).

A presença de um número regular de ovócitos e folículos é considerada um indicador de fertilidade. Desta forma, a avaliação quantitativa destes parâmetros é necessária. Procedimentos padrão de contagem de folículos foram originalmente desenvolvidos em camundongos, mas podem ser prontamente empregados em ratos (Plowchalk et al., 1993; Heindel, 1998).

1.5.2. Útero

Grande parte do desenvolvimento uterino ocorre no período pós-natal em espécies como camundongos e ratos (Wiesner, 1934; Ogasawara et al., 1983; Forsberg, 1988; Branham et al., 1985ª). Ao nascimento, o sistema genital feminino de ratos apresenta o desenvolvimento equivalente ao 100° dia gestacional em humanos (Wiesner, 1934; O'Rahilly, 1989).

No útero de ratos recém-nascidos, apenas o epitélio luminal é diferenciado (cubóide a colunar baixo). A diferenciação da musculatura e do epitélio glandular ocorrem no período pós-natal (Sheehan et al., 1981; Branham et al., 1985ª).

As glândulas endometriais estão presentes em úteros de várias espécies, incluindo os roedores (Ogasawara et al., 1983; Branham e Sheehan, 1995) e a adenogenese e o crescimento uterino iniciais occorem na ausência de hormônios esteroides. Estas glândulas originam-se da invaginação do epitélio luminal em direção ao estroma uterino uma semana após o nascimento (Branham et al., 1985[a]), formando um epitélio cuboidal glandular (Brody et al., 1989; Hu et al., 2004). Dependendo da espécie, as glândulas endometriais apresentam graus variáveis de enovelamento e ramificação. Estas estruturas endometriais

produzem e transportam secreções essenciais para a implantação embrionária (Stewart et al., 1992; Zhu et al., 1998). Desta forma, a ausência destas glândulas pode resultar em infertilidade (Stewart et al., 1992; Gray et al., 2001).

Também após o nascimento, o mesênquima uterino se diferencia em duas camadas de miométrio que envolvem o estroma e duas semanas após o nascimento, o útero de roedores contém a arquitetura similar a de um animal adulto, porém com número de glândulas reduzido (Hu et al., 2004). Os mecanismos que envolvem a diferenciação uterina neonatal e a formação das glândulas são complexos, envolvem comunicações intrínsecas entre o epitélio e o mesênquima, e são mediadas pela composição e distribuição da matriz extracelular, ligação de fatores de crescimento a seus receptores e expressão de receptores de hormônios esteroides (Cunha, 1976; Yamashita et al., 1990; Kurita et al., 1998; Hom et al., 1998).

Na idade adulta, o útero de ratas apresenta a luz dos cornos uterinos completamente separadas e elas se abrem na luz da vagina como orifícios externos pareados. A parede uterina é composta pela mucosa (endométrio), duas camadas de músculo liso (miométrio) e camada adventícia. Uma camada de células epiteliais colunares forma as glândulas uterinas que se projetam para o endométrio (Komárek et al., 2000).

Durante o ciclo estral, o útero de uma rata sexualmente madura passa pelas seguintes modificações: no proestro, o lúmem é distendido com fluido, as células epiteliais são cubóides ao invés de colunares e o estroma e o miométrio demonstram uma intensa infiltração de leucócitos; no estro o endométrio encontra-se hiperhêmico, o lúmen está distendido ao máximo e a infiltração de leucócitos persiste; no metaestro a quantidade de fluido uterino está diminuída, as células do epitélio cuboidal apresentam degeneração vacuolar, e a infiltração de leucócitos no estroma e no miométrio diminui; no diestro o epitélio é regenerado e a infiltração leucocitária é diminuída ao mínimo (Hebel e Stromberg, 1986).

Embora a formação das glândulas ocorra independente de hormônios ovarianos e adrenais, a exposição inapropriada do sistema genital feminino imaturo a esteroides pode resultar em desregulação do desenvolvimento necessário para a fertilidade na idade adulta (Wilson, 1943; Branham et al., 1985ª).

A rápida e sicrônica formação das glândulas uterinas pode ser alterada pela administração de compostos exógenos de uma maneira tempo-específica e dose-específica (Branham et al., 1985[b], 1988[a,b]). Estes fatos devem-se a presença de receptores de estrógeno nas células mesenquimais uterinas logo ao nascimento (Taguchi et al., 1988). Os níveis destes receptores aumentam a partir do DPN 10 (Medlock et al., 1981, 1988; Clark e Gorski, 1970) e interessantemente, os níveis séricos basais de estrógeno endógeno também aumentam no início do período de desenvolvimento uterino (aproximadamente entre os dias pós-natal 9-11) (Dohler e Wuttke, 1975; Meijs-Roelofs et al., 1973; Frawley e Hendricks 1979).

1.5.3. Vagina

Durante a primeira semana após o nascimento, a parte caudal da vagina é preenchida por um epitélio compacto e somente a parte mais cranial possui uma cavidade. Ainda na idade imatura, as células do epitélio compacto da vagina sofrem degeneração e queratinização, e algumas células são absorvidas e não repostas, originando assim, a cavidade caudal vaginal (Del Vecchio, 1992).

A vagina de ratas possui um comprimento de cerca de 20 mm e o diâmetro de 3-5 mm quando distendida (Yuan e Carlson 1987). As paredes da vagina não possuem glândulas e são constituídas por três camadas: mucosa, muscular e adventícia. O muco presente no lúmen desta estrutura origina-se nas glândulas uterinas e a mucosa é constituída por tecido conjuntivo frouxo rico em fibras elásticas, linfócitos e neutrófilos (que, em determinadas fases do ciclo estral, passam para a luz vaginal) (Junqueira e Carneiro, 2008[a]).

Em fêmeas jovens, a vagina é fechada por uma membrana, ou *plug*, até o momento da puberdade. Hebel e Stromberg (1986) descrevem esta membrana como um septo transverso epitelial que começa a degenerar em torno 20-35 dias de idade. O canal da vagina abre-se para o exterior do organismo separadamente da uretra (Del Vecchio, 1992).

A vagina é revestida por um epitélio estratificado pavimentoso, exibindo mudanças características durante os diferentes estágios do ciclo estral (Hebel e Stromberg, 1986; Weiss et al., 2000; Maeda et al., 2000):

Fase do ciclo estral	Lavado vaginal	Histologia vaginal
Proestro	Predominância de células epiteliais nucleadas	Fino epitélio (8-12 camadas), início do desenvolvimento do epitélio queratinizado
Estro	Células epiteliais queratinizadas abundantes	Epitélio da vagina com 6-10 camadas, das quais 3-5 são queratinizadas
Metaestro	Presença de leucócitos, células epiteliais nucleadas e queratinizadas	As camadas de células queratinizadas começam a se destacar e ocupam a luz da vagina
Diestro	Predominância de leucócitos	Epitelio fino; número de camadas celulares aumenta novamente; numerosos leucócitos

1.5.4. Tubas uterinas

Durante a primeira semana pós-natal, as tubas uterinas de roedores crescem lentamente em comprimento e começam a se enrolar entre o ovário e o útero (Del Vecchio, 1992). Em animais adultos, a tuba uterina é provida de grande mobilidade e pode ser dividia em quatro segmentos: infundíbulo, ampola, istmo e porção intramural. Todos os segmentos possuem basicamente uma mucosa, uma camada de músculo liso circular interna e uma camada muscular longitudinal externa e uma serosa formada por lâmina visceral de peritônio (Junqueira e Carneiro, 2008[a]).

O infundíbulo possui o epitélio constituído essencialmente por células ciliadas, com poucas células não ciliadas importantes para mediação de transporte celular e a camada muscular é fina. A ampola apresenta uma camada muscular fina e um amplo lúmen, a mucosa possui numerosas dobras e o epitélio é colunar simples, com células ciliadas e não ciliadas com diferentes funções secretoras. O istmo possui dobras na mucosa e poucas células ciliadas, porém longos e regulares estereocílios, o número e a profundidade das dobras diminuem em direção ao útero e a camada muscular é pronunciada. A porção intramural possui o lúmen mais estreito da tuba uterina; a proporção de células ciliadas e

não ciliadas varia no epitélio e a camada muscular é bem desenvolvida (Del Vecchio, 1992).

As tubas uterinas são essenciais para a gestação: o infundíbulo é necessário para transportar o ovócito do ovário até a região da ampola, onde ocorre a fertilização; o ístmo serve como passagem para ovócito e para espermatozoide e a movimentação dos ovócitos/zigoto nas tubas uterinas ocorre principalmente devido a ação das células cliliadas desta estrutura (Alden 1942).

1.5.5. Cérvix

A cérvix é pequena é contínua com o útero e pouca ou nenhuma alteração cíclica é descrita na literatura para esta porção do trato genital feminino (Del Vecchio, 1992). Esta estrutura apresenta duas cavidades separadas por um septo formado de camadas de músculo liso entre os cornos uterinos (Hebel e Stromberg, 1986).

1.6. Sistema genital masculino

O sistema genital masculino (figura 2) é formado pelos testículos, local onde ocorre a produção de células germinativas masculinas (espermatogênese), por um sistema de ductos extratesticulares responsáveis pela maturação, estocagem e transporte dos gametas, e por órgãos glandulares acessórios, que secretam fluidos que compõem o ejaculado. A integração e o controle destes diversos componentes e funções necessitam interações celulares especializadas e mecanismos de controle hormonal. Algumas destas interações incluem controle endócrino envolvendo o hipotálamo, a hipófise, as células de Leydig e células de Sertoli e também regulações parácrinas entre tipos celulares vizinhos, assim como regulações autócrinas de células individuais (Haschek e Rousseaux, 1998).

Os órgãos genitais no sexo masculino possuem duas funções básicas: produzir espermatozoides (espermatogênese) e hormônios (principalmente testosterona). Além disso, este sistema deve ser capaz de coordenar a liberação espermática e o transporte para o epidídimo, onde a maturação e estocagem destas células ocorrem (Foley, 2001).

O desenvolvimento reprodutivo em machos foi extensamente investigado usando animais da linhagem Wistar (Clermont e Perey, 1957; Robb et al., 1978). Em relação à

espermatogênese, os espermatozoides aparecem primeiramente no testículo em torno do DPN 30-45; aos 40-45 dias, a eficiência da produção espermática aumenta rapidamente e a produção diária espermática/grama de testículo atinge valores adultos aos 75 dias de idade; no DPN 45, somente alguns espermatozoides são encontrados no epidídimo e a reserva espermática neste órgão aumenta significativamente somente após os 100 dias de idade. Considerando-se os pesos dos órgãos, os testículos apresentam um rápido crescimento até os 75 dias de idade, e continuam crescendo até o DPN 100; entre os DPN 40-125, a cabeça e corpo do epidídimo apresentam uma curva de crescimento semelhante aos testículos; já a cauda epididimária aumenta significativamente seu peso entre os dias 100-125. Aos 70 dias de idade, os pesos relativos dos órgãos sexuais acessórios começam a aumentar, e o formato definitivo do pênis inicia sua modificação aos 20-30 dias de idade e é atingido totalmente em torno dos 70 dias. A descida testicular ocorre em torno do DPN 15, e, diferentemente de outras espécies, o canal inguinal permanece aberto nos ratos, permitindo que o macho retraia os testículos para a cavidade abdominal (Wensing, 1986).

Após as publicações de trabalhos demonstrando quedas nos números espermáticos e aumento de incidência de malformações no sistema genital masculino no final do século XX (Topari et al., 1996; US-EPA, 1997; Crisp et al., 1998; National Academic of Science, 1999), o interesse sobre a saúde reprodutiva masculina aumentou. Exposições a agentes ambientais que mimetizam ou antagonizam os hormônios endógenos durante o período de desenvolvimento do sistema genital podem estar relacionadas com estes efeitos (Kavlock, 1999; Clegg et al., 2001).

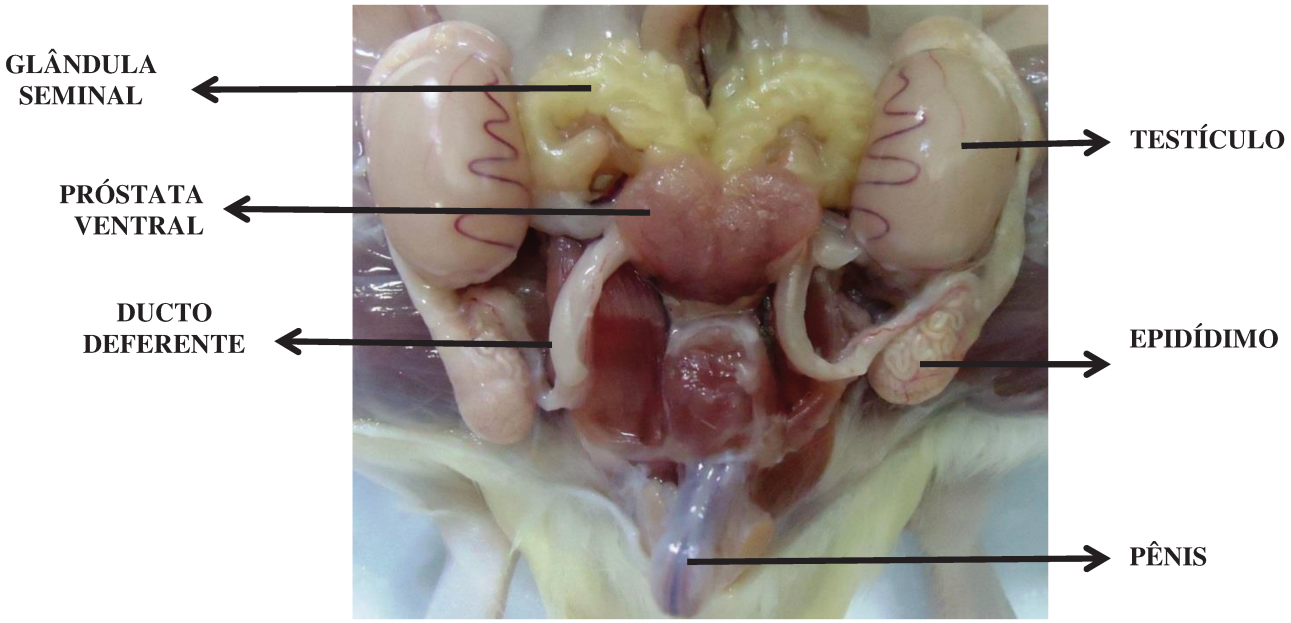

Figura 2. Órgaos sexuais de ratos Wistar (fonte: Laboratório Reprotox).

1.6.1. Testículos

Em mamíferos adultos, os testículos encontram-se no interior do escroto, fora da cavidade abdominal (Haschek e Rousseaux, 1998). Cada testículo é envolvido por uma grossa cápsula de tecido conjuntivo denso, a túnica albugínea (Junqueira e Carneiro, 2008[b]). O parênquima testicular de ratos é composto por cerca de 20 túbulos seminíferos (Russel, 1992; Foley, 2001), onde ocorre a produção dos gametas masculinos, separados por tecido intersticial (tecido conjuntivo frouxo rico em vasos sanguíneos e linfáticos, macrófagos, fibroblastos, nervos e células de Leydig) (Junqueira e Carneiro, 2008[b]). Uma camada de células contráteis mióides envolve a membrana basal dos túbulos seminíferos (Maekawa et al., 1996).

O epitélio dos túbulos seminíferos, também chamado de epitélio germinativo, é composto por células somáticas denominadas células de Sertoli e por populações de células germinativas em diferentes estágios de maturação. De acordo com a maturação das células

germinativas, elas movem-se em direção ao lúmen, onde são finalmente liberadas e transportadas para a rede testis (Haschek e Rousseaux, 1998).

Nas gônadas masculinas (XY), as células germinativas estão presentes nos cordões seminíferos e pausam as divisões mitóticas aproximadamente no dia embrionário 14 (McCarrey, 1993). O processo de espermatogênese começa com uma célula germinativa denominada espermatogônia, situada próximo à lâmina basal do epitélio. Por ocasião da puberdade, grupos de espermatogônias começam a se dividir por mitose e produzem sucessivas gerações de células. As células-filhas podem seguir dois caminhos: continuar se dividindo, mantendo-se como células-tronco de outras espermatogônias, ou diferenciar-se durante sucessivos ciclos de divisão mitótica para se tornarem espermatócitos primários (diploides) (Amann, 1986; Russel et al., 1990; Junqueira e Carneiro, 2008[b]). Durante a fase meiótica, os espermatócitos primários sofrem meiose I e originam espermátócitos secundários (haploides), que por sua vez, sofrem meiose II e formam as espermátides (haploides) (Adler, 1996). As espermátides passam por um processo denominado espermiogênese, que consiste em citodiferenciação celular (condensação cromossômica, formação do acrossoma, reposicionamento mitocondrial, perda de citoplasma e formação de flagelo) para gerar o espermatozoide (Clermont, 1972).

No epitélio seminífero, as gerações de células germinativas formam associações celulares de composição fixa, que correspondem aos estágios do ciclo espermatogênico. Um ciclo da espermatogênese de ratos dura cerca de 12 dias e é composto por 14 estágios. Uma espermatogônia necessita de 4,5 ciclos para formar um espermatozoide, o que significa que a espermatogênese completa de rato tem duração de 52 a 53,2 dias, dependendo da linhagem animal avaliada (Clermont, 1972; Haschek e Rousseaux, 1998).

As células de Leydig, localizadas no espaço intersticial testicular, são as principais produtoras do hormônio esteroide masculino predominante, a testosterona (Marty et al., 2003). Em ratos, este tipo celular é sempre encontrado em associação com vasos linfáticos e sanguíneos (Foley, 2001). A produção de andrógenos por estas células é controlada por múltiplos fatores, principalmente pelo hormônio hipofisário LH (Marty et al., 2003). As células de Leydig são redondas ou polihedricas e, em microscopia óptica, são fortemente eusinofílicas (Haschek e Rousseaux, 1998), apresentam o citoplasma com numerosos

vacúolos lipídicos e retículos endoplasmáticos liso (Zirkin et al., 1980). Estas células são ativas durante o início do período embrionário, diminuem sua atividade durante o desenvolvimento e são reativadas durante a instalação da puberdade (Hooker, 1970).

As células de Sertoli no túbulo seminífero começam a se proliferar aproximadamente no DG 16, atingem o pico de divisão em torno do DG 19 e cessam estas divisões aproximadamente nos DPN 14-16 (Steinberger e Steinberger, 1971; Orth 1982; Van den Dungen et al., 1990; Pryor et al., 2000). Sabe-se que o número de células de Sertoli no testículo permanece constante na idade adulta (Lino, 1971; Hochereau-de Reviers e Courot, 1978).

Em resposta a estimulação com FSH e na presença de testosterona, as céluas de Sertoli secretam fluido e produtos específicos como a proteína ligadora de andrógeno denominada ABP (androgen binding protein) e a inibina (Steinberger 1981). Este tipo celular também é responsável por fornecer suporte estrutural e nutricional para as células germinativas em desenvolvimento, pela formação da barreira hemato-testicular e fagocitose de células germinativas (Foley, 2001).

A formação das junções oclusivas entre as células de Sertoli ocorre entre os DPN 14-19, concomitantemente com a parada de divisão deste tipo celular e com as movimentações dos primeiros espermatócitos para o compartimento adluminal (Haschek e Rousseaux, 1998; Setchell et al., 1969). As junções entre as células de Sertoli formam a barreira hemato-testicular, que divide o epitélio seminífero em compartimentos basal e adluminal. Espermatogônias e espermatócitos imaturos estão presentes no compartimento basal. Esta barreira serve para proteger as células germinativas em desenvolvimento do ataque de células do sistema imune e da exposição a toxinas sistêmicas (Foley 2001). Em animais jovens, as junções celulares no testículo são relativamente permeáveis e o epitélio germinativo pode ser mais vulnerável a insultos quando comparados a animais adultos (Haschek e Rousseaux, 1998).

Os espermatozóides produzidos nos testículos são coletados pela rede testis (Haschek e Rousseaux, 1998). No rato, a rede testis consiste em uma rede subcapsular de canais altamente anastamosados, com aproximadamente 1-2 mm de largura e 7 mm de comprimento, localizada no lado dorsal de cada testículo (Dym, 1976). Esta estrutura é

formada por um epitélio cubóide e seu lúmen contém fluido com espermatozoides. A função da rede testis é pouco entendida, mas além do transposte espermático, ela pode estar relacionada com capacidade de reabsorção e modulação estrogênica (Comhaire e Vermeulen, 1976; Maddocks e Sharpe, 1989; Hess et al., 1997).

Os ductos eferentes surgem da rede testis extratesticular e se extendem através da gordura epididimária para desembocar nas porções iniciais do epidídimo. No rato, em torno de 4 a 8 ductos estão presentes e a maior parte do epitélio é composta por células cubóides não ciliadas (que absorvem fluidos secretados pelos testículos) que se alternam com grupos de células ciliadas, cujos cílios batem em direção ao epidídimo (Junqueira e Carneiro, 2008[b]). Estes ductos coalescem para formar um ducto único, que por sua vez, se liga ao epidídimo. As principais funções dos ductos eferentes são o transporte espermático e a reabsorção de fluidos (Clulow et al., 1998; Newcombe et al., 2000).

1.6.2. Epidídimo

Através do desenvolvimento embrionário e neonatal, o epidídimo de mamíferos muda de um tubo reto para um ducto altamente enovelado e complexo que liga os ductos eferentes ao ducto deferente. Este órgão transforma os espermatozoides em células completamente maduras que conseguem se movimentar, reconhecer e fertilizar ovócitos (Robaire et al., 2006).

Além da maturação espermática, o epidídimo é fundamental no transporte, concentração, proteção e armazenamento de espermatozoides. Um microambiente altamente especializado e específico para cada região é criado ao longo do lúmen epididimário por ativa secreção e absorção de água, íons, solutos orgânicos e proteínas, assim como pela barreira hemato-epididimária. O principal fator de regulação epididimário são os andrógenos, mas existem evidências de que estrógenos, retinóis e outros fatores provenientes do testículo também possuem funções no epidídimo (Robaire et al., 2006).

Sun e Flickinger (1979) classificaramo desenvolvimento epididimário em três períodos: um período indiferenciado: do nascimento ao DPN 15; um período de diferenciação: DPN 16 aos 44; e um período de expansão: após os 44 dias de idade. Tanto a

testosterona, quanto as células germinativas e o fluido testicular provenientes da rede testis contribuem para a diferenciação do epidídimo (Pryor et al, 2000).

Até o dia pós-natal 15, o epidídimo do rato cresce consideravelmente e atinge cerca de 2 metros de comprimento (Hermo et al., 1992; Jiang et al., 1994). As células indiferenciadas diferenciam-se em tipos celulares classicamente descritos para o epidídimo adulto (principais, apicais, claras, basais, halo e estreitas): no dia pós-natal 14 as células halo aparecem; no dia 28 as células colunares diferenciam-se em células basais e principais; a partir do DPN 36 as células estreitas e as células claras aparecem; aproximadamente aos 49 dias de idade todas as células estão completamente diferenciadas (Robaire et al., 2006). A barreira hemato-epididimária é completamente formada em ratos no DPN 21, antes da chegada dos espermatozoides no lúmen do epidídimo (Agarwal e Hoffer, 1989).

O epidídimo é comumente dividido em quatro regiões macroscópicas: o segmento inicial (porção mais proximal do ducto epididimário, diretamente ligada aos ductos deferentes), cabeça (região proximal do ducto epididimário em forma de bulbo), corpo (porção estreita localizada na região média do órgão) e cauda (região mais distal do epidídimo de onde emerge o ducto deferente) (Robaire e Hermo, 1988; Turner, 1995). Em todas as espécies de mamíferos, cada região do epidídimo é organizada em lóbulos separados por septos de tecido conjuntivo. Estes septos não servem somente como suporte interno ao órgão, mas também fornece uma separação funcional que permite a expressão seletiva de genes e proteínas em lóbulos específicos (Turner et al., 2003).

As células principais são as mais abundantes e aparecem em toda a extensão do ducto epididimário, porém com diferenças estruturais em cada região (Hamilton, 1975; Robaire e Hermo, 1988). Este tipo celular possui uma maquinaria de secreção e endocitose bem desenvolvida e representa de 65% a 80% do total de células epiteliais do epidídimo (Trasler et al., 1988). As células apicais são encontradas principalmente na região do segmento inicial (Sun e Flickinger, 1980; Adamali e Hermo, 1996) e estão localizadas apicalmente no epitélio do epidídimo. Pouco se sabe sobre suas funções específicas, com exceção da habilidade em endocitar substâncias presentes no lúmen e apresentar inúmeras enzimas proteolíticas (Adamali e Hermo, 1996).

Células estreitas, no rato e em camundongo, aparecem no epitélio do segmento inicial (Sun e Flickinger, 1980; Adamali e Hermo, 1996) e possuem inúmeras vesículas apicais, envolvidas em endocitose e na secreção de íons H^+ para o lúmen (Hermo et al, 2000). As células claras são células endocíticas presentes nas regiões da cabeça, corpo e cauda do epidídimo, sendo mais numerosas na cauda, e são encontradas em muitas espécies animais, incluindo o homem (Hamilton, 1975; Cooper, 1986; Robaire e Hermo, 1988). Estas células são responsáveis pela acidificação do fluido luminal, importante para manutenção do espermatozoide em estado quiescente para a maturação (Morton et al., 1974; Turner e Howards, 1978; Pholpramool e Chaturapanich, 1979; Wong et al., 1981).

As células basais estão aderidas a membrana basal do epitélio epididimário (Veri et al., 1993) e podem ser encontradas ao longo de todo o ducto. Estas células possuem um papel imune (Seiler et al., 2000), bem como participa da regulação de transporte de água e eletrólitos nas células principais (Cheung et al., 2005) e endocitose mediada por receptores (Robaire et al., 2006). As células halo são pequenas e presentes ao longo do epitélio epididimário (Robaire e Hermo, 1988). Usualmente estão presentes na base do epitélio e contêm um número variável de grânulos densos. Estas células têm sido descritas como linfócitos (Robaire e Hermo, 1988) ou monócitos (Hamilton, 1972). Em condições normais, as células halo são consideradas as principais células imunes no epidídimo (Robaire et al., 2006).

1.6.3. Ducto deferente

O ducto deferente é contínuo com a cauda do epidídimo e se extende até a uretra prostática. Na maioria das espécies, a parte final deste ducto é dilatada para formar a ampola, que se junta com o ducto da vesícula seminal, formando o ducto ejaculatório. O epitélio do ducto deferente é pseudoestratificado com longos estereocílios na superfície apical. Este ducto transfere o espermatozoide que estava estocado no epidídimo para a uretra, onde secreções adicionais são liberadas para produzir o sêmen. A fina camada fibromuscular que envolve o ducto é capaz de se contrair para prover uma propulsão rápida do espermatozoide pelo trato durante a ejaculação (Haschek e Rousseaux, 1998).

1.6.4. Próstata

A próstata é formada por múltiplos lóbos envolta da uretra. É classificada como uma glândula tubuloalveolar que secreta um fluido proteico incolor contendo cálcio e íons citrato e fosfato na uretra através de inúmeros ductos. Em ratos, um par de lobos ventrais e pequenos lobos dorsal e lateral estão situados em volta da bexiga (Sandberg et al., 1980; Marty et al., 2003). Um par de lobos anteriores, também conhecidos como glândulas coaguladoras, encontra-se localizado adjacente à vesícula seminal. As glândulas prostáticas são formadas por um epitélio cubóide ou pseudo-estratificado colunar (Haschek e Rousseaux, 1998; Junqueira e Carneiro, 2008[b]).

A maoir parte do desenvolvimento da próstata de ratos ocorre após o nascimento. Os lobos prostáticos desenvolvem-se entre os DPN 1 ao 7 e o lúmen tubular prostático aparece entre os DPN 7-14. Grânulos secretórios são observados entre os DPN 14-21. A próstata adquire sua aparência adulta entre os DPN 28-35, concomitantemente ao aumento de níveis de testosterona pós-natal (Marty et al., 2003).

1.6.5. Glândula seminal

As glândulas seminais são órgãos pares elongados ocos, preenchidos com fluido viscoso amarelado contendo frutose, ácido cítrico e outras substâncias nutritivas em abundância (Guyton e Hall, 2002[b]). Elas estão situadas distalmente à ampola dos vasos deferentes e esvaziam-se no ducto ejaculatório na uretra. O epitélio é composto por células colunares pseudoestratificadas no camundongo e por células colunares simples no rato (Haschek e Rousseaux, 1998).

Em ratos, o padrão básico de formação da glândula seminal está presente no DPN 10. A formação do lumen ocorre ente os DPN 2-15. O produto de secreção desta glândula é evidente após 16 dias de idade. A glândula seminal aumenta consideravelmente de tamanho entre os DPN 11-24, e continua a crescer até atingir o aspecto adulto e as propriedades secretórias entre os DPN 40 e 50 (Brooks et al., 1973). Desta forma, semelhantemente à próstata, a proliferação e diferenciação das vesículas seminais ocorrem em pararelo ao aumento nos níveis de testosterona circulantes (Marty et al., 2003).

1.6.6. Pênis

Os principais componentes do pênis são a uretra e três corpos cilíndricos eréteis, sendo este conjunto envolvido por pele. Dois desdes cilíndros, os corpos cavernosos, estão localizados na parte dorsal do pênis. O terceiro cilíndro, o corpo esponjoso, está localizado ventralmente e envolve a uretra; na sua extremidade distal ele se dilata e forma a glande do pênis. Os corpos cavernosos são envolvidos por uma camada de tecido conjuntivo denso, a túnica albugínea e o tecido erétil que compõe os três corpos eréteis tem uma grande quantidade de espaços venosos separados por fibras de tecido conjuntivo e células musculares lisas (Junqueira e Carneiro, 2008[b]).

A ereção é um processo hemodinâmico controlado por impulsos nervosos sobre os músculos lisos das artérias do pênis e sobre os músculos lisos que cercam os espaços venosos. Impulsos parassimpáticos causam o relaxamento da musculatura dos vasos penianos e do músculo liso dos corpos cavernosos. A abertura das artérias penianas e dos espaços cavernosos aumenta o fluxo de sangue no local, produzindo a rigidez do pênis (Junqueira e Carneiro, 2008[b]).

A liberação do espermatozoide estocado na cauda do epidídimo é controlada pelo sistema nervoso simpático, que inerva a musculatura lisa epididmária e dos ductos deferentes. A contração destes músculos resulta na propulsão dos espermatozoides pelos ductos até a uretra, onde a secreções dos órgãos sexuais acessórios são adicionadas para a produção do sêmen (Haschek e Rousseaux, 1998).

1.7. Andrógenos e o sistema genital

A presença de andrógenos endógenos em concentrações fisiológicas é essencial para o correto desenvolvimento e função do sistema genital, tanto masculino quanto feminino.

Diversos estudos demonstram a importância de andrógenos circulantes na regulação fisiológica reprodutiva feminina, uma vez que camundongos deficientes em receptores de andrógeno apresentam alterações reprodutivas como, por exemplo, falha ovariana prematura (Shiina et al., 2006). Em ratas, a testosterona e a di-hidrotestosterona séricas variam de acordo com as fases do ciclo estral (Rush e Blake, 1982; Dunlap e Sridaran,

1988) e estas variações são importantes na regulação hormonal da expressão gênica (Haisenleder et al., 1997; Burger et al. 2007).

A presença de andrógenos durante o período de diferenciação sexual é muito importante para a determinação do fenótipo masculino (Jost et al., 1973; Cruz e Pereira, 2012), resultando em comportamento sexual característico e estabelecimento do padrão de secreção de gonadotrofinas (Adkins-Regan, 1988; Bakker, 2003; Piffer etal., 2009). Além disso, a presença de andrógenos é fundamental para a espermatogênese (Meisel e Sachs, 1994) e na diferenciação de órgãos como epidídimo (Pryor et al., 2000), próstata e glândula seminal (Marty et al., 2003; Rang et al., 2004).

Embora a presença de hormônios androgênicos seja imprescindível para o correto funcionamento do sistema genital, a exposição de animais experimentais a estes compostos durante a vida fetal ou pré-púbere pode reprogramar múltiplos tecidos e causar alterações morfológicas, bioquímicas e funcionais permanentes em vários órgãos ou sistemas (Schwartz et al., 1986; Carter et al., 1988).

A exposição androgênica pode ocasionar, no sistema genital de fêmeas, mudanças no desenvolvimento normal da genitália, síndrome de ovário policístico, alterações uterinas, modificações no ciclo estral, no comportamento sexual e esterilidade (Greene et al., 1939; Huffman eHendricks, 1981; Slob et al., 1983; Mena et al., 1992; Rhees et al., 1997; Ryan e Vandenbergh, 2002). Wolf et al. (2002) determinaram, em estudos com exposição ao propionato de testosterona durante o período gestacional, a LOAEL (lowest observable adverse effects level) em fêmeas para o número de mamilos em 0,5mg/kg e 1,0mg/kg para alterações na distância ano-genital. Hotchkiss et al. (2007) demonstraram que, o acetato de trembolona (composto androgênico utilizado na produção de gado de corte) na dose de 0,5mg/kg pode ocasionar, além de alterações no número de mamilos, aumento na distância ano-genital, e alterações na instalação da puberdade.

Em relação ao sistema reprodutor masculino, o tratamento perinatal a andrógenos em machos intactos resulta na disfunção da reprodução sexual (Diamond et al., 1973; Pollak e Sachs, 1975; Zadina et al., 1979; Piacsek e Hostetter, 1984). Doses de 1mg/kg e 2mg/kg de propionato de testosterona, quando administradas durante o período gestacional, podem ocasionar alterações na preferência sexual masculina, na distância ano-genital, no

peso dos órgãos reprodutores, na puberdade e nos níveis de testosterona circulantes (Pollak e Sachs, 1975; Henley et al., 2010; Cruz e Pereira, 2012). Já doses inferiores, como 0,25mg/kg não demostraram ser capazes de alterar o desenvolvimento reprodutivo de machos (Frick et al., 1969).

Compostos com atividade androgênica têm sido encontrados como contaminantes de rios e em animais destinados a alimentação humana nos Estados Unidos e Europa (Davis e Bortone, 1992; FDA, 1996; Vos et al., 2000; Parks et al., 2001; Orlando et al., 2004; Radl et al., 2005; Durhan et al., 2006). Mesmo sob suspeitas desde 1970, a presença de andrógenos de origem antropogênica no ambiente só foi confirmada nos últimos anos (Gray et al., 2006).

Além disso, algumas condições médicas são consideradas áreas de investigação. Mulheres com síndrome do ovário policístico e/ou hiperplasia da adrenal oferecem, durante a gentação, um ambiente com elevados níveis de andrógenos e, desta forma, expondo potencialmente os fetos em desenvolvimento a uma condição hiperandrogência no ambiente intrauterino (Otten et al., 2005; Blank et al., 2006).

Justificativa

2. Justificativa do tema

O correto desenvolvimento do sistema genital, tanto masculino quanto feminino, depende de diversos eventos controlados hormonalmente e geneticamente, que se iniciam na vida intrauterina e continuam após o nascimento. A exposição a determinados compostos químicos durante estes eventos pode ocasionar alterações no desenvolvimento sexual que podem levar à infertilidade.

Desta forma, sabendo-se que a exposição a substâncias presentes no ambiente pode contribuir para várias disfunções reprodutivas, especialmente se ocorrer em períodos críticos de desenvolvimento (como período intrauterino e pós-natal), e que compostos com ação androgênica são encontrados como contaminantes ambientais, justificou-se a realização deste estudo para avaliar os efeitos da exposição a andrógenos (situação que pode ocorrer em conseqüência à exposição a químicos ambientais e industriais com propriedades androgênicas e em condições médicas como síndrome do ovário policístico ou hiperplasia congênita da adrenal) sobre a estrutura do sistema genital e a função reprodutiva feminina e masculina.

O período escolhido para o tratamento – gestacional e lactacional – coincide com o período crítico do desenvolvimento do sistema genital da prole, que continua após o nasceimento.

Objetivos

3. Objetivos

3.1.Objetivo geral

O presente estudo teve por objetivo avaliar os possíveis efeitos da exposição materna a um andrógeno durante a prenhez e lactação sobre a função e a estrutura do trato genital da prole.

3.2.Objetivos específicos

Para alcançar o objetivo acima descrito, foi utilizado o propionato de testosterona como modelo androgênico. Este composto foi administrado a ratas Wistar prenhes durante a prenhez e lactação e os filhotes foram avaliados em diversos parâmetros reprodutivos. Na prole feminina foram realizadas análises das gônadas fetais para determinação de possíveis efeitos nas células germinativas primordiais, distância anogenital e contagem de mamilos para verificação de marculinização, e data da instalação da puberdade; logo após a puberdade e aos 75 dias de idade o peso e histologia de útero e ovário foram avaliados, assim como dosagens hormonais para FSH, LH, estrógeno, progesterona e testosterona, regularidade do ciclo estral, imunomarcação para receptores esteroides e taxa de proliferação e morte celular em tecidos uterinos, comportamento sexual, fertilidade após acasalamentos naturais e teste de sensibilidade da hipófise e do útero ao tratamento com estrógenos. Os filhotes do sexo masculino foram avaliados na idade adulta (90 dias de idade) e na meia-idade (270 dias de idade), e foram analisados parâmetros como peso e histologia de testículo e epidídimo, contagens espermáticas, morfologia e motilidade espermática, dosagens hormonais para FSH, LH, estrógeno, progesterona e testosterona, comportamento sexual e fertilidade após acasalamentos naturais.

Materiais e métodos

4. Materiais e métodos

4.1.Prole feminina

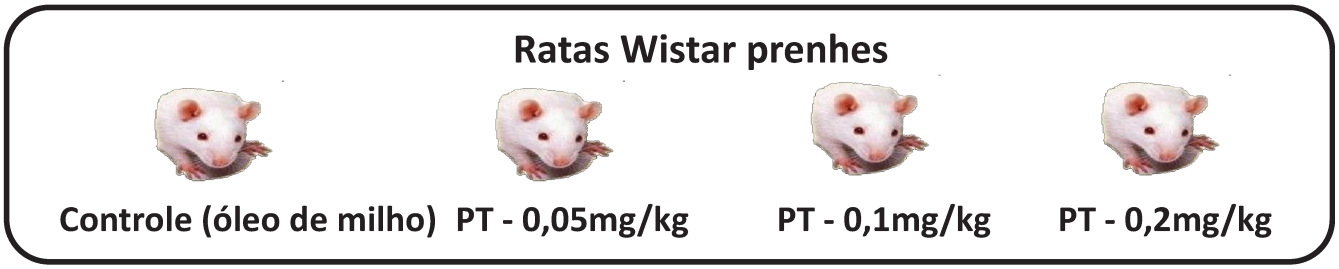

Figura 1. Esquema de tratamento de ratas Wistar para avaliação da prole feminina. Os animais prenhes foram expostos, subcutaneamente, ao PT, do 12° dia gestacional ao 21° dia pós-natal. O grupo controle recebeu somente o veículo (óleo de milho). Grupos experimentais: controle (*n*=8), 0,05mg/kg (*n*=7), 0,1mg/kg (*n*=9) e 0,2mg/kg (*n*=10).

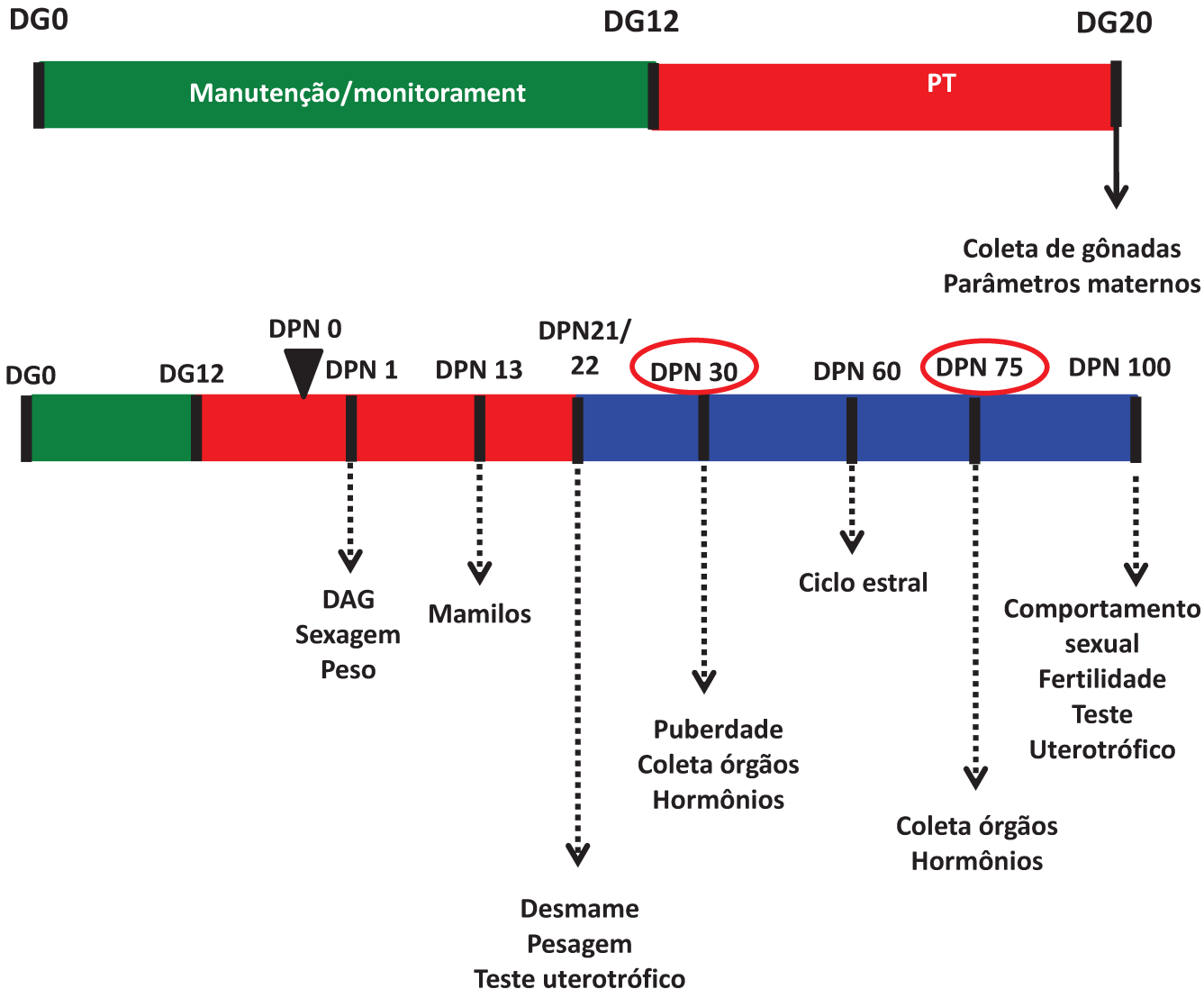

Figura 2. Esquema de avaliação dos filhotes fêmeas de ratas Wistar. Os animais prenhes foram expostos, subcutaneamente, ao PT, do 12° dia gestacional ao 21° dia pós natal. O grupo controle recebeu somente o veículo (óleo de milho). Grupos experimentais: controle (*n*=8), 0,05mg/kg (*n*=7), 0,1mg/kg (*n*=9) e 0,2mg/kg (*n*=10). No DPN 1 foi avaliado a DAG, feita a sexagem e o peso corpóreo dos filhotes; no DPN 13 foi realizada a contagem de mamilos; no DPN 22 os animais foram desmamados e foi realizado o primeiro desafio uterino ao estrógeno. No DPN 30 iniciou-se a avaliação da instalação da puberdade, coleta de órgãos e sangue para dosagens hormonais; No DPN 60 iniciou-se o estudo sobre a regularidade do ciclo estral; aos 75 dias foram coletados novamente órgãos e sangue para dosagens hormonais e aos 100 dias de idade foram realizados testes de comportamento sexual, fertilidade e o segundo desafio uterino ao estradiol.

4.2.Prole masculina

Figura 3. Esquema de tratamento de ratas Wistar para avaliação da prole masculina. Os animais prenhes foram expostos, subcutaneamente, ao PT, do 12° dia gestacional ao 21° dia pós-natal. O grupo controle recebeu somente o veículo (óleo de milho). Grupos experimentais: controle (*n*=8) e 0,2mg/kg (*n*=8).

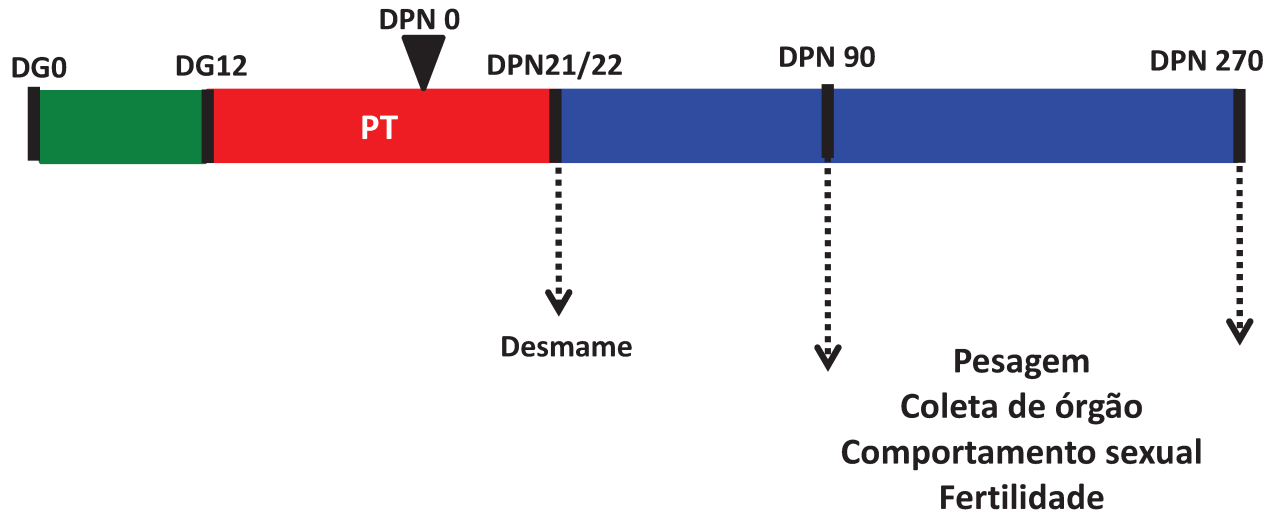

Figura 4. Esquema de avaliação dos filhotes machos de ratas Wistar. Os animais prenhes foram expostos, subcutaneamente, ao PT, do 12° dia gestacional ao 21° dia pós natal. O grupo controle recebeu somente o veículo (óleo de milho). Grupos experimentais: controle (*n*=8) e 0,2mg/kg (*n*=8). No DPN 22 os animais foram desmamados e tanto no DPN 90 (idade adulta) quanto no DPN 270, eles foram pesados, foram coletados órgãos sexuais, fígado, rins e hipófise, foram realizados testes de comportamento sexual e fertilidade.

Capítulos

5. Capítulos

O presente trabalho resultou em quatro manuscritos que serão apresentados a seguir.

5.1. Manuscrito 1

O primeiro manuscrito é intitulado "*Excess androgen during perinatal life alters steroid receptor expression, apoptosis, and cell proliferation in the uteri of the offspring*", e foi submetido à revista Reproductive Toxicology, USA, Elsevier, ISSN:0890-6238, Fator de Impacto: 3,226.

EXCESS ANDROGEN DURING PERINATAL LIFE ALTERS STEROID RECEPTOR EXPRESSION, APOPTOSIS, AND CELL PROLIFERATION IN THE UTERI OF THE OFFSPRING

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Abstract

Exposure to environmental chemicals may contribute to reproductive disorders, especially when it occurs in critical periods of development. The female reproductive system can be a target for androgens derived from environmental contaminants or pathological conditions. The purpose of this study was to assess the long-term effects of androgens on uterine tissue after maternal exposure limited to the time of gestation and lactation. Pregnant Wistar rats were treated with testosterone propionate (TP) at 0.05mg/kg, 0.1mg/kg, 0.2mg/kg or corn oil (vehicle), s.c., from gestational day 12 until the end of lactation. The results show changes in the pattern of expression of receptors for estrogen, progesterone, and androgen at all doses tested, and decreases in both apoptosis and cell proliferation indices at 0.1 and 0.2mg/kg. We conclude that early TP exposure, under these experimental conditions, causes changes in cellular and molecular parameters that are essential for normal uterine function in the adult.

Key words: Female reproduction, hyper-androgenic condition, uterus, immunohistochemistry, steroid receptors

1. Introduction

Several studies demonstrated a strong association between exposure to environmental chemicals *in utero* or during early extrauterine life and the development of chronic diseases during adulthood [1]. In many animal species, these substances reach the fetus via the mother/placenta and/or the offspring via the mother's milk [2].

Several synthetic chemicals sold commercially (e.g., pesticides, animal nutritional supplements) are known to mimic hormones. They are called *endocrine-disrupting compounds* (EDC) and have been shown previously to interfere with the physiology of normal endocrine-regulated events to disease onset [3, 4].

Androgens are well known to play a pivotal role in the differentiation and development of the male reproductive system and secondary sex characteristics [5]; however, exposure to abnormally high levels may result in permanent damage to reproductive functions, especially in the female [7-9]. Androgenic activity has been described in industrial and agricultural environments [10, 11] and there are reports of premature breast enlargement and increased incidence of ovarian cysts due to high hormone levels in meat [12]. Further, in humans, the lasting effects of prenatal androgens due to medical conditions such as congenital adrenal hyperplasia remain an active area of investigation [13, 14].

The exposure of experimental animals to high doses of androgens during fetal or early postnatal life appears to reprogram female tissues and cause morphological, biochemical and/or functional alterations in several organs or systems [15-18]. Androgenic compounds, when given prenatally, can disrupt sexual differentiation and reproductive function of female offspring [7, 19, 20].

We now understand that uterine tissues are epigenetically programmed during perinatal life [21]. This is the most likely mechanism by which early exposure to androgens can alter the response of the adult uterus to estrogens [22, 23]. Perinatal androgenization may modify the normal development of estrogen receptors [24] in the uterus and/or alter post-receptor stages of the response to estrogens [17].

Progesterone is an essential regulator of the reproductive events, including uterine development [25]. Estrogen receptor (ER) and progesterone receptor (PR) signals provide

the basis for the cyclic changes in the uterine tissues during the estrous cycle [26]. Regarding androgenic action, Weihua et al. [27] reported the essential role of androgen receptor (AR) in uterine epithelial cell proliferation induced by estrogens and indicated that stromal AR can amplify the effects of ERα. Identification of regional and cellular AR and PR localization allows a better understanding not only of the role of AR and PR, but also the mechanism of estrogen action in female reproductive tract [28]. In female, apoptosis and cell proliferation have an important role in the regulation of reproductive tract function and steroid hormones are regulatory keys from these processes [29]. Based on this information, we can suppose that variations on uterine steroidal receptors may cause a misbalance in uterine tissue growth and regression.

Earlier studies addressing the effects of neonatal androgenization on uterine estrogen receptors yielded inconsistent results [30, 31, 32]. Others used relatively high doses and short periods of treatment [7-9, 33]. Against this background, our aim was to evaluate the possible effects of low and protracted doses, as would occur with natural exposure to EDCs, of the androgenic compound testosterone propionate (TP) on the uterine pattern of the expression of steroid receptors and the dynamics of cell proliferation and apoptosis in female rats whose mothers were exposed to TP during pregnancy and lactation. The treatment was designed to coincide with the critical period of reproductive development of the offspring both before and after birth.

2. Material and methods

2.1. Animals and treatment

Adult female (60 days of age) and male (90 days of age) Wistar rats were supplied from the Central Biotherium of State University of São Paulo (UNESP) and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu. Animals were housed in polypropylene cages (43 cm x 30 cm x 15 cm) with laboratory grade pine shavings as bedding. Rats were maintained under controlled temperature (23±1°C) and lighting conditions (12:12-h photoperiod). Standard rodent chow and filtered tap water were provided *ad libitum*.

Two non-gravid females were mated with one male during the dark portion of the light cycle. Vaginal smears were performed the next day for sperm detection, and day of sperm detection was considered day zero of gestation (gestational day 0 - GD0). The pregnant females were randomly assigned to the experimental groups and housed individually in cages. At post-natal day 1 (PND1 - considering the day of birth as PND0), the number per litter was reduced to eight, maintaining, preferentially, female offspring [34, 35]. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol no. 104/2009-CEEA).

Pregnant rats were administered either corn oil (Control, *n*=8) or testosterone propionate (TP - imported from India by DEG Importation of Chemical Products Ltd., CAS 57-85-2, 97% of purity) at doses of 0.05mg/kg (0.05mg, *n*=7), 0.1mg/kg (0.1mg, *n*=9) or 0.2mg/kg (0.2mg, *n*=10), subcutaneously, from GD12 to the end of lactation (PND21). The beginning of the mother's treatment coincided with the critical period of reproductive system development, which continues after birth [36].

2.2. Maternal body weight

Dams were weighed on alternative days from GD12 until the end of lactation (PND 21) to adjust the volume of TP to be administered and to evaluate possible maternal toxicity.

2.3. Female pups

At PND1, all pups were weighed and sexed. At PND22 they were weaned, female offspring was separated from males and the respective mothers were euthanized by CO_2 inhalation followed by decapitation. After the end of lactation, the parameters below were performed to assess the development of uterine tissues.

2.3.1. *Immunohistochemistry*

After puberty onset (determined by vaginal opening and first estrus) and at PND75, one female offspring per litter (control=8; 0.05mg=7; 0.1mg=9; 0.2mg=10), was euthanized on estrus phase by CO_2 inhalation followed by decapitation and uteri (with fluid) was collected, weighed, fixed in formaldehyde in PBS (10%), dehydrated in ethanol and embedded in Paraplast.

To perform the immunohistochemistry for estrogen receptor alpha (ERα - 6F11: sc-56836, Santa Cruz, 1:1,000) and cell proliferation (PCNA – PC10: sc-56, Santa Cruz, 1:10,000), biotinylated anti-mouse IgG (1:100) and normal horse serum were used, and, for androgen (AR – ab3510, Abcam, 5µg/mL) and progesterone (PR – H190:sc-7208, Santa Cruz, 1:100) receptors, biotinylated anti-rabbit IgG(1:200) and normal goat serum.

Uterine transverse sections (6µm) were dewaxed using toluene and hydrated using decreasing concentrations of ethanol, followed by antigen recovery with citrate buffer (0.01M, pH 6.0 – microwave, 650 Watts, high temperature, 3 x 5 minutes) [37, 38]. After this step, slides (one for each animal) were maintained at room temperature for 15 minutes to cool down, and after that, washed for 5 minutes in PBS (0.01M, pH 7.5). After PBS wash, the slides were incubated for 10 minutes with blocking solution 10% (normal serum (1%) diluted in PBS) to avoid unspecific reactions. Then, sections were incubated overnight at 4°C with primary antibody.

Following two 5 min washes in PBS and 10 min incubation with block solution, the sections were incubated for 1 hour at room temperature with appropriate secondary biotinylated antibody (Vector Laboratories, Inc.). Then, slides were incubated with ABC solution (1:400) for 1 hour at room temperature (VECTASTAIN ABC KIT, PK 4000, Vector laboratories, Inc.) to evidence the immunocomplex formed by secondary antibody linked to the primary antibody.

After three 2 min washes in PBS, peroxidase activity was reveled using diaminobenzidine solution (DAB) (Vector Laboratories, Inc., according to the manufacturer's instructions) for 10 minutes at room temperature. The sections were washed in distillated water for 5 minutes and then in PBS for another 5 minutes, counterstained with toluidine blue, dehydrated by applying increasing concentrations of ethanol, cleared

by xylene and coverslipped with Permount. For the steroid receptors, sections were analyzed under light microscope and the evaluation was performed by semi-quantitative scale, according to intensity of staining at luminal and glandular epithelium, stroma and myometrium. For PCNA, results were expressed as percentage of stained cells at different uterine compartments.

The results of immunohistochemical staining for ERα, AR and PR were evaluated semiquantitatively based on Petrusz et al. [39]: overstained (diffusion of the reaction product and/or non-specific background), strong (black or dark brown), light (medium brown), very light staining (usually restricted to a smaller number of stained cells) or no visible staining. To measure the percentage of stained cells in the PCNA-assay, one hundred cells were analyzed in each uterine compartment (luminal epithelium, glandular epithelium, stroma and myometrium) and the number of stained cells was divided by total number of cells.

2.3.2. Apoptosis

The free 3'OH DNA termini from uterine cells were labeled in situ by terminal deoxynucleotidyl transferase (TdT), forming an oligomer composed of digoxigenin-conjugated nucleotide. This complex is able to bind to an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule. The bound peroxidase antibody conjugate enzymatically generates a permanent, intense, localized stain from chromogenic substrates, providing sensitive detection in immunohistochemistry. This methodology was performed using Apoptag® peroxidase in situ Apoptosis detection kit (Millipore™), according to the manufacturer's instructions. Following apoptosis staining, sections were counterstained with toluidine blue and mounted under glass coverslips with permount. The sections were analyzed under light microscope and results were expressed as percentage of stained cells at luminal epithelium, glandular epithelium, stroma and myometrium. To measure the percentage of stained cells one hundred cells were analyzed in each uterine compartment and the number of stained cells were divided by total number of cells.

2.4. *Statistical analysis*

For comparison of results among the experimental groups, ANOVA with an a posteriori Dunnett's test (mean ± S.E.M) (for body and uterine weights and litter size) or non-parametric Kruskal-Wallis with an a posteriori Dunn (Bonferroni) test [median (Q_1-Q_3)] (for the percentage of stained cells) was performed, according to the characteristics of each variable, using Instat 3.0 software (GraphPad software, San Diego, CA, USA). Differences were considered significant when $p<0.05$.

3. Results

3.1. *Effects on dams*

None of the experimental groups showed visible signs of toxicity during pregnancy or lactation period. No alterations were observed in health conditions or behavior of pregnant or nursing rats. At the dose levels tested, TP treatment had no effects on maternal body weight or body weight gain during gestational and lactational period (data not shown).

3.2. *Effects on female pups*

On PND1, all experimental groups presented comparable female offspring body weight (Control=6.87 ± 0.2; 0.05mg= 6.74 ± 0.2; 0.1mg= 6.53 ± 0.1; 0.2mg= 6.36 ± 0.1; Anova with an a posteriori Dunnett's test, $p>0.05$) and there was no death (pups or dams) during the period of treatment. The mean litter size was not different among experimental groups (control=11.33 ± 0.79; 0.05mg=9.86 ± 0.70; 0.1mg=11.44 ±0.71; 0.2mg=11.50 ± 0.70, ANOVA with an a posteriori Dunnett's test). After the end of lactation, the parameters below were performed to assess the development of uterine tissues in one female offspring per litter (control=38 pups in 8 litters; 0.05mg=30 pups in 7 litters; 0.1mg=38 pups in 9 litters, 0.2mg=31 pups in 10 litters).

At necropsy of female offspring (after puberty onset and PND75), the uterine weight was not affected by the administration of TP during gestation and lactation (table 1).

Analysis of immunohistochemistry from ERα, AR and PR show alterations in the intensity of staining in all groups treated with TP (table 2). At puberty, experimental groups

exposed to 0.05 and 0.1mg of TP presented a decrease in glandular epithelium PR labeling; the 0.2mg group revealed a weak staining at luminal and glandular epithelium for ERα and contrarily, an increase in AR staining at the same uterine compartments (figure 1). At PND 75, 0.05mg group showed an increase in PR and a decrease in ERα staining (luminal and glandular epithelium); the 0.1mg revealed a decrease in PR (luminal and glandular epithelium) and AR (luminal epithelium) staining. The 0.2mg group showed an increase in AR (luminal epithelium) and PR immunostaining (luminal and glandular epithelium) (figure 2).

The evaluation of apoptosis showed a decrease in labeled cells at 0.2mg group at adult age in myometrium and stromal compartments, when compared to control group (figure 3). The analysis of cell proliferation through PCNA immunohistochemistry revealed a decrease in the percentage of stained cells at 0.1 and 0.2mg groups at 75 days of age in myometrium (table 3).

4. Discussion

Endocrine disrupting compounds may mimic, antagonize, or modify the effects of steroid hormones such as estrogens and androgens in the organism. Although suspected since the 1970s, the presence of androgens of anthropogenic origin in the environment was only confirmed recently [40].

It is well known that experimental administration of androgens to rats either before or shortly after birth results in profound, permanent changes in their reproductive system [33, 41, 42]. Androgenic compounds can disrupt sexual differentiation and reproductive function of female offspring when given prenatally [7, 18-20].

Most studies of this type used relatively high doses of androgens and short periods of exposure [7-9, 33, 42]. Under such conditions, female rats develop a male-like phenotype, continuous (instead of cyclic) gonadotropin secretion, infertility due to lack of ovulation, polycystic ovaries, and persistent vaginal estrus (“androgen sterilization”). The uterus of such animals is smaller than normal and shows a reduced response to exogenously administered estrogens [22, 43, 44]. In contrast, in the present work we attempted to mimic a more realistic scenario using low-dose, prolonged exposure, as might occur with

environmental contaminants, and study the effects of such exposure in the female offspring. The doses of testosterone, low enough not to induce persistent vaginal estrus and masculinization of external genitalia, were established in preliminary experiments (not reported in detail).

The significantly reduced staining intensities for ERα in the luminal and glandular epithelium in the 0.2 mg group at puberty and the 0.05 mg group in the adult (PND 75) indicate reduced ERα expression in these groups and, in turn, may be expected to result in reduced estrogen responsiveness of the uterus [17, 24, 30, 45].

However, the uterine weights of all TP-treated animals were similar to those of the controls. This suggests that lower than normal ERα levels can still mediate physiological (baseline) estrogen action on the uterus. Wrenn et al. [43] and Gellert et al. [31] reported similar results after a single TP dose of 250μg on PND 0 and 100μg on PND 3, respectively. It remains to be established whether the lower ERα levels found after the low-dose, protracted exposure used in our study are sufficient to mediate a normal uterine weight response (i.e., increase) to exogenously administered estrogens.

Estrogens act together with progesterone to maintain the sequential events of a normal uterine cycle [46]. The gene that codes for the PR is controlled by estrogen [47, 48]; thus, expression of PR protein is regarded as an indicator of previous estrogen action [49]. We found only one report on the effects of early androgen treatment on PR expression in the uterus [50]. These authors reported that the concentration of PR, measured by biochemical techniques, was abnormally low in the uteri of neonatally treated rats. Our immunohistochemical results (Figs. 1 and 2, Table 3), although they indicate changes in PR expression in several groups, do not reflect any consistent changes in PR expression following perinatal androgenization, nor any discernible correlation between the expression of ER and PR.

Estrogen action in the adult uterus is also supported by androgens acting through the androgen receptor (AR) [51, 52]. AR-knockout mice show a severely diminished uterine response to exogenous gonadotropin treatment [53]. To our knowledge, the present report is the first one on adult uterine expression of AR in rats after perinatal androgenization. As with PR, changes in the pattern and intensity of labeling were observed (Figs. 1 and 2,

Table 3). Although the dose-response relationships are not clear at this time, such changes may reflect involvement of both PR and AR in the diminished uterine response to estrogen reported earlier, when higher doses of androgen were used [22, 23, 30, 43].

The female reproductive tract undergoes dramatic fluctuations of growth and regression during the reproductive cycle. Steroid hormones are key regulators of these processes [54]: estrogen functions as a mitogenic factor, whereas progesterone inhibits proliferation and apoptosis [55, 56]. Thus, changes in steroid receptor levels may be expected to be followed by changes in the rates of proliferation and/or apoptosis. Alternatively, early androgen exposure may directly affect the cellular machinery regulating proliferation and apoptosis in the adult. Our findings of decreased apoptotic index in the 0.2mg TP group and decreased proliferation indices in both the 0.1mg and 0.2mg groups at PND75 (see Table 3 and Fig. 3) are consistent with both of these possibilities. Further studies will be needed to decide which of the two mechanisms are involved.

A still larger issue that remains to be resolved is the molecular mechanism(s) by which inappropriate developmental exposure to androgens causes permanent functional-pathological changes in female reproductive tissues. Sexually dimorphic programing of tissues during development is well established and has been studied most extensively with regard to the hypothalamus [e.g., [57, 58]. An epigenetic mechanism has been proposed for PCOS [59] and examination of epigenetic markers after developmental exposure to diethylstilbestrol provided actual evidence of this possibility [60, 61]. Specific epigenetic markers in the uterus remain to be identified after environmental or experimental exposure to androgens during development.

In conclusion, early TP exposure in female rats, under these experimental conditions, causes changes in cellular and molecular parameters that are essential for normal uterine function in the adult. We are now evaluating the uterine response capacity to estradiol stimulation by uterotrophic test and assessing the sexual development and fertility from these early androgenized females to analyze the physiological meaning of these parameters.

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6. References

[1] Lucas A. Programming by early nutrition in man. In: Bock GR, Whelan J, editors. The childhood environment and adult disease, Chichester (UK): CIBA Foundation Symposium 156. Wiley; 1991, p.38-55.

[2] Silveira PP, Portella AK, Goldani MZ, Barbieri MA. Developmental origins of health and disease (DOHaD). J Pediatr 2007; 83(6):494-504.

[3] Guillette LJJr, Crain DA. Endocrine disrupting contaminants: an evolutionary perspective. Philadelphia: Taylor and Francis; 2000.

[4] McLachlan JA. Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocr Rev 2001; 22:319-41.

[5] Wilhelm D, Koopman P. The makings of maleness: towards an integrated view of male sexual development. Nat Rev Genet 2006; 7(8):620-31.

[7] Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA, Gray LEJr. Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study. Toxicol Sci 2002; 65:71-86.

[8] Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenbergh JG, Gray Jr LE. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol Sci 2007; 96(2):335-45.

[9] Welsh M, Sharpe RM, Walker M, Smith LB, Saunders PTK. New insight into the role of androgens in wolffian duct stabilization in male and female rodents. Endocrinology 2009; 150(5):2472-80.

[10] Parks LG, Lambright CS, Orlando EF, Guillette LJJr, Ankley GT, Gray LEJr. Masculinization of female mosquitofish in Kraft mill effluent-contamined Fenholloway River water is associated with androgen receptor agonist activity. Toxicol Sci 2001; 62:257-67.

[11] Orlando EF, Kolok AS, Binzcik GA, Gates JL, Horton MK, Lambright CS et al. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel sensitive species, the fathead minnow. Environ Health Perspect 2004; 112:353-8.

[12] Sáenz de Rodriguez CA, Bongiovanni AM, Conde de Borrego, L. An epidemic of precocious development in Puerto Rican children. J Pedriatr 1985; 107:393-6.

[13] Otten BJ, Stikkelbroeck MM, Claahsen-van Der Grinten HL, Hermus AR. Puberty and fertility in congenital adrenal hyperplasia. Endocr Dev 2005; 8:54-66.

[14] Blank SK, McCartney CR, Marshall JC. The origins and sequelae of abnormal neuroendocrine function in polycystic ovary syndrome. Hum Reprod Update 2006; 12:351-61.

[15] Greene RR, Burrill MW, Ivy AC. Experimental intersexuality. The effect of antenatal androgens on sexual development of female rats. Am J Anat 1939; 65:415-69.

[16] Slob AK, den Hamer R, Woutersen PJ, van der Werff ten Bosch JJ. Prenatal testosterone propionate and postnatal ovarian activity in the rat. Acta Endocrinol 1983; 103:420-7.

[17] Schwartz Z, Guest JF, Elder MG, White JO. Enzyme activities in the androgenized rat uterus refractory to oestrogenic stimulation. J Steroid Biochem 1986; 25:491-6.

[18] Ryan BC, Vandenbergh JG. Intrauterine position effects. Neurosci Biobehav Rev 2002; 26:665-78.

[19] Food and Drug Administration [FDA]. NADA summary #138-612. http://www.fda.gov/cvm/efoi/section1/138612070286.html; 1986. [acessed may.2011].

[20] Wilson VS, Lambright C, Ostby J, Gray LEJr. In vitro and in vivo effects os 17-beta-trenbolone: a feedlot effluent contaminant. Toxicol Sci 2002; 70:202-11.

[21] Bartol FF, Wiley AA, Bagnell CA. Uterine development and endometrial programming. Soc Reprod Fertil Suppl 2006; 62:113-30.

[22] Petrusz P, Flerkó B. Effect of ovariectomy and oestrogen administration on the pituitary and uterurs weight in androgen-sterilized rats. Acta biol Acad Sci hung 1967; 19(2):159-62.

[23] Lobl RT, Maenza RM. Androgenization: alterations in uterine growth and morphology. Biol Reprod 1975; 13:255-68.

[24] Csaba G, Inezefi-Gonda A, Dobozy O. Hormonal imprinting by steroids: a single neonatal treatment with diethylstilbestrol or allylestrenol gives rise to a lasting decrease in the number of rat uterine receptors. Acta Physiologica Hungarica 1986; 67:207-12.

[25] Clarke CL, Sutherland RL. Progestin regulation of cellular proliferation. Endocrine Rev 1990; 11:266-300.

[26] Kraus WL, Montano MM, Katzenellenbogen BS. Identification of multiple widely spaced estrogen-responsive regions in the rat progesterone receptor gene. Mol Endocrinol 1994; 8:952-69.

[27] Weihua Z, Ekman J, Almkvist A, Saji S, Wang L, Warner M et al. Involvement of androgen receptor in 17β-estradiol-induced cell proliferation in rat uterus. Biol Reprod 2002; 67:616-23.

[28] Okada A, Sato T, Ohta Y, Iguchi T. Sex steroid hormone receptors in the developing female reproductive tract of laboratory rodents. J Toxicol Sci 2005; 30(2):75-89.

[29] Kerr JF, Searle J. Deletion of cells by apoptosis during castration-induced involution of the rat prostate. Virchows Arch B Cell Pathol 1973; 13:87-102.

[30] Lobl RT. Andorgenization: alterations in the mechanism of oestrogen action. J Endocr 1975; 66:79-84.

[31] Gellert RJ, Lewis J, Pétra PH. Neonatal treatment with sex steroids: relationship between the uterotropic response and the estrogen “receptor” in prepubertal rats. Endocrinology 1977; 100(2):520-8.

[32] Campbell PS. Impaired prepubertal uterine capacity of response after neonatal exposure to steroid hormone esters. J Exp Zool 1980; 214:345-53.

[33] Hoepfner BA, Ward IL. Prenatal and neonatal androgen exposure interact to affect sexual differentiation in female rats. Behav Neurosci 1988; 102:61-5.

[34] Guerra MT, Scarno WR, Toledo FC, Franci JA, Kempinas WG. Reproductive developmental and function of female rats exposed to di-ɳ-butyl-phthalete (DBP) in utero and lactation. Reprod Toxicol 2010; 29(1):99-105.

[35] Guerra MT, Toledo FC, Kempinas WG. In utero and lactational exposure to fenvalerate disrupts reproductive function in female rats. Reprod Toxicol 2011; 32(3):298-303.

[36] Wolf CJ, LeBlanc GA, Ostby JS, Gray Jr L.E. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 2000; 55:152-61.

[37] Balaton AJ, Ochando F, Painchaud MH. Use of microwaves for enhancing or restoring antigens before immunohistochemical staining. Ann Pathol 1993; 13:188-9.

[38] Cuevas EC, Bateman AC, Wilkins BS, Johnson PA, Williams JH, Lee AHS et al. Microwave antigen retrieval in immunocytochemistry: a study of 80 antibodies. J Clin Pathol 1994; 47:448-52.

[39] Petrusz P, DiMeo P, Ordronneau P, Weaver C, Keefer DA. Improved immunoglobulin-enzyme bridge method for light microscope demonstration of hormone-containing cells of the rat adenohypophysis. Histochemistry 1975; 46:9-26.

[40] Gray LEJr, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N et al. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int J Androl 2006; 29:96-104.

[41] Bukovsky A, Ayala ME, Dominguez R, Keenan JA, Wimalasena J, McKenzie PP et al. Postnatal androgenization induces premature aging of rat ovaries. Steroids 2000; 65:190-205.

[42] Wolf CJ, LeBlanc GA, Gray LE Jr. Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat. Toxicol Sci 2004; 78:135-43.

[43] Wrenn TR, Wood JR, Bitman J. Oestrogen responses of rats neonatally sterilized with steroids. J Endocr 1969; 45:415-20.

[44] Lobl RT, Trotta P, Brumberger B Oestrogen-induced proteins and uterine growth in androgenized female rat. Endocr 1974; 60:371.

[45] Campbell PS, Modlin PS. Uterine glucose metabolism in the prepubertal rat treated neonatally with androgen, estrogen and antihormones. Experientia 1987; 43:309-10.

[46] Sahlin L, Masironi B, Åkerberg S, Eriksson H. Tissue-and hormone- dependent progesterone receptor distribution in the rat uterus. Reprod Biol Endocrinol 2006; 4:47.

[47] Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H et al. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 1990; 9:1603-14.

[48] Conneely OM, Lydon JP. Progesterone receptors in reproduction: functional impact of the A and B isoforms. Steroids 2000; 65:571-7.

[49] Kraus WL, Montano MM, Katzenellenbogen BS. Identification of multiple widely spaced estrogen-responsive regions in the rat progesterone receptor gene. Mol Endocrinol 1994; 8:952-69.

[50] White JO, Moore PA, Elder MG, Lim L. The relationship of the oestrogen and progestin receptors in the abnormal utero of the adult anovulatory rat. Effets of neonatal treatment with testosterone propionate or clomiphene citrate. Biochem J 1981; 196(2):557-65.

[51] Armstrong DT, Moon YS, Leung PC. Uterotrophic effects of testosterone and 5alpha-dihydrotestosterone in intact and ovariectomized immature female rats. Biol Reprod 1976; 15:107-14.

[52] Weihua Z, Ekman J, Almkvist A, Saji S, Wang L, Warner M et al. Involvement of androgen receptor in 17β-estradiol-induced cell proliferation in rat uterus. Biol Reprod 2002; 67:616-23.

[53] Hu YC, Wang PH, Yeh S, Wnag RS, Xie C, Xu Q et al. Subfertility and defective folliculogenesis in female mice lacking androgen receptor. Prc Natl Acad Sci USA 2004; 101(31): 11209-14.

[54] Kerr JF, Searle J. Deletion of cells by apoptosis during castration-induced involution of the rat prostate. Virchows Arch B Cell Pathol 1973; 13:87-102.

[55] Terada N, Yamamoto R, Takada T, Miyake T, Terakawa N, Wakimoto H et al. Inhibitory effect of progesterone on cell death of mouse uterine epithelium. J Steroid Biochem 1989; 33:1091-6.

[56] Sato T, Fukazawa Y, Kojima H, Enari M, Iguchi T, Ohta Y. Apoptotic cell death during the estrous cycle in hte rat uterus and vagina. Anat Rec 1997; 248:76-83.

[57] Robinson J. Prenatal programming of the female reproductive neuroendocrine system by androgens. Reproduction 2006; 132(4):539-47.

[58] Williams TM, Carroll SB. Genetic and molecular insights into the development and evolution of sexual dimorphism. Nat Rev Genet 2009; 10(11):797-804.

[59] Li Z, Huang H. Epigenetic abnormality: a possible mechanism underlying the fetal origino f polycystic ovary syndrome. Med Hypotheses 2008; 70(3):638-42.

[60] Li S, Washburn KA, Moore R, Uno T, Teng C, Newbold RR et al. Developmental exposure do diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 1997; 57(19):4356-9.

[61] Ho SM, Tang WY, Belmont de Fausto J, Prins GS. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer Res 2006; 66(11):5624-32.

7. Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

8. Legends of figures

Figure 1. Immunostaining of ERα (A-D), AR (E-H) and PR (I-L) in pubertal rat uterus (at estrus phase; one female offspring per litter) from groups exposed to corn oil (control group, *n*=8 animals – A, E, I), or to TP at doses of 0.05mg/kg (*n*=7 animals – B, F, J), 0.1mg/kg (*n*=9 animals – C, G, K) and 0.2mg/kg (*n*=10 animals – D, H, L). Observe nuclear staining at ERα and PR and cytoplasmic labeling at AR. Arrows represent alterations at luminal epithelium and asterisks, glandular epithelium. Final magnification, 600X.

Figure 2. Immunostaining of ERα (A-D), AR (E-H) and PR (I-L) in adult rat uterus (PND 75, at estrus phase; one female offspring per litter) from groups exposed to corn oil (control group, *n*=8 animals – A, E, I), or to TP at doses of 0.05mg/kg (*n*=7 animals – B, F, J), 0.1mg/kg (*n*=9 animals – C, G, K) and 0.2mg/kg (*n*=10 animals – D, H, L). Observe nuclear staining at ERα and PR and cytoplasmic labeling at AR. Arrows represent alterations at luminal epithelium and asterisks, glandular epithelium. Final magnification, 600X.

Figure 3. Apoptotic index (percentage of stained cells) in uterine compartments, in one female offspring per litter, at estrus phase, whose mothers were exposed to vehicle (Control, *n*=8 animals) or TP at 0.05mg/kg (*n*=7 animals), 0.1mg/kg (*n*=9 animals) and 0.2mg/kg (*n*=10 animals). A: at puberty onset; B: at PND75. Values are expressed as median (Q_1-Q_3). Kruskal-Wallis with an a posteriori Dunn (Bonferroni) test. **p<0.01; ***p<0.001.

Figure 1

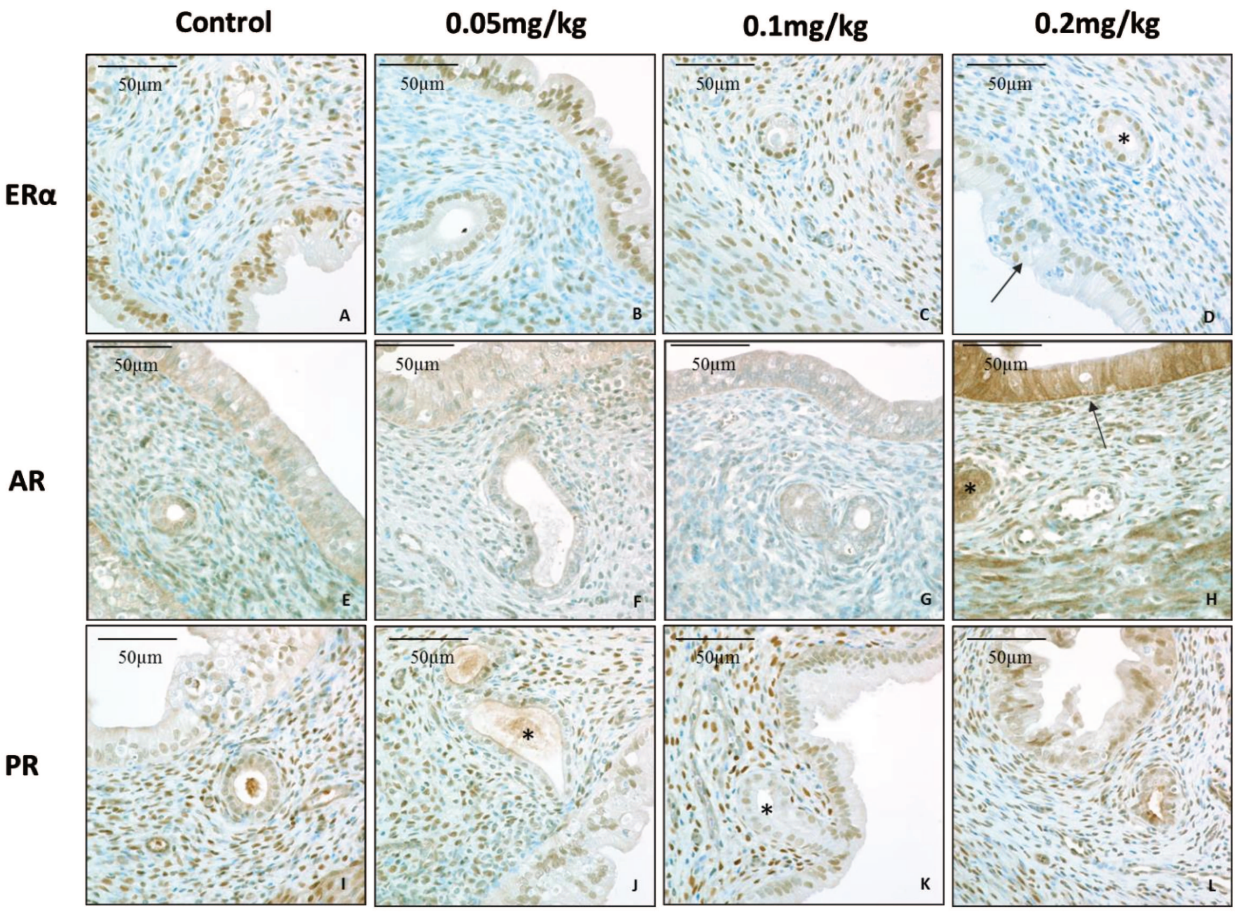

Figure 2

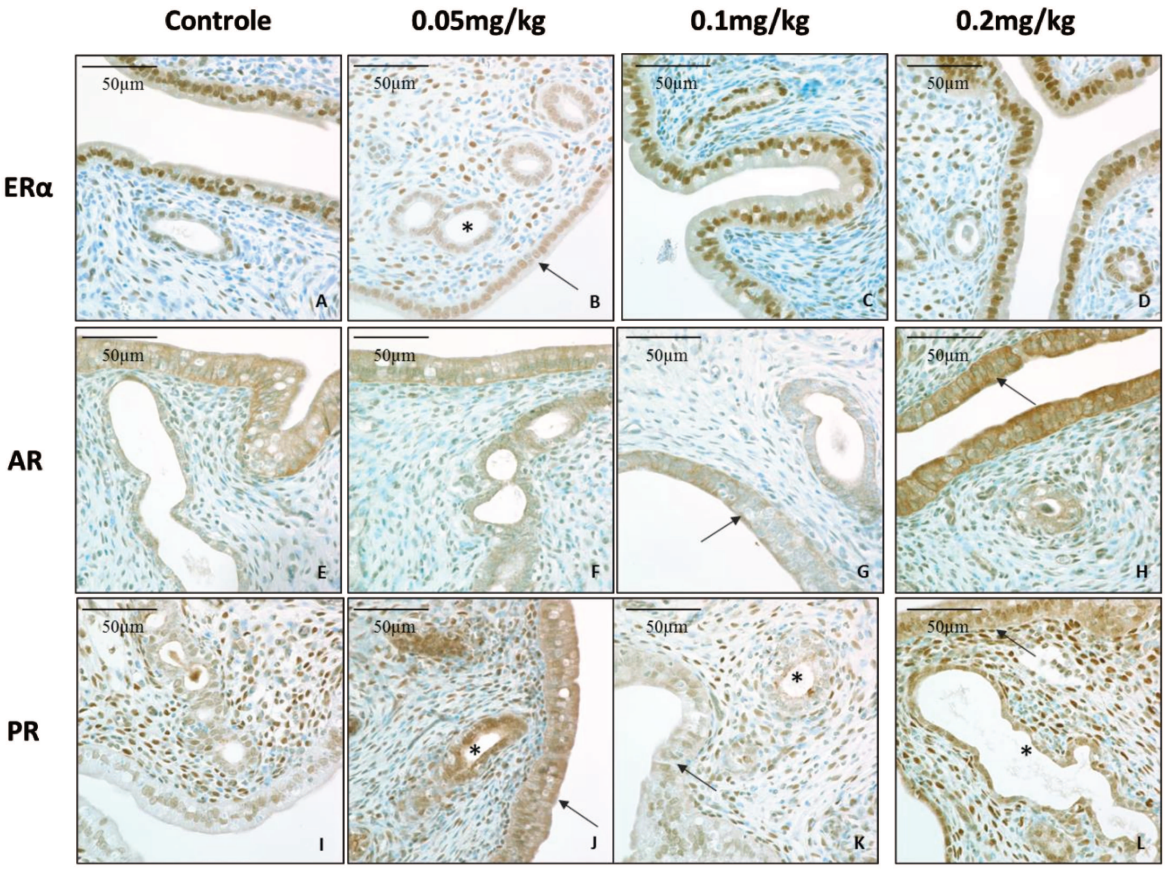
Controle
0.05mg/kg
0.1mg/kg
0.2mg/kg
ERα
AR
PR
50µm
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3

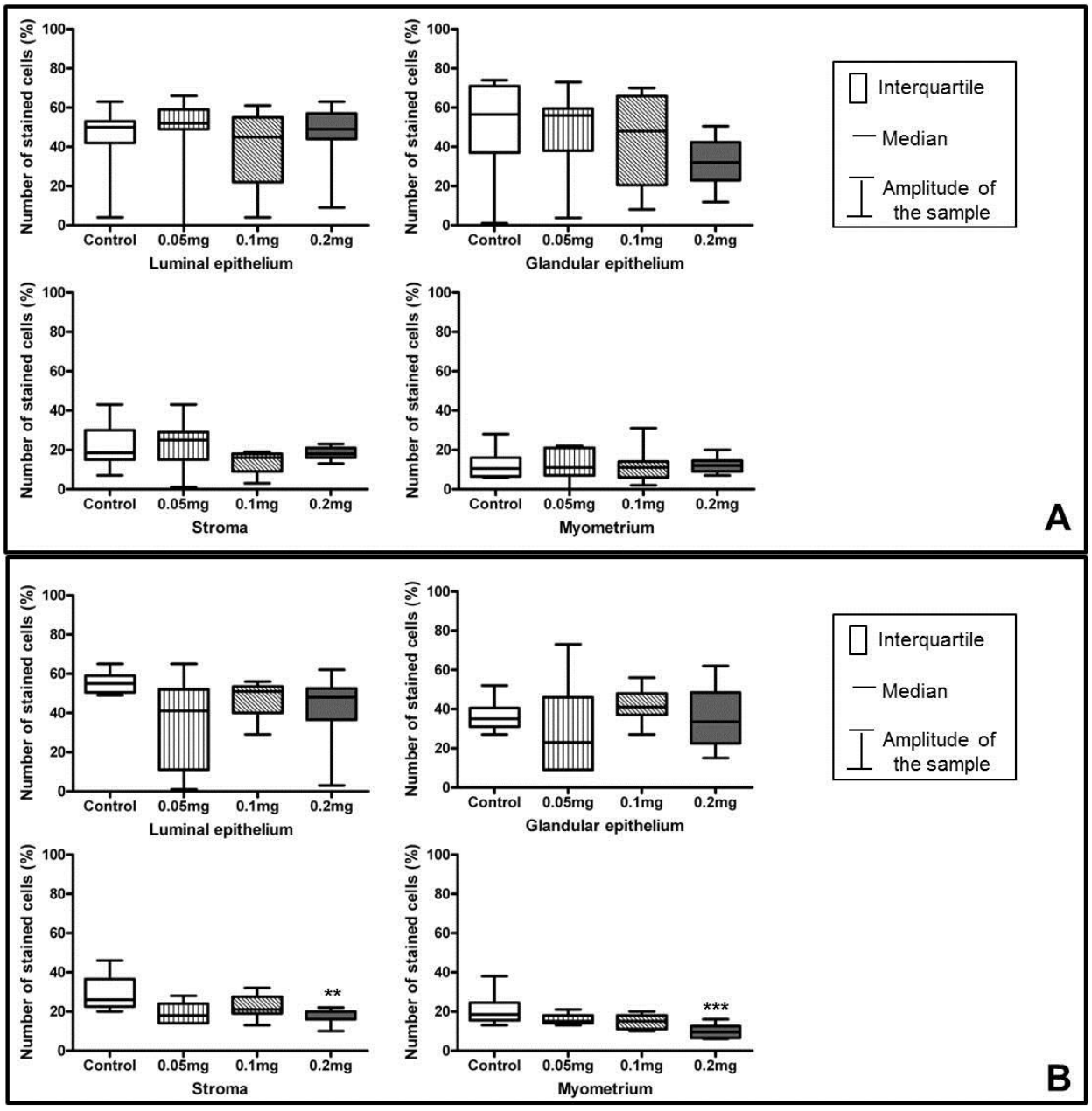
Number of stained cells (%)
Control
0.05mg
0.1mg
0.2mg
Luminal epithelium
Glandular epithelium
Stroma
Myometrium
Interquartile
Median
Amplitude of the sample
A
B
**
***

Table 1. Body and uterine weights, at estrus phase, after puberty onset and PND75, from control and TP-treated groups.

Parameters	Puberty onset			
	Control (n=8 animals)	**0.05mg (n=7 animals)**	**0.1mg (n=9 animals)**	**0.2mg (n=10 animals)**
Body weight (g)	117.14 ± 3.96	112.20 ± 4.34	114.01 ± 5.24	105.99 ± 3.01
Uterine weight + fluid (mg)	211.90 ± 12.65	214.10 ± 19.83	231.40 ± 26.83	175.1 ± 5.51
	PND75			
	Control (n=8 animals)	**0.05mg (n=7 animals)**	**0.1mg (n=9 animals)**	**0.2mg (n=10 animals)**
Body weight (g)	222.00 ± 4.97	225.63 ± 4.11	218.16 ± 5.05	215.37 ± 3.42
Uterine weight + fluid (mg)	417.93 ± 14.15	529.23 ± 92.63	392.24 ± 22.73	414.96 ± 43.03

Values are expressed as mean ± S.E.M. ANOVA with a *posteriori test* of Dunnet.

Table 2. Summary of the results from immunohistochemistry analysis. Data from groups treated with TP were evaluated together with control group.

Antibody	Uterine Compartmet	Puberty			PND75		
		0.05mg/kg	**0.1mg/kg**	**0.2mg/kg**	**0.05mg/kg**	**0.1mg/kg**	**0.2mg/kg**
Erα	Luminal	n.o.e.	n.o.e.	↓	↓	n.o.e.	n.o.e.
	Glandular	n.o.e.	n.o.e.	↓	↓	n.o.e.	n.o.e.
AR	Luminal	n.o.e.	n.o.e.	↑	n.o.e.	↓	↑
	Glandular	n.o.e.	n.o.e.	↑	n.o.e.	↓	n.o.e.
PR	Luminal	n.o.e.	n.o.e.	n.o.e.	↑	↓	↑
	Glandular	↓	↓	n.o.e.	↑	↓	↑

n.o.e= no observed effect – similar to control group; ↑: increase of staining when compared to control group; ↓ decrease of staining when compared to control group.

Stroma and myometrium are not present at this table due to absence of alterations in all tested doses when compared to control group.

Table 3. Percentage of proliferative cells by PCNA immunohistochemistry, on uterine tissue from female rats at estrus phase, on puberty onset and at PND75, from control and TP-treated groups.

	Puberty onset			
Uterine Compartment	**Control (*n*=8 animals)**	**0.05mg (*n*=7 animals)**	**0.1mg (*n*=9 animals)**	**0.2mg (*n*=10 animals)**
Luminal epithelium	14.50 (8.50 - 16.75)	9.00 (8.00 - 11.00)	11.00 (8.00 - 12.00)	12.00 (8.75 - 15.5)
Glandular epithelium	23.50 (20.00 - 28.75)	18.33 (17.00 - 24.00)	21.84 (20.00 - 24.00)	25.00 (19.00 - 33.00)
Stroma	35.00 (31.75 - 37.50)	38.00 (30 - 44.50)	27.00 (25.00 - 31.00)	26.50 (21.00 - 35.75)
Myometrium	31.00 (26.50 - 38.50)	29.00 (27.50 - 34.50)	28.00 (26.00 - 32.00)	29.00 (23.75 - 32.75)
	PND75			
	Control (*n*=8 animals)	**0.05mg (*n*=7 animals)**	**0.1mg (*n*=9 animals)**	**0.2mg (*n*=10 animals)**
Luminal epithelium	18.50 (15.5 - 22.00)	18.00 (13.00 - 25.50)	18.00 (16.00 - 21.00)	20.00 (15.25 - 24.50)
Glandular epithelium	18.50 (15.75 - 22.25)	24.00 (18.50 - 29.00)	18.00 (16.00 - 18.00)	17.00 (14.50 - 21.50)
Stroma	31.50 (28.88 - 35.50)	28.00 (23.00 - 36.50)	26.00 (24.00 - 30.00)	28.00 (27.25 - 32.25)
Myometrium	29.50 (27.75 - 33.00)	27.00 (25.00 - 29.00)	23.00 (19.00 - 25.00)*	23.50 (21.00 - 26.75)*

Values are expressed as median (Q_1-Q_3). Kruskal-Wallis with a *posteriori test* of Dunn. *p<0.05.

5.2.Manuscrito 2

O segundo manuscrito é intitulado "*Androgenic exposure during critical periods of development and reproductive health effects on the female rat offspring*", que será submetido à revista Reproductive Toxicology, USA, Elsevier, ISSN:0890-6238, Fator de Impacto: 3,226.

ANDROGENIC EXPOSURE DURING CRITICAL PERIODS OF DEVELOPMENT AND REPRODUCTIVE HEALTH EFFECTS ON THE FEMALE RAT OFFSPRING

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ABSTRACT

Environmental contaminants known as endocrine disrupting compounds (EDCs) have been associated with adverse effects on the reproductive processes. They may mimic or antagonize endogenous hormones, disrupting the reproductive system. Although first researches have focused on environmental estrogens, the presence of compounds with androgenic activity has been described recently. The present study aimed to evaluate the exposure of female pregnant and lactating rats to low doses androgen and assesses the possible effects on the female offspring. Pregnant Wistar rats were exposed to testosterone propionate (TP) at doses of 0.05mg/kg, 0.1mg/kg, 0.2mg/kg or corn oil (vehicle), s.c., from gestational day 12 until gestational day 20 or the end of lactation to evaluate the possible effects on the reproductive health of female offspring. Perinatal exposure to TP caused an increased anogenital distance after birth and diminished height of uterine glandular epithelium at puberty in animals exposed to 0.2mg/kg. At 0.05mg/kg, TP caused an increase in estradiol levels at 75-days old animals. These alterations, however, were not enough to impair sexual differentiation and normal physiology of the reproductive tract from these animals.

Key words: Female reproduction, rat, androgens, uterus, ovary, estrous cycle.

1. INTRODUCTION

Since the 1990s, there has been a rising scientific and regulatory interest in environmental chemicals capable of interfering with the endocrine systems of wildlife species and humans [1]. These substances, known as endocrine disrupting compounds (EDCs), have been associated with adverse effects on the populations and reproductive processes [2-6]. They may mimic or antagonize endogenous hormones, disrupting the reproductive system [6]. A number of substances with endocrine activity are known to cause adverse reproductive effects in humans following exposure during pregnancy, occupational and accidental exposures [7].

Exposure of developing tissues to an adverse stimulus or insult can permanently reprogram normal physiologic responses, and may give rise to metabolic and hormonal disorders later in life [8-12]. The developing fetus and children are uniquely sensitive to the effects of biohazards environmental toxicants that alter endocrine function [13].

Although first researches has focused on environmental estrogens, androgenic activity has been described in water from kraft pulp and paper mills and concentrated animal feed operations in the U.S. and Europe [14-17]. The presence of androgens of anthropogenic origin in the environment was only confirmed within the last few years [18].

The female reproductive tract has been shown to be a target for developmental programming as a result of environmental hormone exposure [19]. Human females exposed prenatally to higher than normal levels of androgens, may develop ambiguous internal and external reproductive organs, male-like external genitalia and male play behavior [20-23]. In human species, prenatal or postnatal exposure to androgen causes alterations of personality, gender-related behavior and sexual orientation [24-27].

Previous studies demonstrated that exposure of experimental animals to high levels of androgens during the perinatal age causes permanent morphological, biochemical, and functional alterations in several organs and systems [28, 29]. Prenatal exposure of female rodents to exogenous androgens results in both physiological and behavioral masculinization although the effects vary with the timing of exposure [30-32].

Testosterone propionate (TP), among others compounds with androgenic activity, masculinize and defeminize female rats producing a partial male phenotype, absence of

vagina and presence of male sex accessory tissues [30, 31, 33-35]. Besides, prenatal administration of exogenous androgens to the rat induces in the female delayed puberty, early constant estrus, and delayed anovulatory syndrome [30, 36-40].

Uterine tissues can be epigenetically reprogramed during perinatal life [41] and androgen exposure can compromise the endometrial adenogenesis [42] and decrease uterine response to estrogenic stimulus [43-46]. In the ovary, disturbance in the initial steps of ovarian differentiation also results in incomplete sexual development and may contribute to childhood and adult diseases [47].

Previous work from our lab demonstrated that female offspring whose mothers were exposed to TP during gestational and lactational life presented alterations in the pattern of expression of uterine steroidal receptors and proliferative/death cell index [48] and such alterations may suggest an influence in the physiological function of the uterus.

Literature shows that most part of the studies are performed using relatively high doses and short periods of treatment [31, 32, 49, 50]. The present study, on the other side, aims to evaluate the exposure of female pregnant and lactating rats to low doses of TP and assesses the possible effects on the female offspring. TP has been chosen to act as an environmental contaminant with androgenic action.

2. MATERIAL AND METHODS

2.1 Animals and treatment

Adult female (60 days of age) and male (90 days of age) Wistar rats were supplied by Central Biotherium of State University of São Paulo (UNESP) and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu. Animals were housed in polypropylene cages (43cm×30cm×15 cm) with laboratory grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1◦C) and lighting conditions (12:12h photoperiod). Standard rodent chow and filtered tap water were provided *at libitum*. Two non-gravid female rats were mated with one male, during the dark portion of the lighting cycle, and the day of sperm detection in the vaginal smear was considered day 0 of gestation (gestational day – GD 0). The gravid females were randomly assigned among the experimental groups and housed

individually in cages. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol no. 104/2009-CEEA).

In order to perform the analysis of maternal endpoints and fetal gonads, gravid rats were administered either corn oil (Control, *n*=7) or testosterone propionate (TP- DEG Ltd., CAS 57-85-2, 97% of purity) at doses of 0.05mg/kg (*n*=9), 0.1mg/kg (*n*=9) or 0.2mg/kg (*n*=8), subcutaneously, from GD 12 until GD 20, when the dams were sacrificed and caesarean section was performed. Reproductive performance data were assessed and the gonads of one female offspring from each litter were collected for histological analysis.

An additional group of pregnant rats (Control, *n*=8; 0.05mg/kg, *n*=7; 0.1mg/kg, *n*=9; 0.2mg/kg, *n*=10) were exposed to either TP or vehicle from GD 12 to the end of lactation (post-natal day 21—PND 21) to evaluate the possible effects of the treatment on the reproductive function of the female offspring. The beginning of the mothers' treatment coincided with the critical period of reproductive system development, which continues after birth [6, 51]. Dams were weighed on alternate days, during the treatment period to calculate the volume of TP to be administered and to evaluate possible signs of maternal toxicity. On the next day after the last administration of TP, pups were weaned (PND 22) and the respective mothers were euthanized by CO_2 inhalation followed by decapitation.

2.2 Evaluation of maternal endpoints and histological analysis of fetal gonads

Pregnant rats from control (*n*=7) and treated with different doses of TP (0.05mg/kg=9; 0.1mg/kg=9; 0.2mg/kg=8) were killed by CO_2 inhalation followed by decapitation on GD 20. After the uterus and ovaries were removed, the number of corpora lutea were examined by gross morphology, and the number of implantation sites, resorptions and live and dead fetuses were recorded. From female fetuses (one per litter) the ovaries were collected, fixed in Karnovsky (2.5% glutaraldehyde, 8% paraformaldehyde), included in historesin, processed for histological analysis, stained with hematoxylin and eosin and germ cells were counted, in 3 non-consecutive sections per animal (5µm - 50 µm of distance among them). Using a light microscope (Leica

microscope DMLB 100x) coupled to a digital camera and a PC with software Leica Q-Win (version 3 for Windows TM), the total area of each section was measured and pictures were obtained to proceed germ cell counting. Results were expressed as number of cells per unit area (no. germ cells/mm^2).

2.3 Analysis of the female offspring

2.3.1 Anogenital distance (AGD) and nipple counting

On PND 1 (considering the day of birth as PND0), the anogenital distance (AGD, the distance between the anus and the genital tubercle) was measured in female pups, using a manual pachymeter and results were expressed as mm / $\sqrt[3]{g}$ (relative AGD) [52-54]. On PND 13, the number of nipples/areolas was recorded. Observations were scored based on the presence or absence of a nipple bud or a discoloration of the skin surrounding the nipple [55].

2.3.2 *External Signs of puberty onset*

Beginning on PND 30, all female pups were evaluated daily for complete vaginal opening (V.O.). At the momento of V.O., female rats were weighted and daily vaginal fluid was collected as described by Marcondes et al. [56], to detect the day of first estrus. 10 µL of 0.9% saline was instilled into the vagina and subsequently aspirated. Vaginal fluids were placed in a slide and analyzed under a light microscopy (Leica MicroStar IV) at 200×magnification. The estrus phase was characterized by the predominance of cornified epithelial cells.

2.3.3 Estrous cycle

On PND 60, in all experimental groups, the estrous cyclicity of female rats was assessed on cells from daily vaginal fluid, collected over a period of 15 days (as described previously by first estrus detection) and the estrous cycle phase was determined by cytology: predominance of nucleated epithelial cells (proestrus); predominance of cornified epithelial cells (estrus); the presence of cornified and nucleated epithelial cells and leukocytes (metaestrus); predominance of leukocytes (diestrus). The total frequency of each

phase for every rat observed in this period was used to calculate the total length of the proestrus, estrus, metaestrus and diestrus (in days), the estrous cycle length and the number of cycles during the evaluated period.

2.3.4 Collection and analysis of organs

At puberty onset and at PND 75, one female rat from each litter was euthanized by CO_2 inhalation followed by decapitation, between 8:00 and 10:00 a.m., on estrus phase. Pituitary, liver, kidneys, ovaries and uteri (with fluid) were collected and weighed on a precision balance. The histological evaluation was performed on the reproductive organs; uterus and ovaries were fixed in Alfac's solution, dehydrated in ethanol and embedded in paraplast. Three sections (5 µm) per animal, separated by 50 µm distance, were obtained, mounted on glass slides and stained with hematoxylin and eosin.

In each ovary, ovarian follicles and corpora lutea were counted and the area of each section was measured. To obtain the total area of each section, all the slides were scanned by a digital light microscope (COOLSCOPE II, Nikon Inc., Japan) and the measurements were performed at Image J 1.45e software. Follicles were classified according to Borgeest et al. [57] and Talsness et al. [58]. Primordial and primary follicles were enumerated together; oocytes surrounded by a single layer of either squamous or cuboidal epithelial cells were included. Follicles were classified as preantral when containing 2–4 layers of granulosa cells with no antral space. Antral follicles were classified when containing three or more layers of granulosa cells and a clearly defined antral space. Characteristics of atretic follicles included pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte and detachment from the basement membrane. The results were divided by the total area of the section.

In the uterus, morphometrical analyses were made to assess the height of endometrium, luminal and glandular epithelium. For this, a Leica microscope DMLB (100×) coupled to a digital camera and a PC with software Leica Q-win (version 3 for WindowsTM) were used. In each section, five different regions were analyzed, resulting in a total of 15 measurements per animal by each parameter. The number of glands presented in each section was also recorded.

2.3.5 Hormonal analysis

After decapitation, trunk blood was collected and allowed to clot on a refrigerator (4 ◦C) for 30 min. Serum was collected after centrifugation (20 minutes at 2.400rpm) and stored at −20◦C until analysis. Serum FSH, LH, estrogen, progesterone and testosterone concentrations were measured using a double-antibody radioimmunoassay (RIA) kit (National Institute of Arthritis, Diabetes and Kidney Diseases–NIADDK, USA). All the samples were analyzed at the same assay to avoid inter-assay variability.

2.3.6 Sexual behavior

During the first proestrus after PND 100, females from control and treated with different doses of TP groups, one or two per litter, were used for the mating test. After the end of estrous cycle analysis, rats were maintained in controlled temperature conditions under an inverted 12h light–dark cycle, for at least two weeks for acclimation. To evaluate female sexual behavior, sexually experienced male rats were allowed ten mounts on the female and the presence of lordosis was measured. Results were expressed as the lordosis quotient (LQ, number of lordosis/ten mounts × 100). All females were used only once.

2.3.7 Fertility performance

This analysis was performed by natural mating. Female offspring (PND 100, immediately after sexual behavior test) from control and treated groups (one or two per litter) were placed with sexually experienced males (1:1), for up to 3 sexual cycles (3 proestrus), in the beginning of the morning, during a dark period of the cycle. At the end of the afternoon, males and females were separated and vaginal smears were collected, in which initial sperm detection was determined to be GD 0. On GD 20 females were euthanized by CO_2 inhalation followed by decapitation. After the uterus and ovaries were collected, the numbers of corpora lutea (by gross morphology), implantation sites, early or late resorptions, live fetuses and fetal weights were determined. Early resorption is defined as a conceptus in which it is not grossly evident that organogenesis has occurred; a late resorption is a fetus which it is grossly evident that organogenesis had occurred. A live fetus is defined as one that responds to stimuli; a dead fetus is defined as a term fetus not

demonstrating marked to extreme autolysis. Fetuses with extreme autolysis are considered to be late resorption [59]. From these results, the following parameters were determined: the mating index: number of females with mating/number of cohabitated females × 100; the gestation rate: number of pregnant females/number of inseminated females × 100; implantation rate (efficiency of implantation): implantation sites/corpora lutea × 100; pre-implantation loss rate: number of corpora lutea − number of implantations/number of corpora lutea × 100; post-implantation loss rate: number of implantations − number of live fetuses/number of implantations × 100; sex ratio: number of male fetuses/number of female fetuses × 100.

2.4 Statistical analysis

Values are expressed as mean ± S.E.M. and medians (Q1 − Q3), according to the characteristics of each variable. For comparison of inter-group results, Anova with a *post hoc* Dunnet or Kruskall-Wallis with *a post hoc* Dunn were utilized. Differences were considered significant when $p < 0.05$ and the litter was utilized as the statistical unit. The statistical analyses were performed by GraphPad InStat (version 3.02).

3. RESULTS

None of the experimental groups showed visible signs of toxicity during pregnancy or lactation periods. At the tested doses, TP treatment had no effects on maternal body weight or body weight gain, during pregnancy and lactation and the maternal endpoints recorded at GD 20 were also comparable among experimental groups (data not shown). The fetal gonad analysis revealed no significant difference in relation to germ cells number, as shown in figure 1.

On PND 1, the analysis of the number of delivered pups (males and females) from all pregnant rats did not differ among experimental groups (control=11.33±0.76; 0.05mg/kg=9.86±0.70; 0.1mg/kg=11.44±0.71; 0.2mg/kg=11.50±0.70; Anova with a *post hoc* Dunnet). The body weight of female offspring from all TP treatments was similar to the control group but the relative AGD, on the other side, was significantly increased by the

treatment with 0.2mg/kg of TP. At PND 13, the evaluation of the number of nipples showed no difference among experimental groups (table 1).

Although the 0.2mg/kg group presented lower body weight, the evaluation of the external sings of puberty onset (V.O. and first estrus) showed that TP treatment, at any tested dose, was not able to interfere in this parameter (figure 2). In the same manner, hormonal analyses, organs weight and AGD, after puberty onset and at PND 75, showed no alterations after maternal TP exposure on the female offspring, with exception of a decrease in the weight of kidneys from 0.2mg/kg group at PND 75 and and an increase of 65.8% of estrogen levels in female rats from 0.05mg/kg group at 75 days of age (table 2 and 3). The estrous cycle regularity, evaluated from PND 60 to PND 75, also showed that all TP-treated animals presented no alterations in the pattern of estrous cyclicity when compared to control animals (table 4).

In order to evaluate the histology of reproductive organs, uterus and ovary were analyzed. Regarding the uterus, the height of endometrium and luminal epithelium and number of glands were similar among experimental groups (figure 3 and table 5), but the height of glandular epithelium from 0.2mg/kg group at puberty was significant diminished by the treatment with TP (figures 3 and 4). The assessment of the number of follicles or corpora lutea per area of the ovary revealed no alterations in TP-treated animals when compared to control (figure 5).

At PND 100, in proestrus, the sexual behavior was assessed followed by the fertility performance. The lordosis quotient (LQ) was similar among all experimental groups (figure 6) and parameters evaluated by the fertility test showed no alterations in pregnancy period, fetuses intrauterine growth and implantational indexes (table 6).

4. DISCUSSION

Recent work on endocrine disruptors has demonstrated the existence of environmental substances with androgenic actions are potential cause of abnormal fetal androgenization [18, 60, 61]. This present study demonstrated that, in this experimental condition, administration of TP to pregnant rats during gestation and lactation in relatively low doses, despite causing increased AGD only at PND 1 and diminished uterine glandular

epithelium height at 0.2mg/kg and increased estradiol levels at 0.05mg/kg, causes no alteration in female offspring reproductive function in rats.

Although previous researchers pointed to reduced maternal gestational body weight, litter size and body weight of offspring when high doses of androgenic compound is used [31, 32, 62, 63],this experiment showed that the body weight gain from dams and size/weight of the offspring were not altered by TP treatment.

The AGD and number of nipples, considered biomarkers to androgen exposure [64, 65], may be impaired by female testosterone exposure during intrauterine life. Wolf et al. [31, 66] and Hotchkiss et al. [32] showed a significant increase in the AGD and reduction in nipple counting in female pups after androgen administration. Thus, TP can be considered to cause partial male phenotype (indicated by increased AGD) [35] and it was confirmed in our study, whereas female pups receving 0.2mg/kg of TP presented a longer AGD at PND 1, when compared to control group. On the other side, in this present study, the treatment with TP during gestation and lactation did not cause adverse effect in nipple counting, suggesting that the levels of testosterone that reached the developmental fetuses were not enough to prevent nipple formation in female offspring [31,67, 68].

The stablishment of puberty in rats results from a cascade of events that defines the pattern of liberation of LH, after the fourth week post-natal (approximately 30 days of age) that leads to ovarian maturation [69]. After that, blood levels of estradiol increases and puberty onset occurs. The age of vaginal opening and first estrus are external indicative of puberty installation [70, 71] and before this period, female reproductive tract is considered inactive [72].

Depending on the dose and time of exposure, androgens may compromise the puberty onset in female rats. Hotchkiss et. al. [32, 60] exposed female rats to androgen compounds during intrauterine period at doses of 1,5mg/kg and 2mg/day and and found a delay in the vaginal opening, agenesis of vaginal orifice and hypospadias. Doses as 250 µg of TP, on the day of birth, prevents vaginal opening [44]. At doses as 0.1 and 0.5mg/day, intrauterine exposed females presented no alterations in this parameter [31]. In our experiment, all experimental groups showed similar values in the vaginal opening and first

estrous evaluation, indicating that TP exposure at low doses is not able to alter puberty in females.

The endocrine secretions of hypothalamus, pituitary and gonads control the reproductive system [56, 73, 74]. Exposure to androgens may alter the release of GnRH (gonadotropin release hormone), and then, affect the secretion of gonadotropins and steroid hormones. Additionally, androgens may modify hypothalamus and pituitary response to ovarian steroids feedback, leading to a disruption in the secretion of GnRH and LH [75-79].

In the hormonal levels analysis, we observed, despite not significant, an increase of 65.8% in the serum levels of estrogens at PND 75 from 0.05mg/kg group. This group presented lower immunointensity in the uterine estrogen receptor alpha in previous study [48] and this increased estrogen levels may suggest a compensatory mechanism due to lack of these receptors.

Even with this alteration in the estrogenic levels, others hormones as LH, FSH, progesterone and testosterone were not altered, and these results togheter with normal estrous cycle and pituitary weight shows that the doses of TP used in this experiment were not capable of interferes with sexual cycle. The extreme low levels of detected testosterone may explain the cytoplasmic staining found in our previous work [48]. Apparently this amount of androgens was not able to bind or activate androgen receptors in the uterine tissues.

In female mammals, reproductive capacity is limited by the size of the non-renewable pool of oocytes, which is established during the fetal life [80]. The follicular formation during fetal development and the activation of follicles during post-natal period are potential target to hormonal alterations [42], and then, pre-natal exposure to androgens may impact foliculogenesis [81].

Exposure to excess of androgens during fetal or pre-puberty life may cause alterations in the ovary [82-84] as multifollicular morphology [85, 86], increased follicular recruitment [86, 87] and follicular persistence [88, 89]. Sotomayor-Zárate et al [90] showed that exposure to TP at dose of 1.0mg to female rats during the first 12 hours of life causes reduction of ovarian follicles number, increases the cystic follicles and causes absence of corpora lutea.

Germ cell counting in the fetal gonad, as well as the weight of ovaries and the morphological analysis of this organ revealed absence of alterations, suggesting that the female gonad was not susceptible to TP action in these experimental doses.

The principal period of differentiation and maturation from rodent uterus occurs after birth [91] and the exposure to steroid hormones in this period may compromise the endometrial adenogenesis [42]. Endocrine disruptors with androgenic activity also may cause uterine abnormalities when administered to female rats during intrauterine period [31, 46, 60, 78].

The uterus from all TP treated rats did not show any sign of distention or fluid retention and the weight of this organ was comparable to control group. The histological analyzes also demonstrated no alterations in TP-treated animals. The decreased height of glandular epithelium at puberty from 0.2mg/kg group was reversed at PND75 and did not represent impairment to the uterine physiology.

Some compounds, as endocrine disruptors, administered to pregnant females or neonates, may interfere with the hypothalamic sexual differentiation of the offspring [92, 93]. Female rats exposed to testosterone during perinatal period presented an increased expression of typically male sexual behavior [94, 95]. According to Hoepfner e Ward [49], TP treatment at 2mg during gestation followed by a single dose of 5µg of TP at birth, did not cause alterations in the lordosis coefficient in the female offspring. In our experiment, the treatment with different doses of TP during intrauterine and lactation periods were not capable to cause significant alterations in the pattern of female sexual behavior, expressed by LQ. Nevertheless, the experimental group exposed to 0.2mg/kg of TP showed a more variable pattern in this response when compared to other animal, suggesting a possible evidence of damage in the hypothalamic sexual differenctiation process.

The abnormal uterine development may compromise gestation due implantation impairment [96, 97]. Kramen [98,99] demonstrated that female rats exposed to 50 µg of TP on PND 5 presented normal uterine decidual response and sensibility to blastocyst implantation. Our results showed that TP-treated female rats, besides normal uterine morphology, presented regular implantations and normal fetal and placental development.

Although our previous study has shown that exposure to TP, at the same doses used in this experiment, is capable of cause alterations in the levels of steroid receptors and proliferation/death cell index in the uterine tissue from female rats whose mothers were treated during gestation and lactation, this present work reveled that these changes were not able to impair sexual differentiation and normal physiology of the reproductive tract from these animals.

5. ACKNOWLEDGMENTS

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6. REFERENCES

[1] Colborn T, Clement C. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Advances in Modern Toxicology, XXI. Princeton Publishing Co. Inc., Princeton, NJ.2002.

[2] Gibbs PE, Pascoe PL, Bryan GW. Tributyltin-induced imposex in stenoglossan gastropods: Pathological effects on the female reproductive system. Comp Biochem Physiol 1991; 100:231-6.

[3] Moore CG, and Stevenson JM. The occurrence of intersexuality in harpacticoid copepods and its relationship with pollution. Mar Pollut Bull 1991; 22:72-4.

[4] Facemire CF, Gross TS, Guillette LJ Jr. Reproductive impairment in the Florida panther: Nature or nurture? Environ Health Perspect 1995; 103(4):79-86.

[5] Feyk LA, and Giesy JP. Xenobiotic modulation of endocrine function in birds. In: Kendall R, Dickerson R, Giesy J, Suk W, editors. Principles and Processes for Evaluating Endocrine Disruption in Wildlife. Pensacola, Fl: Setac Press; 1998.p. 121-40.

[6] Wolf CJ, LeBlanc GA, Ostby JS, Gray Jr LE. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 2000; 55:152-61.

[7] Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, et al. Effects of environmental antiandrogens on reproductive development in experimental animals. Hum Reprod Update 2001; 7: 248-64.

[8] Frankel S, Elwood P, Sweetnam P, Yarnell J, Smith GD. Birthweigth, body-mass index in middle age, and incident coronary heart disease. Lancet 1996; 348:1478-80.

[9] Hattersley AT, Tooke JE. The fetal insulin hypothesis: an alternative explanation of the association of low birthweight with diabetes and vascular disease. Lancet 1999; 353: 1789-92.

[10]Barker DJ. Fetal programming of coronary heart disease. Trends Endocrinol Metabol 2002; 13:364-8.

[11] Couzin J. Quirks of fetal environmental felt decades later. Science 2002; 296:2167-9.

[12] Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. Science 2004; 305: 1733-6.

[13] Foster GW. Fetal and early postnatal enviromental contaminant exposures and reproductive health effects in the female. Fertil Steril 2008; 89(1):e53-e54.

[14] Parks LG, Lambrigth CS, Orlando EF, Guillette LJJr, Ankley GT, Gray LEJr. Masculinization of female mosquitofish in Kraft mill effluent-contamined Fenholloway River water is associated with androgen receptor agonist activity. Toxicol Sci 2001; 62:257-67.

[15] Orlando EF, Kolok AS, Binzcik GA, Gates JL, Horton MK, Lambrigth CS et al. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel sensitive species, the fathead minnow. Environ Health Perspect 2004; 112:353-8.

[16] Radl V, Pritsch K, Munch JC, Schloter M. Structural and functional diversity of microbial communities from a lake sediment contaminated with trenbolone, an endocrine-disrupting chemical. Environ Pollut 2005; 137(2): 345-53.

[17] Durhan EJ, Lambright CS, Makynen EA, Lazorchak J, Hartig PC, Wilson VS et al. Identification of metabolites of trembolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect 2006; 114(1): 65-8.

[18] Gray LEJr, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N et al. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int J Androl 2006; 29:96-104.

[19] Woodruff TK, Walker CL. Fetal and early postnatal environmental exposures and reproductive health effects in the female. Fertil Steril 2008; 89(1):e47-e51.

[20] Ammini AC, Sunderaraman PG, Gupta R, Karmarkar MG, Buckshee K, Ahuja MM. Congenital adrenal hyperplasia among peripubertal girls with hyperandrogenism. Indian Pedriatr 1992; 29:79-83.

[21] Schardein JL. Hormones and hormonal antagonists. In: J. Schardein, editor. Chemically Induced Birth Deffects. Marcel Dekker, New York; 1993. p.271-339.

[22] New MI, Wilson RC. Steroid disorders in children: Congenital adrenal hyperplasia and apparent mineralocorticoid excess. Proc Nat Acad Sci U.S.A. 1999; 96: 12790-7.

[23] Berenbaum SA, Duck SC, Bryk K. Behavioral effects of prenatal versus postnatal androgen excess in children with 21-hydroxylase-deficient congenital adrenal hyperplasia. J Clin Endocrinol Metab 2000; 85:727-33.

[24] Reinisch JM. Prenatal exposure of human fetuses to synthetic progestin and oestrogen affects personality. Nature 1977; 266:561-2.

[25] Ehrhardt AA, Meyer-Bahlburg HFL. Effects of prenatal sex hormones on gender-related behavior. Science 1981; 211:1312-18.

[26] Rubin RT, Reinisch JM, Haskett RF. Postnatal gonadal steroid effects on human behavior. Sience 1981; 211:1318-24.

[27]Reinisch JM, Sanders SA. Prenatal gonadal steroidal influences on gender-related behavior. Prog Brain Res 1984; 61:407-16.

[28] Schwartz Z, Guest JF, Elder MG, White JO. Enzyme activities in the androgenized rat uterus refractory to oestrogenic stimulation. J Steroid Biochem 1986; 25:491-6.

[29] Carter DA, Saridaki E, Lightman SL. Sexual differentiation of oxytocin stress responsiveness: effect of neonatal androgenization, castration and luteinizing hormone—releasing hormone antagonist. Acta Endocrinol 1988; 117: 525-30.

[30] Greene RR, Burrill MW, Ivy AC. Experimental intersexuality. The effect of antenatal androgens on sexual development of female rats. Am J Anat 1939; 65:415-69.

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

[32] Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenbergh JG, Gray JrLE. Prenatal testosterone exposure permanently masculinizes anogenital distance nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol Sci 2007; 96(2):335-45.

[33] Swanson HE, van der Werff ten Bosch JJ. The "early androgen" syndrome: effects of pre-natal testosterone propionate. Acta Endocrinol 1965; 50:379-90.

[34] Food and Drug Administration [FDA]. NADA summary #138-612. Avaliable at http://www.fda.gov/cvm/efoi/section1/138612070286.html; 1996. [Acessed may.2011].

[35] Wilson VS, Lambright C, Ostby J, Gray LEJr. In vitro and in vivo effects os 17-beta-trenbolone: a feedlot effluent contaminant. Toxicol Sci 2002; 70:202-11.

[36] Barraclough CA. Production of anovulatory, sterile rat by single injections of testosterone propionate. Endocrinology 1961; 68:62-7.

[37] Kawashima K, Nakaura S, Nagao S, Tanaka S, Kuwamura T, Omori Y. Virilizing effect of methyltestosterone on female descendants in the rat. Endocrinol Jpn 1978; 25:1-6.

[38] Slob AK, den Hamer R, Woutersen PJ, van der Werff ten Bosch JJ. Prenatal testosterone propionate and postnatal ovarian activity in the rat. Acta Endocrinol 1983; 103:420-7.

[39] McCoy SJ, Shirley BA. Effects of prenatal administration of testosterone and cortisone on the reproductive system of the female rat. Life Sci 1992; 50:621-8.

[40] Rhees RW, Kirk BA, Sephton S, Lephart ED. Effects of prenatal testosterone on sexual behavior, reproductive morphology and LH secretion in the female rat. Dev Neurosci 1997; 19:430-7.

[41] Bartol FF, Wiley AA, Bagnell CA. Uterine development and endometrial programming. Soc Reprod Fertil Suppl 2006; 62:113-30.

[42] Crain AD, Janssen SJ, Edwards TM, Heindel J, Ho S, Hunt P, Iguchi T, Juul A et al. Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing. Fertil Steril 2008; 90(4):911-40.

[43] Petrusz P, Flerkó B. Effect of ovariectomy and oeestrogen administration on the pituitary and uterine weight in androgen-sterilized rats. Acta Biol Acad Sci Hung 1967; 19:159-62.

[44] Wrenn TR, Wood JR, Bitman J. Oestrogen responses of rats neonatally sterilized with steroids. J Endocr 1969; 45:415-20.

[45] Lobl RT, Gorski RA. Neonatal intrahypothalamic androgen administration: the influence of dose and age on androgenization of female rats. Endocrinology 1974; 94:1325-30.

[46] Lobl RT, Maenza RM. Androgenization: alterations in uterine growth and morphology. Biol Reprod 1975; 13:255-68.

[47] de Boo HA, Harding JE. The developmental origins of adult disease (Barker) hypothesis. Aust N Z J Obstet Gynaecol 2006; 46:4-14.

[48] Guerra MT, Sanabria M, Grossman G, Petrusz P, Kempinas WG. Excess androgen during perinatal life alters steroid receptor expression, apoptosis, and cell proliferation in the uteri of the offspring. Manuscript 1, submitted to Reprod Toxicol, 2013.

[49] Hoepfner BA, Ward IL. Prenatal and neonatal androgen exposure interact to affect sexual differentiation in female rats. Behav Neurosci 1988; 102:61-5.

[50] Welsh M, Sharpe RM, Walker M, Smith LB, Saunders PTK. New insight into the role of androgens in wolffian duct stabilization in male and female rodents. Endocrinology 2009; 150(5):2472-80.

[51] McIntyre BS, Barlown NJ, Foster PM. Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. Toxicol Sci 2002; 65(1):62–70.

[52] Wise LD, Vetter CM, Anderson CA, Antonello JM, Clark RL. Reversible effects of triamcinolone and lack of effects with aspirin or L-656,224 on external genitalia of male Sprague-Dawley rats exposed in utero. Teratology 1991; 44:507-20.

[53] Gallavan Jr RH, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterantions in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 1999; 13:383-90.

[54] Clark RL. Endpoints of reproductive system development. In: An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington DC: ILSI Press; 1999.p. 10-27.

[55] Mylchreest E, Wallace DG, Cattley RC, Foster PMD. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(*n*-butyl) phthalate during late gestation. Toxicol Sci 2000; 55:143-51.

[56] Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002; 62(4A):606-14.

[57] Borgeest C, Symonds D, Mayer LP, Hoyer PB, Flaws JA. Methoxychlor may cause ovarian follicular atresia and proliferation of the ovarian epithelium in the mouse. Toxicol Sci 2002;68:473–8.

[58] Talsness CE, Shakibaei M, Kuriyama SN, Grande SW, Sterner-Kock A, Schnitker P, et al. Ultrastructural changes observed in rat ovaries following in utero and lactational exposure to low doses of a polybrominated flame retardant. Toxicol Lett 2005;157:189–202.

[59] Christian MS. Test methods for assessing female reproductive and developmental toxicology. In: Wallace Hayes A, editor. Principles and methods of toxicology. New York: Informa Healthcare; 2008. p. 1673.

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

[61] Chen J, Ahn K, Gee N, Ahmed M, Duleba A, Zhao L et al. Triclocarban enhances testosterone action: a new type of endrocrine disruptor? Endocrinology 2008; 149:1173-9.

[62] Slob AK, Goy RW, van der Werff Ten Bosch JJ. Sex differences in growth of guinea pigs and their modification by neonatal gonadectomy and prenatally administered androgen. J Endocrinol 1973; 58:11-9.

[63] De Haan KC, Berger LL, Kesler DJ, McKeith FK, Thomas DL, Nash TG. Effect of prenatal androgenization on lamb performance, carcass composition, and reproductive function. J anim Sci 1987; 65:1465-70.

[64] Vandenbergh JG, Huggett CL. The anogenital distance index, a predictor of the intrauterine position effects on reproduction in female house mice. Lab Anim Sci 1995; 45:567–73.

[65] Gray LEJr, Wolf C, Lambright C, Mann P, Price M, Cooper R L et al. Administration of potentially antiandrogenic pesticide (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health 1999; 15:94–118.

[66] Wolf CJ, LeBlanc GA, Gray Jr LE. Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat. Toxicol Sci 2004; 78:135-43.

[67] Goldman AS, Shapiro B, Neumann F. Role of testosterone and its metabolites in the differentiation of the mammary gland in rats. Endocrinology 1976; 99:1490-5.

[68] Imagawa W, Yang J, Guzman R, Nandi S. Control of mammary gland development. In: Knobil E, Neill JD, editors. The Physiology of Reproduction. New York: Raven Press; 1994. p.1033-63.

[69] Andrews WW, Ojeda SR. A detailed analysis of the serum LH secretory profiles of conscious free-moving female rats during the time of puberty. Endocrinology 1981; 109:2032-9.

[70] US EPA - United State Environmental Protection Agency, Guidelines for Reproductive Toxicity Risk assessment. U.S. Environmental Protection Agency. Federal Register. 1996; 61:212.

[71] Guyton AC, Hall JE. Fisiologia Feminina antes da Gravidez e os Hormônios Femininos. In: Guyton AC, Hall JE, editors. Tratado de fisiologia médica. Rio de Janeiro: Guanabara Koogan; 2002.p.869-82.

[72] Westwood, F.R. The female rat reproductive cycle: a practical histological guide to staging. Toxicol Pathol 2008; 36:375-84.

[73] Smith MS, Freman ME, Neil JD. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 1975; 96: 219-26.

[74] Sportnitz UM, Socin CD, David AA. Estrous stage determination in rats by means of scanning electron microscopic images of uterine surface epithelium. The Anat Rec 1999; 254: 116-26.

[75] Johnson DC, Brown EB Jr. Serum follicle-stimulating hormone (FSH) in normal and androgenized male and female rats. Proc Soc Exp Biol Med 1971; 138(1):140-4.

[76] Korenbrot CC, Paup DC, Gorski RA. Effects of testosterone propionate or duhydrotestosterone propionate on plasma FSH and LH levels in neonatal rats and on sexual differentiation of the brain. Endocrinology 1975; 97(3):709-17.

[77] Goomer N, Saxena RN, Sheth AR. Effect of neonatal testosterone and oestradiol treatment on the development of the hypothalamo-hypophysial axis in the female rat. J Reprod Fert 1977; 50:239-43.

[78] Pinilla L, Trimiño PG, Bellido C, Aguilar R, Gaytán F, Aguilar E. Changes in pituitary secretion during the early postnatal period and anovulatory syndrome induced by neonatal oestrogen or androgen in rats. J Reprod Fert 1993; 97:13-20.

[79] Foecking EM, McDevitt MA, Acosta-Martínez M, Horton TH, Levine JE. Neuroendocrine consequences of androgen excess in female rodents. Horm Behav 2008; 53:673-92.

[80] Sarraj MA, Drummond AE. Mammalian foetal ovarian development: consequences for health and disease. Reproduction 2012; 143:151-63.

[81] Forsdike RA, Hardy K, Bull L, Stark J, Webber LJ, Stubbs S et al. Disordered follicle development in ovaries of prenatally androgenized ewes. J Endocrinol 2007; 192:421-8.

[82] Abbott DH, Dumesic DA, Fanks S. Developmental origin of polycystic ovary syndrome – a hypothesis. J Endocrinol 2002; 174:1-5.

[83] Abbott DH, Barnett DK, Buns CM, Dumesic DA. Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome? Hum Reprod Update 2005; 11:357-74.

[84] Xita N, Tsatsoulis A. Review: fetal programming of polycystic ovary syndrome by androgen excess: evidence from experimental, clinical, and genetic association studies. J Clin Endocrinol Metab 2006; 91:1660-6.

[85] West C, Foster DL, Evans NP, Robinson J, Padmanabhan V. Intra-follicular activin availability is alterated in prenatally-androgenized lambs. Mol Cell Endocrinol 2001; 185:51-9.

[86] Smith P, Steckler TL, Veiga-Lopez A, Padmanabhan V. Developmental programming: differential effects of prenatal testosterone and dihydrotestosterone on follicular recruitment, depletion of follicular reserve, and ovarian morphology in sheep. Biol Reprod 2009; 80:726-36.

[87] Steckler T, Wang J, Bartol FF, Roy SK, Padmanabhan V. Fetal programming: prenatal testosterone treatment causes intrauterine growth retardation, reduces ovarian reserve and increases ovarian follicular recruitment. Endocrinology 2005; 146:3185-93.

[88] Manikkam M, Steckler TL, Welch KB, Inskeep EK, Padmanabhan V. Fetal programming: prenatal testosterone treatment leads to follicular persistence/luteal defects; partial restoration of ovarian function by cyclic progesterone treatment. Endocrinology 2006; 147:1997-2007.

[89] Steckler T, Manikkam M, Inskeep EK, Padmanabhan V. Developmental programming: follicular persistence in prenatal testosterone-treated sheep is not programmed by androgenic actions of testosterone. Endocrinology 2007; 148: 3532-40.

[90] Sotomayor-Zárate R, Tiszavari M, Cruz G, Lara HE. Neonatal exposure to single doses of estradiol or testosterone programs ovarian folicular development – modified hypothalamic neurotransmitters and causes polycystic ovary during adulthood in the rat. Fertil Steril 2011; 96:1490-6.

[91] Brody JR, Cunha GR. Histologic, morphometric, and immunocytochemical analysis of myometrial development in rats and mice: II. Efects of DES on development. Am J Anat 1989; 186:21-42.

[92] Arena AC, Pereira OCM. Neonatal inhalatory anesthetic exposure: reproductive changes in male rats. Comp Biochem Physiol C 2002; 133:633-40.

[93] Gerardin DCC, Pereira OCM. Reproductive changes in male rats treated with an aromatase inhibitor. Pharmacol Biochem Behav 2002; 71:309-13.

[94] Ward IL. Differential effect of pre- and postnatal androgen on the sexual behavior of intact and spayed female rats. Horm Behav 1969; 1:25–36.

[95] Pollak EI, Sachs BD. Masculine sexual-behavior and morphology paradoxical effects of perinatal androgen treatment in male and female rats. Behav Biol 1975; 13(4):401–11.

[96] Jauniaux E, Poston L, Burton GJ. Placental-related diseases of pregnancy: involvement of oxidative stress and implications in human evolution. Hum Reprod Update 2006; 12:747-55.

[97] St-Louis J, Brochu M. The cardiovascular paradox of pregnancy. Med Sci (Paris) 2007; 23:944-9.

[98] Kramen MA. Reproduction and ovum viability in the anovulatory, androgen-induced, persistent-estrus rat. Thesis, University of Kansas, Kansas City, 1969.

[99] Kramen, M.A. Implantation in rats treated neonatally with testosterone propionate. J Reprod Fert 1974; 38:461-3.

LEGENDS OF FIGURES

Figure 1. Number of fetal germ cells from female rats whose mothers were exposed to corn oil (control, *n*=7) or TP at different doses (0.05mg/kg, *n*=9; 0.1mg/kg, *n*=9; 0.2mg/kg, *n*=8) from GD 12 to GD 20. Values expressed as mean ± S.E.M. Anova with a *post hoc* Dunnet.

Figure 2. Evaluation of external signs of puberty onset from female rats whose mothers received corn oli (control, *n*=8 litters/46 females) or TP at doses of 0.05mg/kg (*n*=7 litters/37 females), 0.1mg/kg (*n*=9litters/47 females) or 0.2mg/kg (*n*=10 litters/ 41 females) during gestation and lactation periods. **A**: age of vaginal opening and first estrus; **B**: body weight immediately after vaginal opening. Values are expressed as mean ± S.E.M. Anova with *a post hoc* Dunnet.*$p<0.05$.

Figure 3. Luminal and glandular epithelial height of uterine tissues from female rats, in estrus, whose mothers were exposed to TP during gestation and lactation. Experimental groups: control (*n*=8 animals), and exposed to TP at 0.05mg/kg of TP (*n*=7 animals), 0.1mg/kg (*n*= 9 animals) and 0.2mg/kg (*n*= 10 animals) **A**: at puberty and **B**: at 75 days of age. Values expressed as mean ± S.E.M. Anova with *a post hoc* Dunnet, *$p<0.05$.

Figure 4. Morphological aspect of transverse section from rat uterus after puberty onset. (A) Control group. (B) 0.05mg/kg group. (C) 0.1mg/kg group. (D) 0.2mg/kg group. Arrow: Glandular epithelium. 200×, H.E.

Figure 5. Ovarian follicles and corpora lutea counting per unit area (number/mm^2) from female rats from control (*n*=8 animals) and treated with TP at 0.05mg/kg (*n*=7 animals), 0.1mg/kg (*n*=9 animals) or 0.2mg/kg (*n*=10 animals). **A**: at puberty onset; **B**: at PND75. Values are expressed as mean ± S.E.M. Anova with a *post hoc* Dunnet.

Figure 6. Lordosis quotient (LQ), obtained by sexual behavior test, from female rats at PND 100, one or two per litter, at proestrus phase. Values are expressed as median (Q_1-Q_3). Kruskall-Wallis with a *post hoc* Dunn. Control (*n*=16 animals), 0.05mg/kg (*n*=13 animals), 0.1mg/kg (*n*=17 animals), 0.2mg/kg (*n*=16 animals).

Figure 1

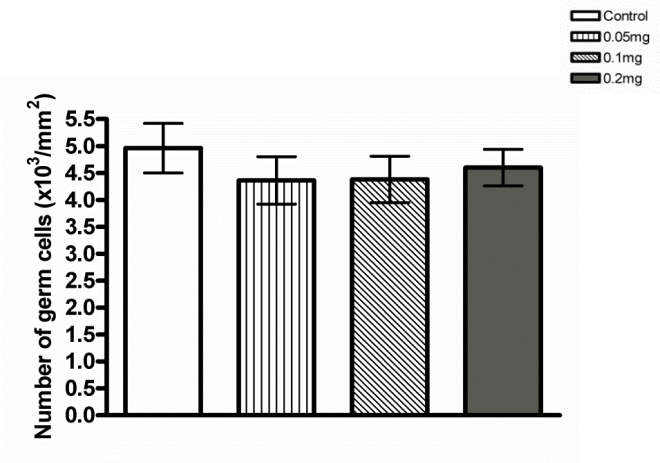
Control
0.05mg
0.1mg
0.2mg
Number of germ cells (x10^3/mm^2)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

Figure 2

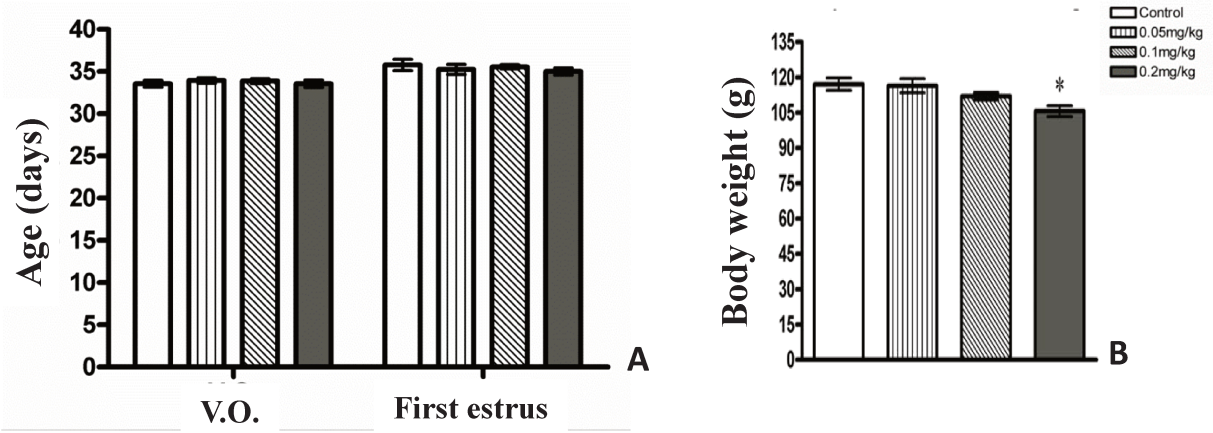
Age (days)
40
35
30
25
20
15
10
5
0
V.O.
First estrus
A
Body weight (g)
135
120
105
90
75
60
45
30
15
0
*
B
Control
0.05mg/kg
0.1mg/kg
0.2mg/kg

Figure 3

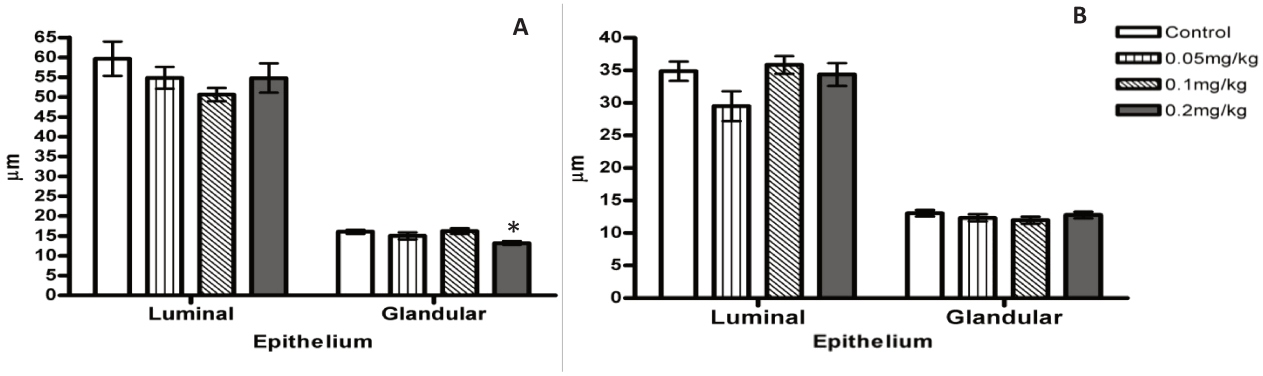
A
B
µm
Luminal
Glandular
Epithelium
Control
0.05mg/kg
0.1mg/kg
0.2mg/kg
*

Figure 4

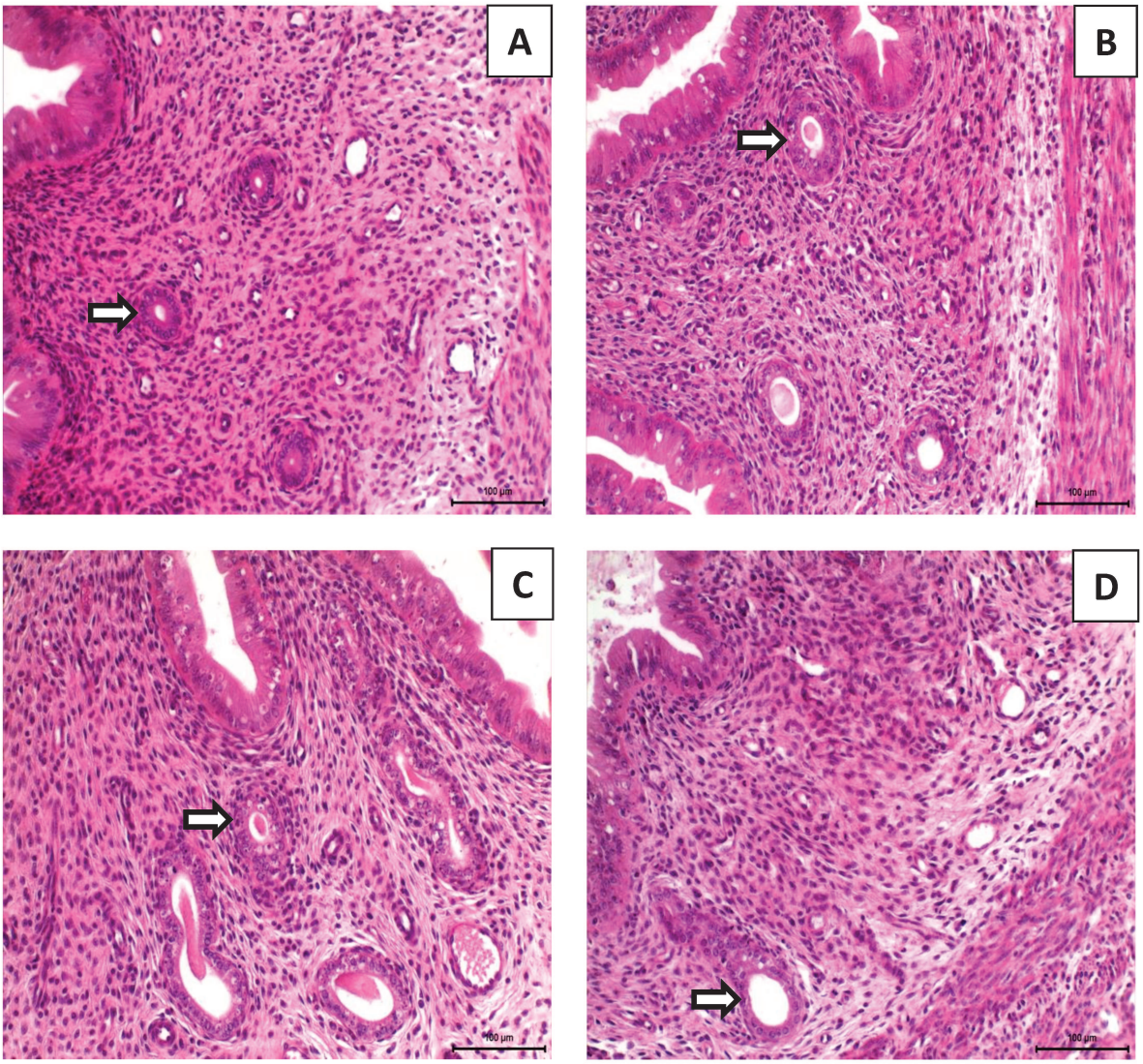

Figure 5

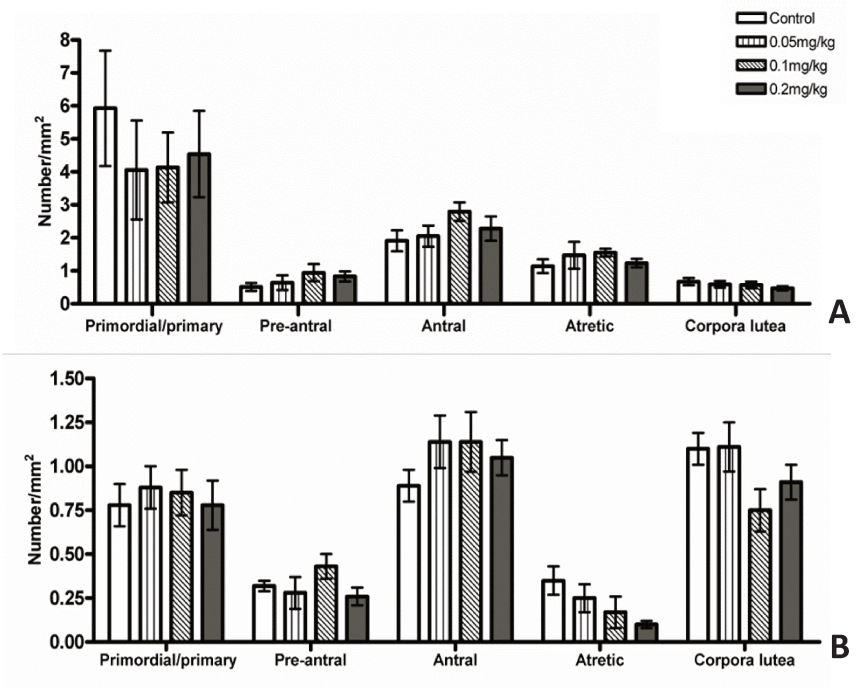
Control
0.05mg/kg
0.1mg/kg
0.2mg/kg
Number/mm²
Primordial/primary
Pre-antral
Antral
Atretic
Corpora lutea
A
B

Figure 6

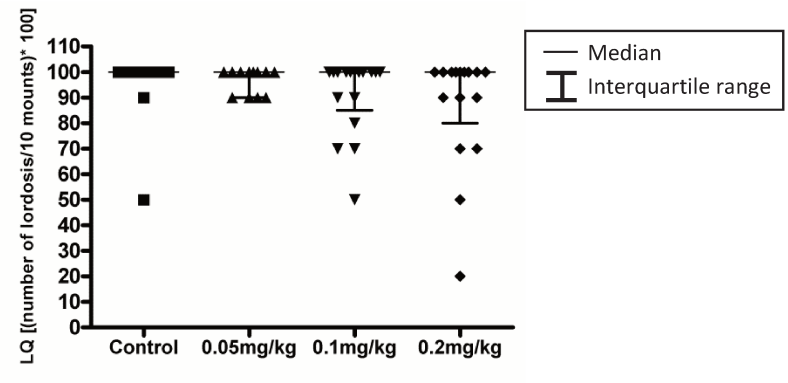
LQ [(number of lordosis/10 mounts)* 100]
110
100
90
80
70
60
50
40
30
20
10
0
Control
0.05mg/kg
0.1mg/kg
0.2mg/kg
Median
Interquartile range

Table 1. Body weight and relative AGD, evaluated at PND 1, and nipple counting at PND 13, from female offspring whose mothers were exposed to corn oil (control group) or TP at 0.05mg/kg, 0.1mg/kg or 0.2mg/kg, during gestation and lactation.

	Experimental groups			
	Control (n=8)	0.05mg (n=7)	0.1mg/kg (n=9)	0.2mg/kg (n=10)
Body weight (g)	6.87 ± 0.20	6.74 ± 0.23	6.53 ± 0.06	6.36 ± 0.11
Relative AGD ($mm/g^{1/3}$)	0.68 ± 0.01	0.71 ± 0.03	0.68 ± 0.01	0.78 ± 0.02*
Number of nipples[A]	12 (12 - 12.12)	12 (12 - 12.21)	12.14 (12 - 12.25)	12.25 (12 - 12.31)

Values are expressed as mean ± S.E.M. Anova with a *post hoc* Dunnet. *$p<0.05$.
[A] Values are expressed as median (Q_1-Q_3). Kruskal-Wallis with a *post hoc* Dunn.

Table 2. Effects of prenatal and lactation (GD 12 – PND 21) exposure to TP at different doses on body and organ weights in the female offspring at the estrus phase, at puberty onset and PND75.

	Puberty onset			
Parameters	**Control (n=8 animals)**	**0.05mg/kg (n=7 animals)**	**0.1mg/kg (n=9 animals)**	**0.2mg/kg (n=10 animals)**
Body weight (g)	117.14 ± 3.96	112.20 ± 4.34	114.01 ± 5.24	105.99 ± 3.01
Relative AGD (mm/$g^{1/3}$)	0.19 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
Liver (g)	5.69 ± 0.27	5.46 ± 0.14	5.38 ± 0.31	4.82 ± 0.16*
Liver (g/100g body weight)	4.85 ± 0,14	4.89 ± 0.15	4.70 ± 0.08	4.55 ± 0.10
Kidneys (g)	1.22 + 0.04	1.15 ± 0.02	1.16 ± 0.05	1.11 ± 0.04
Pituitary (mg)	6.40 ± 0.36	5.66 ± 0.28	5.78 ± 0.30	5.58 ± 0.18
Ovaries (mg)	37.14 ± 3.07	33.40 ± 1.77	36.83 ± 4.81	35.38 ± 2.20
Uterus + fluid (mg)	211.90 ± 12.65	214.10 ± 19.83	231.40 ± 26.83	175.10 ± 5.51
	PND75			
	Control (n=8 animals)	**0.05mg/kg (n=7 animals)**	**0.1mg/kg (n=9 animals)**	**0.2mg/kg (n=10 animals)**
Body weight (g)	222.00 ± 4.97	225.63 ± 4.11	218.16 ± 5.05	215.37 ± 3.42
Relative AGD (mm/$g^{1/3}$)	0.20 ± 0.01	0.20 ± 0.01	0.22 ± 0.01	0.20 ± 0.01
Liver (g)	8.55 ± 0.22	8.48 ± 0.36	8.48 ± 0.27	8.09 ± 0.20
Kidneys (g)	1.83 ± 0.05	1.73 ± 0.06	1.66 ± 0.05	1.56 ± 0.05*
Kidneys (g/100g body weight)	0.82 ± 0.02	0.77 ± 0.02	0.76 ± 0.02	0.73 ± 0.03*
Pituitary (mg)	11.80 ± 0.51	11.19 ± 0.63	10.67 ± 0.56	11.21 ± 0.29
Ovaries (mg)	71.50 ± 3.66	70.00 ± 3.34	66.72 ± 3.42	69.01 ± 2.25
Uterus + fluid (mg)	417.93 ± 14.15	529.23 ± 92.63	392.24 ± 22.73	414.96 ± 43.03

Values are expressed as mean ± S.E.M. Anova with a *post hoc* Dunnet. *p<0.05.

Table 3. Serum hormonal levels of female rats, at estrus phase, from control group and treated with different doses of TP.

	Puberty onset			
Hormonal levels	Control (*n*=8 animals)	0.05mg/kg (*n*=7 animals)	0.1mg/kg (*n*=9 animals)	0.2mg/kg (*n*=10 animals)
FSH (ng/ml)	4.84 ± 0.58	10.59 ± 3.91	3.28 ± 0.32	3.10 ± 0.39
LH (ng/nl)	1.53 ± 0.15	1.77 ± 0.29	1.60 ± 0.22	1.40 ± 0.14
E_2 (ρg/ml)	121.26 ± 14.66	130.00 ± 3.68	142.72 ± 12.64	122.93 ± 4.69
P_4 (ng/ml)	14.64 ± 6.62	8.90 ± 1.54	11.41 ± 3.28	20.70 ± 4.00
T (ng/ml)	0.025 ± 0.011	0.012 ± 0.001	0.018 ± 0.005	0.029 ± 0.006
	PND75			
	Control (*n*=8 animals)	0.05mg/kg (*n*=7 animals)	0.1mg/kg (*n*=9 animals)	0.2mg/kg (*n*=10 animals)
FSH (ng/ml)	3.56 ± 0.42	2.71 ± 0.28	3.61 ± 0.50	3.47 ± 0.24
LH (ng/ml)	1.47 ± 0.10	1.43 ± 0.30	1.48 ± 0.12	1.45 ± 0.10
E_2 (ρg/ml)	137.29 ± 5.63	227.66 ± 67.10	141.35 ± 6.71	194.08 ± 66.43
P_4 (ng/ml)	16.40 ± 3.89	19.11 ± 2.38	21.10 ± 5.55	23.45 ± 4.67
T (ng/ml)	0.018 ± 0.007	0.042 ± 0.015	0.036 ± 0.013	0.031 ± 0.008

FSH: Follicle stimulating hormome; LH: Luteinizing hormone; E_2: Estradiol; P_4: Progesterone; T: Testosterone.
Values are expressed as mean ± S.E.M. Anova with *a post hoc* Dunnet.

Table 4. Assessment of estrous cycle length (days), number of cycles and frequency of each phase (days) over a 15-day period of evaluation in the female offspring (PND 60) from control and TP-treated groups.

Estrous cycle	**Control**	**0.05mg/kg**	**0.1mg/kg**	**0.2mg/kg**
Number of evaluated animals/litters	38/8	30/7	38/9	31/10
Proestrus	2.75 ± 0.23	2.59 ± 0.12	2.22 ± 0.27	2.93 ± 0.34
Estrus	4.43 ± 0.17	4.23 ± 0.33	4.35 ± 0.13	4.17 ± 0.15
Metaestrus	3.60 ± 0.40	4.10 ± 0.35	4.33 ± 0.58	4.66 ± 0.39
Diestrus	4.21 ± 0.55	4.09 ± 0.50	4.07 ± 0.46	3.36 ± 0.42
Number of estrous cycles	2.45 ± 0.16	2.18 ± 0.16	2.71 ± 0.19	2.90 ± 0.12
Estrous cycle lenght	4.31 ± 0.35	4.55 ± 0.39	4.51 ± 0.33	4.32 ± 0.27

Values are expressed as mean ± S.E.M. ANOVA with a *post hoc* Dunnet.

Table 5. Height of endometrium and glandular counting in uterine tissue from female rats, in estrus, at puberty and 75 days-old, exposed to vehicle or different doses of TP during intra uterine and lactational life.

	Puberty		**PND 75**	
Experimental Groups	Height of uterine endometrium (µm)	Number of glands	Height of uterine endometrium (µm)	Number of glands
Control (*n*=8 animals)	308.79 ± 15.40	5.83 ± 1.04	569.01 ± 13.26	32.21 ± 4.54
0.05mg/kg (*n*=7 animals)	305.02 ± 14.67	5.95 ± 1.05	534.16 ± 34.94	36.38 ± 5.29
0.1mg/kg (*n*=9 animals)	276.25 ± 33.53	5.67 ± 0.71	508.09 ± 30.62	29.70 ± 3.19
0.2mg/kg (*n*=10 animals)	290.84 ± 14.06	6.05 ± 0.73	544.65 ± 17.18	34.07 ± 3.61

Values are expressed as mean ± S.E.M. Anova with *a post hoc* of Dunnet.

Table 6. Fertility performance of adult female rats (PND100 – one or two per litter) whose mothers received corn oil (control group) or PT at different doses.

Parameters	Control (*n*=16 animals)	0.05mg/kg (*n*= 13 animals)	0.1mg/kg (*n*= 17 animals)	0.2mg/kg (*n*= 16 animals)
Pregnancy rate (%)	100	100	100	100
Fertility potential (%)	100 (92.56 - 100)	100 (94.74 - 100)	93.33 (92.86 - 100)	97.06 (86.16 - 100)
Initial body weight (GD 0 - g)	259.31 ± 5.18	273.27 ± 6.22	261.34 ± 4.10	259.58 ± 5.12
Final body weight (GD 20 - g)	385.61 ± 7.24	402. 74 ± 5.98	382.28 ± 4.86	372.49 ± 7.72
Body weight gain (g)	126.13 ± 3.94	129.47 ± 4.82	120.93 ± 4.24	117.29 ± 4.99
Uterine + fetal weight (g)	64.33 ± 2.56	66.00 ± 1.61	63.35 ± 1.78	61.34 ± 3.08
Rat weight - (uterus + fetal weight) (g)	321.27 ± 5.82	336.64 ± 5.57	318.83 ± 4.73	311.15 ± 5.67
Fetal weight (g)	3.27 ± 0.06	3.19 ± 0.07	3.25 ± 0.05	3.23 ± 0.04
Female pups (g)	3.21 ± 0.06	3.13 ± 0.08	3.18 ± 0.05	3.15 ± 0.04
Male pups (g)	3.35 ± 0.05	3.29 ± 0.07	3.30 ± 0.05	3.29 ± 0.05
Sex ratio (M:F)	1.13 ± 0.25	1.56 ± 0.27	1.47 ± 0.23	1.09 ± 0.13
Placenta weight (g)	0.51 ± 0.02	0.48 ± 0.01	0.46 ± 0.01	0.49 ± 0.02
Number of corpora lutea	14.75 ± 0.88	14.54 ± 0.55	15.18 ± 0.75	14.44 ± 0.77
Number of implantations	13.94 ± 0.93	14.23 ± 0.52	13.41 ± 0.35	13.12 ± 0.58
Number of live fetuses	12.44 ± 0.53	13.15 ± 0.44	12.59 ± 0.34	12.06 ± 0.58
[a]Pre-implantational loss (%)	0 (0 - 7.44)	0 (0 - 5.26)	6.67 (0 - 7.14)	2.94 (0 - 13.85)
[a]Post-implantational loss (%)	6.67 (0 - 9.32)	7.69 (0 - 8.33)	7.14 (0 - 8.33)	7.69 (6.08 - 9.09)

Values are expressed as mean ± S.E.M. Anova with a *post hoc* Dunnet.
[a]Values are expressed as median (Q_1-Q_3). Kruskall-Wallis with a *post hoc* Dunn.

5.3.*Manuscrito 3*

O terceiro manuscrito é intitulado "*Perinatal exposure to excess of androgen and the effects on the rat uterine estradiol responsiveness*", que será submetido à revista Contraception, Elsevier, ISSN: 0010-7824, Fator de Impacto: 2,72.

PERINATAL EXPOSURE TO EXCESS OF ANDROGEN AND THE EFFECTS ON THE RAT UTERINE ESTRADIOL RESPONSIVENESS

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ABSTRACT

INTRODUCTION: Exposure to excess of androgens during critical periods of female sexual development induces permanent alterations in steroidal target tissues and may affect the responsiveness of the tissues to estrogen stimulation. The objective of this study was to evaluate the uterine resposiveness to estradiol stimulation after perinatal androgenization in female rats at prepubertal and adult ages.

MATERIALS and METHODS: For this, pregnant Wistar rats were exposed to corn oil or testosterone propionate (TP) at doses of 0.05mg/kg, 0.1mg/kg or 0.2mg/kg from DG 12 until PND 21. After weaning (PND 22) and at 100 days of age, the female offspring was treated with estradiol to evaluate the uterine responsiveness to this hormone.

RESULTS: Our results showed that TP did not cause damage in the uterine ability to respond to estradiol stimulation at prepubertal age. Comapred to animals that received only corn oil at adulthood, 0.1mg/kg TP group only respond to 10µg/day estrogen, control and 0.05mg/kg TP groups presented a uterine weight increment with the dose of 100µg/day and the 0.2mg/kg TP animals showed increased uterine weight at both estradiol treatment. The dosage of 0.1 and 0.2mg/kg showed opposite response to 100µg/day estradiol exposure, but both may reflect in physiological alterations in the uterine tissues. The pituitary weight was not affected by TP exposure and responded similarly after estradiol stimulation.

DISCUSSION: We can conclude that TP exposure during specific phases of development, induces, at adulthood, an abnormal stimulation of uterine tissue growth by estrogen stimulus without affecting pituitary response. The uterine condition may reflect in fertility decline and more studies should be performed in order to clarify whether these alterations are capable of impair the reproductive capacity of female tract.

Key words: Androgenization, uterus, estradiol, female rat.

1. INTRODUCTION

Over the last 70 years, more than 80,000 chemicals have been released into the environment in wildlife through human activity and more than 85% of these substances have never been assessed for possible effects on human health. Recently, there has been considerable concern about one specific category of these compounds - the endocrine disruptors chemicals [1]. These substances may interfere with production, release, transport, metabolism, binding, action or elimination of the natural hormones in the organism, altering the critical hormonal balance required from proper health and development. Endocrine disruptors may bind to hormonal receptors and impair funtions in the body [2,3].

It is well known that exposure of fetuses to some hormonally active agents during critical periods of their development imprints permanent changes [4,5] that can be detected later in adulthood as a modification in the hormone activity or hormone receptors and in the intensity of responses mediated by them [6,7].

In steroid target tissues, the presence of androgens during critical periods of sexual differentiation induces permanent alterations that may affect the responsiveness of the tissue to estrogen [8,9]. There are several studies showing that androgen exposure can decrease the uterine response to estrogenic stimulation [10,11] and induce changes in the hormone-induced uterine growth [12].

The effects of androgen treatment on uterine responsiveness to estradiol are controversial. Although Gellert et al. [13] did not detect any significant effect of neonatal exposure to androgens on uterine responsiveness to estrogen in prepupertal rats, others have reported a decrease in the responsiveness of the uterus to estrogen treatment in androgenized rats [9, 12, 14-16]. Such discording results may be due to the fact that androgenization exerts different effects according to the stage of development at which fetuses or neonatal animals are exposed [17-19].

Althoug extensive research has been performed on the uterine responsiveness in adults, little is known about the age-related onset of the uterine events. Based on previous studies that revealed that uterine tissues are able to respond to estrogen before puberty [20] and on the importance of normal uterine physiology for reproduction in adulthood, this

present study evaluated the capacity of perinatal androgenized female rats uterus to respond to estrogen stimulation during prepubertal period (before ovulation or the onset of estrous cycles) and at adult age.

Female rats exposed to androgen excess during perinatal life presented alteration in the pattern of steroid immnostaining in the uterine tissue [21] and it may give rise to an abnormal response of this organ to a hormonal stimulation. Differently from most of the available estudies using high doses of androgens and short periods of exposure [22-24], the present work aimed to evaluate the exposure of testosterone propionate on female offspring during perinatal life and possible effects in the uterine response to estradiol treatment at prepuberty and adulthood.

2. MATERIAL AND METHODS

2.1. Animals and androgen exposure

Adult female (60 days of age) and male (90 days of age) Wistar rats were supplied by Central Biotherium of State University of São Paulo (UNESP) and maintained under controlled temperature (23 ± 1°C) and lighting conditions (12:12h photoperiod), in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu. Standard rodent chow and filtered tap water were provided *at libitum*. Two non-gravid female rats were mated with one male, during the dark portion of the lighting cycle, and the day of sperm detection in the vaginal smear was considered day 0 of gestation (gestational day - GD0). The gravid females were randomly assigned among the experimental groups and housed individually in cages. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Biosciences Institute/UNESP Ethics Committee for Animal Experimentation (protocol no. 104/2009-CEEA).

Pregnant rats were administered either corn oil (Control, n=7) or testosterone propionate (TP- DEG Ltd., CAS 57-85-2, 97% of purity) at doses of 0.05mg/kg TP (n=9), 0.1mg/kg TP (n=9) or 0.2mg/kg TP (n=7), subcutaneously, from GD 12 to the end of lactation (Post-natal day 21 – PND 21). The beginning of the mother's treatment coincided with the critical period of the offspring's reproductive system development, which

continues after birth [25, 26]. On the next day after the last administration of TP pups were weaned (PND 22) and the respective mothers were euthanized by CO_2 inhalation followed by decapitation.

2.2. Estradiol challenge

The uterus of the female offspring was challenged with different doses of estradiol benzoate (E_2, β-estradiol 3- benzoate, Sigma®, E-8515), in two periods of life, to assess the ability of uterine tissues to respond to estrogenic stimulation.

2.2.1. First estrogenic assay

This first sensibility test to estrogen was performed based on the methodology of uterotropic assay (with modifications – no evaluation of uterine weight without fluid) [20], as follows: after female pups were weaned, at 22 days of age [27, 28, 29], five animals from each of the four experimental groups (one or two per litter) represented the "challenged group" and were destined to estradiol treatment. Other five pups per experimental group were used to represent the "corn oil group" of this assay and received corn oil only (vehicle). Control animals that received corn oil were called negative control and control animais that received estradiol represented the positive control.

The animals from challenged group were treated, by gavage (orally), during three consecutive days (from PND 22 to PND 24) with E_2 at dose of 0.4mg/kg, strong enough to promote uterine growth [30]. Animals from corn oil group receved corn oil by the same route and period of exposure. Twenty-four hours after the final dose (PND 25), female rats were weighted and killed by CO_2 inhalation followed by decapitation. Uteri were excised [27] and trimmed free of fat. After that, the organ (with fluid) was weighted in precision balance.

2.2.2. Second estrogenic assay

The second treatment with E_2 was performed in adult mature female animals based on Petrusz and Flerkó [11]. At 100 days of age, the response of the uterus and pituitary to estradiol were evaluated. All four experimental groups presented three animals per litter,

and each animal was intramuscularly injected with corn oil, 10µg/day or 100µg/day of E_2 dissolved in corn oil for a period of one month (30 days). Control animais that received corn oil were named negative control and control rats that received estradiol (10 ou 100µg/day) represented the positive control. Twenty-four hours after the last day of exposure, female rats were weighted and killed by CO_2 inhalation followed decapitation, and uteri and pituitary were collected and weighted.

2.3. Statistical analysis

For comparison of results among the experimental groups, according to the characteristics of each variable, ANOVA with a *posteriori test* of Tukey (mean ± S.E.M) or Kruskal-Wallis with a *posteriori test* of Dunn was performed, using Instat 3.0 software (GraphPad software, San Diego, CA, USA). Differences were considered significant when $p<0.05$.

3. RESULTS

The uterine weight of prepubertal animals from corn oil group – all four experimental groups with no E_2 treatment – revealed to be similar (figure 1A), suggesting that TP exposure during perinatal life did not alter the normal weight of this organ at this age. The body weight from all groups (treated wiyh corn oil or estradiol) was not affected (data not shown). The treatment with E_2, in the first uterine assay at dose of 0.4mg/kg/day – approximately 24µg/day, was effective, since all groups presented a significant increase in uterine weight compared to negative control animals (figure 1B). However, no difference in the uterine responsiveness to E_2 was reported, since all animals from challenged group showed similar uterine weight (figure 1B).

In the second estrogenic assay, all experimental groups exposed to corn oil at adulthood presented comparable values regarding uterine weight (figure 2). In the experimental groups exposed to estradiol, we noted that this treatment caused, apparently in a dose-dependent manner and in all tested groups, a heterogeneous distribution of the data resulted from a variable inter-animal response to this treatment (figure 3). In all experimental conditions, body weight was similar among animals (data not shown).

When uterine weights of rats submitted to 10µg/day estradiol were compared to the negative control condition (control group exposed to corn oil), only 0.1 and 0.2mg/kg TP groups revealed a significative increase in this parameter (figure 3A). On the other hand, in all experimental groups, except for 0.1 mg/kg TP, the exposure to 100µg/day estradiol promoted significative uterine growth when compared to the negative control animals, and 0.2mg/kg TP group presented an higher amplitude in this response (figure 3B).

The results obtained after pituitary evaluation showed that the weight of this organ was similar among experimental groups exposed to corn oil and the treatment with 10 and 100µg E_2 were able to cause a significant weight increment. Besides, all groups exposed to TP presented equal pituitaty response after estradiol stimulation when compared to control animals (figure 4).

4. DISCUSSION

In this study, the effect of androgen excess exposure to female rat during perinatal life on uterine estrogen-induced growth and pituitary weight were evaluated. Before puberty the animals demonstrated no alterations in uterine responsiveness to estradiol. On the other hand, at adulthood, the estradiol exposure revealed that animals exposed to 0.1 and 0.2mg/kg of TP respond abnormally to estrogen challenge.

Although the major developmental period of the rodent uterus occurs after birth [31] and is independent of estrogens [32-34], exposure to endocrine disruptors with androgenic activity during intrauterine period may cause uterine abnormalities when administered to female rats [12, 22, 35, 36].

Between PND 12-16 the uterus begins to acquire estrogen response as a preparation for puberty installation [33] and the cellular response to this hormone is mediated, at least in part, by specific estradiol receptors. A diminution in the uterine sensitivity to estradiol occurs naturally during ageing and it has been associated with changes in the capacity of estrogen receptor to interact with cell nuclei [37,38].

The studies on uterine response to estradiol show some contradictory results and this may be due to, at least in part, in the different experimental designs utilized. According to Arriaza et al. [19] and Mena et al. [39], estrogen-induced growth in uterine weight was

completely blocked by androgenization. Cytosol proteins which inhibit estrogen receptor translocation from cytosol to nucleus are increased by androgenization and changes in estrogen receptor levels or possible variations in the concentration of growth factors induced by androgen imprinting explain the alteration in uterine response [39]. Lobl [9] demonstrated that androgenized female rats (with TP at 1.25mg on PND 3) presented a decrease in the total nuclear uptake of estradiol in the uterus, decreased availability of specific estradiol-binding sites and decreased amount of estrogen-induced proteins.

During the first evaluation of estrogen sensitivity in prepubertal animals, uterine weight of females from the corn oil group were similar among experimental groups and this result is in agreement with data from Guerra et al. [40], in which uterine weights from female rats treated with TP in the same doses were also similar among experimental groups. A significantly increase in the weight of uterus in all experimental groups that received 0.4mg/kg of E_2 (challenged group x negative control animals) was noted. However, when only the animals that were injected with E_2 (challenged group) were compared, the estradiol-stimulated uterine growth amplitude was not affected by TP administration, indicating that, at this point, the uterine physiology was not compromised yet. Corroborating our results, Gellert et al. [13] showed that female rats exposed to TP at PND 3 presented no alterations in the uterine responsiveness to estradiol treatment in immature age.

Several studies showed that adult rats neonatally treated with androgens have reduced uterine responsiveness [8, 11, 12, 41, 42] and estradiol binding capacity at adult age [43-48]. Contrarily, Wrenn et al.[49] showed that female exposed to a single dose of TP at 250µg on PND3 had no significant weight alteration in response to estradiol at adulthood.

In the second protocol to study the uterine responsiveness to E_2, performed at adulthood, females from corn oil group presented similar uterine weights, showing that TP did not interfere with normal development of this organ, as shown in Guerra etal. [40]. Only the dose of 100µg/day of estradiol was able to promote a significant uterine weight increase when compared to the negative control animals.

Uterine weights, after estradiol treatment, varied greatly in all experimental groups, and this fact may be explained, at least in part, by the degree of uterine enlargement and fluid accumulation in some individuals, following perinatal androgenization. Wolf et al. [22] described previously a similar situation in androgenized females that received 1 to 5mg of TP. In our results, this abnormal physiology of the uterus in producing exacerbated fluid, especially in animals from 0.2mg/kg group, was only revealed after estradiol challenge in the adulthood; in normal situations – negative control animals – uteri from all groups showed similar and homogeneous values.

Models based on mechanisms of receptor-mediated processes may produce dose-response curves that are non-monotonic [50]. Data from animals exposed to 100 µg of estradiol suggest a multiphasic dose-response curve for uterine condition, despite the absence of statistical significance. This estrogen dose caused, in the 0.2mg/kg TP group, an increase of 44.85% in uterine growth compared to 0.05mg/kg TP animals and 128.32% when compared to control; 0.1mg/kg TP group was not able to respond to estrogen stimulation as other experimental groups and only showed increased uterine weight at 10µg/day E_2. In agreement with data obtained from 0.2mg/kg TP group, this increment in uterine response to estradiol stimulus after androgenization was previously reported by Takewaki [51].

When higher than physiological levels of estradiol are administeredto females , there is an abruptly increase in the level of LH (by six to eight times) and this may be to due to the fact that estrogen may exert, in this peculiar situatin, a positive feedback , stimulating LH pituitary secretion [52].

Previous studies demonstrated that pituitary weight responded to a lesser degree to estradiol after early androgen exposure [11] and androgenization impairs the anterior pituitary response to estradiol [53-55] and these results may be explained by the reducion on the response of pituitary LH content to estradiol after androgenization [56]. Contradicting data from prior experiments, we observed that both estrogen doses were able to increase pituitary weight in all groups, showing that pituitary from TP exposed groups were able to respond similarly to control animals to estrogen stimulation.

Androgens are known to participate in uterine cell proliferation induced by estrogens [57-59]. Although our previous work [21] showed that animals that received TP at doses of 0.1 and 0.2mg/kg during perinatal life presented, at adulthood, normal uterine pattern of ERα expression, the imunostainig for androgen receptor (AR) was diminished in the 0.1mg/kg dose and increased in the higher dose. The ability of AR to cause uterotrophic effects in the presence of estrogenic compounds and amplify the estrogen receptor signals was possibly responsible for the exacerbated increase in the uterine weight at 0.2mg/kg group after 30-days treatment with estradiol and, the lack of this receptor in adequate levels was responsible for the low response found in the 0.1mg/kg TP group.

Based in all the results presented here, we can conclude that female rat developmental exposure to TP alters uterine, but not pituitary, response to estradiol stimulation. This uterine condition may have implications in fertility and more studies should be performed in order to clarify whether these alterations are capable of impairing the reproductive capacity of female tract.

5. ACKNOWLEDGMENTS

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6. REFERENCES

[1]Hormone disruptors and women's health – Reasons for concern. 2008. Collaborative on Health and Environment, University of Florida and University of California San Francisco, Obstetrics, Gynecology and Reproductive Sciences. www.healthandenvironment.org/iniciatives/fertility/pubs. [Acessed on 24.08.12.]

[2]Kavlock RJ. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA – Sponsored workshop. Environ Health Perspect 1996; 104:715-40.

[3]Colborn T, Clement C. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Advances in Modern Toxicology, XXI. Princeton Publishing Co. Inc., Princeton, NJ.2002.

[4]Csaba G, Nagy SU. Plasticity of hormone receptors and possibility of their deformation in neonatal age. Experientia 1976; 32:651-2.

[5]Csaba G. Phylogeny and ontogeny of the hormone receptors: the selection theory of receptor formation and hormonal imprinting. Biol Rev Camb Philos Soc 1980; 55(1):47-63.

[6]Dobozy O, Csaba G, Hetényi G, Shahin M. Investigation of gonadotropin-thyrotropin overlapping and hormonal imprinting in the rat testis. Acta Physiol Hung 1985; 66:169-75.

[7]Csaba G, Inczefi-Gonda A, Dobozy O. Hormonal imprinting by steroids: a single neonatal treatment with diethylstilbestrol or allylestrenol gives rise to a lasting decrease in the number of rat uterine receptors. Acta Physiol Hung 1986; 67:207-12.

[8]Lobl RT, Trotta P, Brumberger B. Oestrogen-induced proteins and uterine growth in the androgenized female rat. J Endocr 1974; 60:371.

[9]Lobl RT. Andorgenization: alterations in the mechanism of oestrogen action. J Endocr 1975; 66:79-84.

[10]Harris GW, Levine S. Sexual differentiation of the brain and its experimental control. J Physiol (Lond.) 1965; 181:379-400.

[11]Petrusz P, Flerkó B. Effect of ovariectomy and oestrogen administration on the pituitary and uterine weight in androgen-sterilized rats. Acta Biol Acad Sci Hung 1968; 19(2):159-62.

[12]Lobl RT, Maenza RM. Androgenization: alterations in uterine growth and morphology. Biol Reprod 1975; 13:255-68.

[13]Gellert RJ, Lewis J, Pétra PH. Neonatal treatment with sex steroids: relationship between the uterotropic response and the estrogen “receptor” in prepubertal rats. Endocrinology 1977; 100(2):520-8.

[14]Campbell PS. Impaired prepubertal uterine capacity if response after neonatal exposure to steroid hormone esters. J Exp Zool 1980; 214:345-53.

[15]Schwartz Z, Guest JF, Elder MG, White JO. Enzyme activities in the androgenized rat uterus refractory to oestrogenic stimulation. J Steroid Biochem 1986; 25:491-6.

[16]Campbell PS, Modlin PS. Uterine glucose metabolism in the prepubertal rat treated neonatally with androgen, estrogen and antihormones. Experientia 1987; 43:309-10.

[17]Lobl RT, Gorski RA. Neonatal intrahipotalamic androgen administration: the influence of dose and age on androgenization of female rats. Endocrinology 1974; 94:1325-30.

[18]MacLusky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science 1981; 211:1294-1303.

[19]Arriaza CA, Mena MA, Tchernitchin AN. Prenatal androgenization selectively modifies some responses to estrogen in the prepubertal rat uterus. J Endocrinol 1988; 120:379-84.

[20]EDSTAC – Endocrine Disruptor Screening and Testing Advisory Committee. 1998. EPA/743/R-98/003: Final Report.

[21]Guerra MT, Sanabria M, Grossman G, Petrusz P, Kempinas WG. Excess androgen during perinatal life alters steroid receptor expression., apoptosis, and cell proliferation in the uteri of the offspring. Manuscript 1.

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

[23]Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenbergh JG, Gray JrLE. Prenatal testosterone exposure permanently masculinizes anogenital distance nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol Sci 2007; 96(2):335-45.

[24]Welsh M, Sharpe RM, Walker M, Smith LB, Saunders PTK. New insight into the role of androgens in wolffian duct stabilization in male and female rodents. Endocrinology 2009; 150(5):2472-80.

[25]Wolf CJ, LeBlanc GA, Ostby JS, Gray Jr LE. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 2000; 55:152-61.

[26]McIntyre BS, Barlown NJ, Foster PM. Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. Toxicol Sci 2002; 65(1):62–70.

[27]Odum J, Lefevre PA, Tittensor S, et al. The rodent uterotrophic assay: Critical protocol features, studies with phenols, and comparision with a yeast estrogenicity assay. Regul Toxicol Pahrmacol 1997; 25:176-88.

[28]Ashby J, Lefevre PA, Odum J, et al. Failure to confirm estrogenic activity for benzoic acid and clofibrate: Implications for lists of endocrine-disrupting chemicals. Regul Toxicol Pharmacol 1997; 26:96-101.

[29]Kang KS, Kim HS, Ryu DY, Che JH, Lee YS. Immature uterotrophic assay is more sensitive than ovariectomized uterotrophic assay for the detection of estrogenicity of p-nonyphenol in Sprague-Dawley rats. Toxicol Lett 2000; 118:109-15.

[30]Andrade AJM, Araújo S, Santana GM, Ohi M, Dalsenter PR. Screening for (anti) estrogenic and (anti)androgenic activities of technical and formulated deltamethrin. Regul Toxicol Pharmacol 2002; 35(3):379–82.

[31]Brody JR, Cunha GR. Histologic, morphometric, and immunocytochemical analysis of myometrial development in rats and mice: II. Efects of DES on development. Am J Anat 1989; 186:21-42.

[32]Ojeda SR, Urbanski HF. Puberty in the rat. In: Knobil E, Neill J.D., editors**.** The physiology of reproduction. Philadelphia: Lippincott Williams & Wilkins; 1994.

[33]Branham WS, Sheehan DM. Ovarian and adrenal contributions to post-natal growth and differentiation of the uterus. Biol Reprod 1995; 53:863-72.

[34]Davis BJ, Travlos G, McShane T. Reproductive endocrinology and toxicological pathology over the life span of the female rodent. Toxicol Pathol 2011; 29:77-83.

[35]Pinilla L, Trimiño PG, Bellido C, Aguilar R, Gaytán F, Aguilar E. Changes in pituitary secretion during the early postnatal period and anovulatory syndrome induced by neonatal oestrogen or androgen in rats. J Reprod Fert 1993; 97:13-20.

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

[37]Haji M, Chuknyiska RS, Roth GS. Isolated uterine nuclei and cytosol receptors of aged rats exhibit impaired estrogenic stimulation of RNA polymerase II. Proc Natn Acad Sci U.S.A. 1984; 81:7481-4.

[38]Chuknyiska RS, Haji M, Foote RS, Roth GS. Age-associated changes in nuclear binding of rat uterine estradiol receptor complexes. Endocrinology 1985; 116:537-51.

[39]Mena MA, Camilo AA, Tchernitchin AN. Early postnatal androgenization imprints selective changes in the action of estrogen in the rat uterus. Biol Reprod 1992; 46:1080-5.

[40] Guerra MT, Silva RF, Luchiari HR, Sanabria M, Kempinas WG. Androgenic exposure during critical periods of development and possible reproductive health effects on the female rat offspring. Manuscript 2.

[41]Wilson JG, Hamilton JB, Young WC. Influence of age and presence of the ovaries on reproductive function in rats injected with androgens. Endocrinology 1941; 29(5):784-9.

[42]Gerall AA, Stone LS, Hitt JC. Neonatal androgen depresses female responsiveness to estrogen. Physiol Behav 1972; 8(1):17-20.

[43]Maurer RA, Woolley DE. Distribution of 3H-estradiol in clomiphene-treated and neonatally androgenized rats. Endocrinology 1971; 88(6):1281-7.

[44]Maurer RA, Woolley DE. Demonstration of nuclear 3H-estradiol binding in hypothalamus and amygdala of female, androgenized-female, and male rats. Neuroendocrinology 1974; 16(3-4):137-47.

[45]Maurer RA, Woolley DE. 3H-estradiol distribution in female, androgenized female, and male rats at 100 and 200 days of age. Endocrinology 1975; 96(3):755-65.

[46]Flerkó B, Mess B. Reduced oestradiol-binding capacity of androgen sterilized rats. Acta Physiol Acad Sci Hung 1968; 33(1):111-3.

[47]Vértes M, King RJB. The mechanism of oestradiol binding in rat hypothalamus: effects of androgenization. J Endocrinol 1971; 51(2):271-82.

[48]Vértes M, Barnes A, Lindner HR, King RJB. Studies on androgen and estrogen uptake by rat hypothalamus. Adv Exp Med Biol 1973; 36:137-73.

[49]Wrenn TR, Wood JR, Bitman J. Oestrogen responses of rats neonatally sterilized with steroids. J Endocrinol 1969; 45:415-20.

[50] National Toxicology Program's Report of the Endocrine disruptors low-dose Peer Review. 2001. Available online at: http://ntp.niehs.nih.gov/ntp/htdocs/liason/LowDosePeerFinalRpt.pdf . Acessed 01.15.2013.

[51]Takewaki K. Uteri of adult rat given androgen injections for thirty neonatal days. Annot Zoo Japon 1966; 39(4): 173-8.

[52] Guyton AC, Hall JE. Fisiologia Feminina antes da Gravidez e os Hormônios Femininos. In: Guyton AC, Hall JE, editors. Tratado de fisiologia médica. Rio de Janeiro: Guanabara Koogan; 2002. p. 869–82.

[53]Flerkó B, Mess B, Illei-Donhoffer A. On the mechanism of androgen sterilization. Neuroendocrinology 1969; 4:164-9.

[54]Tuohimma P, Johansson R. Decreased estradiol binding in the uterus and anterior hypothalamus of androgenized female rats. Endocrinology 1971; 88:1159-64.

[55]Petrusz P, Nagy É. On the mechanism of sexual differentiation of the hypothalamus; decreased hypothalamic oestrogen sensitivity in androgen-sterilized female rats. Acta biol Acad Sci Hung 1967; 18:21-6.

[56]Van Rees GP, Gans E. Effect of gonadectomy and oestrogen on pituitary LH-content and organ weights in androgen-sterilized female rats. Acta Endocrinol 1966; 52:471-77.

[57]Armstrong DT, Moon YS, Leung PC. Uterotrophic effects of testosterone and 5alpha-dihydrotestosterone in intact and ovariectomized immature female rats. Biol Reprod 1976; 15:107-14.

[58]Okada A, Sato T, Ohta Y, Iguchi T. Sex steroid hormone receptors in the developing female reproductive tract of laboratory rodents. J Toxicol Sci 2005; 30(2):75-89.

[59]Hirai M, Hirata S, Osada T, Hagihara K, Kato J. Androgen receptor mRNA in the rat ovary and uterus. J Steroid Biochem Molec Biol 1994; 49:1-7.

LEGENDS OF FIGURES

Figure 1. Uterine weight, at PND 25 of **A**: female offspring from corn oil group; and **B**: animals exposed to corn oil and from challenged group (exposed to 0.4mg/kg of E_2). Values are expressed as mean ± S.E.M. ANOVA with a *posteriori test* of Tukey. Diffent letters indicate statistical difference, with $p<0.05$. Each experimental group was composed by 5 animals.

Figure 2. Uterine weight of female offspring from corn oil group at adulthood (PND 130). Experimental groups: Control (*n*=7), 0.05mg/kg (*n*=9), 0.1mg/kg (*n*=9), 0.2mg/kg (*n*=7). Values are expressed as mean ± S.E.M. ANOVA with a *posteriori test* of Tukey.

Figure 3. Uterine weights of control group exposed to corn oil (basal condition) and of all experimental groups exposed to estradiol **A**: at 10µg/day and **B**: at 100µg/day. Experimental groups (*n* for each estradiol treatment): Control (*n*=7), 0.05mg/kg TP (*n*=9), 0.1mg/kg TP (*n*=9) and 0.2mg/kg TP (*n*=7). Kruskal-Wallis with a *posteriori test* of Dunn. Different letters indicate statistical significance, with $p<0.05$.

Figure 4. Pituitary weight after different doses of estradiol treatment, from female rats at PND 100, from control (*n*=7 animals) or treated with 0.05mg (*n*=9 animals), 0.1mg (*n*=9 animals) or 0.2mg (*n*=7 animals) of TP. Values expressed as mean ± S.E.M. ANOVA with a *posteriori test* of Tukey. Different letters indicate statistical significance, with $p<0.05$.

Figure 1

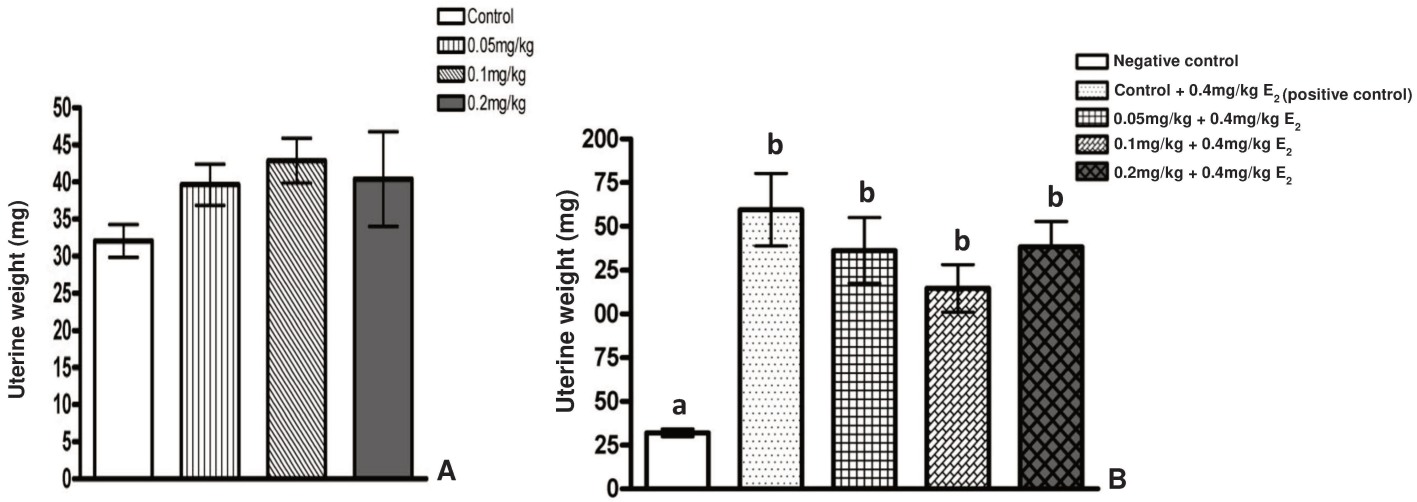
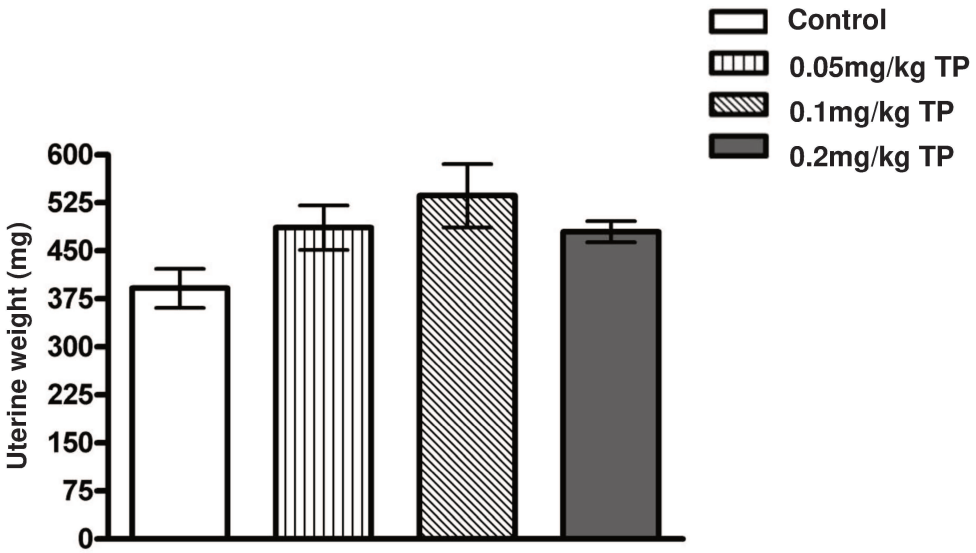

Control
0.05mg/kg
0.1mg/kg
0.2mg/kg
Uterine weight (mg)
50
45
40
35
30
25
20
15
10
5
0
A
Negative control
Control + 0.4mg/kg E2 (positive control)
0.05mg/kg + 0.4mg/kg E2
0.1mg/kg + 0.4mg/kg E2
0.2mg/kg + 0.4mg/kg E2
Uterine weight (mg)
200
75
50
25
00
75
50
25
0
a
b
b
b
b
B

Figure 2


Control
0.05mg/kg TP
0.1mg/kg TP
0.2mg/kg TP
Uterine weight (mg)
600
525
450
375
300
225
150
75
0

`

Figure 3

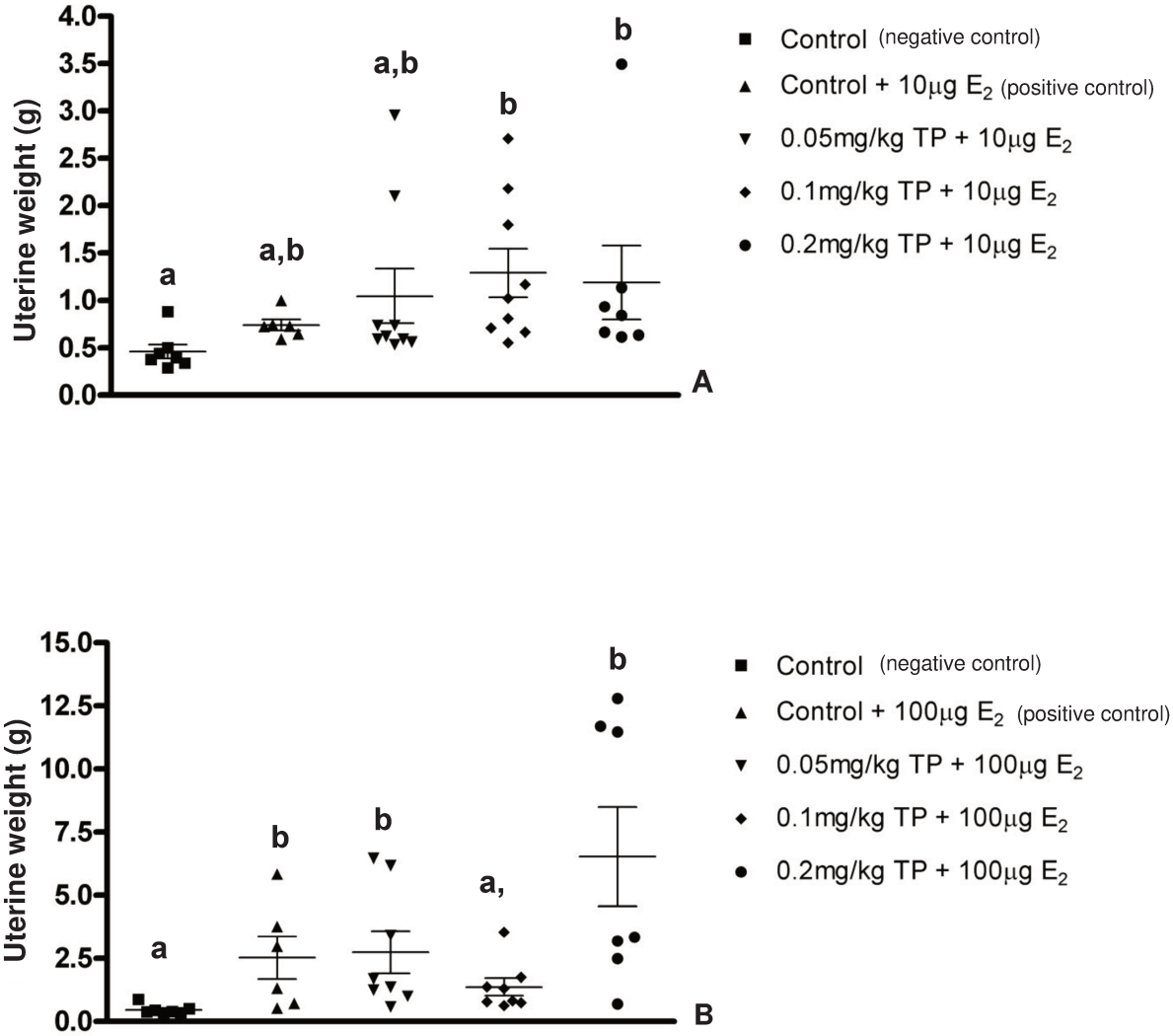
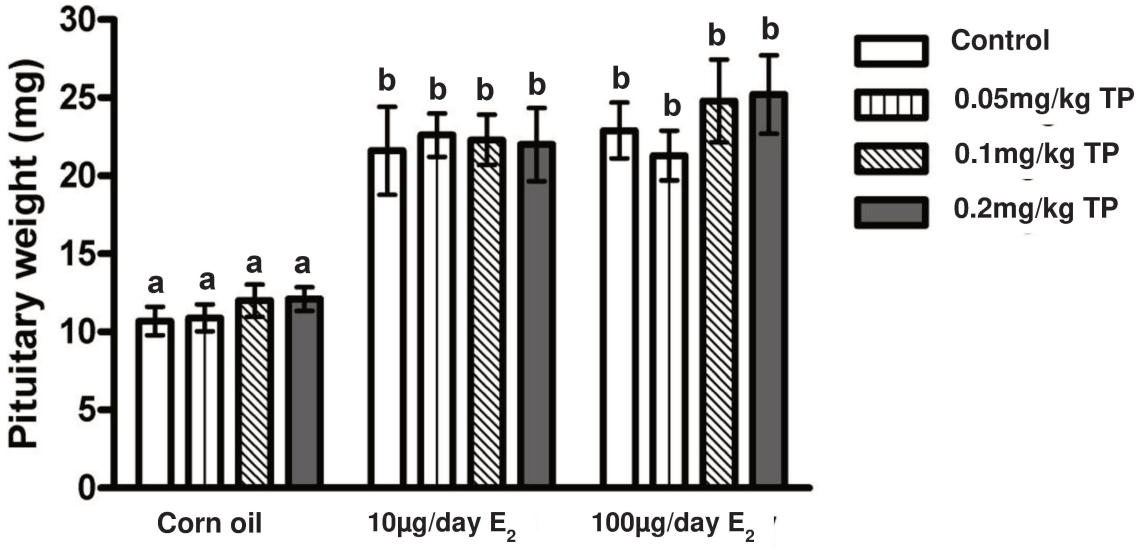

Uterine weight (g)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b
a,b
b
b
A
Control (negative control)
Control + 10μg E2 (positive control)
0.05mg/kg TP + 10μg E2
0.1mg/kg TP + 10μg E2
0.2mg/kg TP + 10μg E2
Uterine weight (g)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
a
b
b
a,
b
B
Control (negative control)
Control + 100μg E2 (positive control)
0.05mg/kg TP + 100μg E2
0.1mg/kg TP + 100μg E2
0.2mg/kg TP + 100μg E2

Figure 4


Pituitary weight (mg)
30
25
20
15
10
5
0
a
a
a
a
b
b
b
b
b
b
b
b
Corn oil
10μg/day E2
100μg/day E2
Control
0.05mg/kg TP
0.1mg/kg TP
0.2mg/kg TP

5.4. Manuscrito 4

O quarto manuscrito é intitulado “Long-term effects of perinatal androgenization on reproductive parameters of male rat offspring”, e foi aceito para publicação na revista Hormone and Metabolic Reseach, Thieme, ISSN:0018-65043, Fator de Impacto: 2,19.

LONG-TERM EFFECTS OF PERINATAL ANDROGENIZATION ON REPRODUCTIVE PARAMETERS OF MALE RAT OFFSPRING

SHORT RUNNING TITLE: ANDROGENIZATION IN MALE RAT REPRODUCTION

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ABSTRACT

It is known that, during sex differentiation, fetal androgens are critical determinants of the male phenotype. Although testosterone is necessary for normal development of male sexual behavior, perinatal androgen treatment can results in disruption of normal male sexual reproduction. Pregnant Wistar rats were administered either corn oil (vehicle) or testosterone propionate at 0.2mg/kg from gestational day 12 until the end of lactation; the reproductive function of male offspring was evaluated at 90 (adulthood) and 270 (middle age) days of age. Perinatal androgenization in the rat provoked a reduction in sperm production and reserves in adulthood that did not affect fertility and did not persist at more advanced ages, as shown by the results at post natal day 270. If perinatal androgenization promotes similar effects in humans of reproductive age, the results of the present work can impact male reproduction health, given the less efficient spermatogenesis and lower sperm reserves in the human epididymis, compared to rodents.

Key words: rat male offspring, perinatal androgenization, sperm counts, sexual behavior, fertility.

INTRODUCTION

It is known that the presence of androgens during sex differentiation is critical for the determination of the male phenotype [1]. In males, testosterone secreted by fetal and neonatal testis [2] reaches two important peaks: first between gestational day 18-19 [3] and again during the first hours after birth [4]. These surges have been shown to be important for sexual differentiation, resulting in male-typical sexual behavior in adulthood, establishment of gonadotropin secretion patterns, and also for various morphological indices [5].

Several researchers reported that although testosterone is necessary for normal development of male sexual behavior, perinatal androgen treatment of an intact male results in disruption of normal male sexual reproduction [6, 7]. In humans and rodents, exposure to hormonally active chemicals during sex differentiation can produce a wide range of abnormal sexual phenotypes [8]. Among contaminant substances, preliminary studies have focused only on environmental estrogens, but there is a growing awareness that androgenic chemicals are widespread in the environment [9].

Structural and/or functional reproductive deficiencies subsequent to male neonatal androgen administration have been reported previously [10]. These detrimental effects include not only reduced testicular, prostate and seminal gland weights but also impairment of sexual activity. Although some studies demonstrated that androgenized male rats presented alterations in hormone levels [11], others showed no variations in this parameter [12].

Based on the previous evidences that early excess exposure to androgen may cause alterations in the sexual development of male rats, the present study aimed to evaluate the exposure of pregnant and lactating female rats to a low dose of TP and assesses the possible long-term effects on sexual parameters of the androgenized male offspring.

Besides sex organ weights, fertility and sexual behavior, commonly evaluated on previous studies, our experiment includes a more complete assessment of male reproductive function by measuring sperm quality, testicular and epididymal histology and hormone assays and a different window of exposure. The exposure period coincides with the critical window of reproductive system development, which continues after birth [13].

During the developmental period, fetuses presented an incomplete machinery of DNA repair, lack of detoxification enzymes, primitive hepatic metabolism and high sensitivity to epigenetic alterations [14]. These facts generate in the fetuses a higher susceptibility to the action of environmental chemicals [15].

MATERIALS AND METHODS

Animals and androgen exposure

Adult female (60 days of age) and male (90 days of age) Wistar rats were supplied by the Central Biotherium of the State University of São Paulo (UNESP). Two non-gravid female rats were mated with one male and the day of sperm detection in the vaginal smear was considered gestational day – GD 0. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Institute of Biosciences /UNESP Ethics Committee for Animal Experimentation (protocol no. 104/2009-CEEA).

Pregnant rats (n=16) were randomly allocated into two experimental groups: exposed group (TP-exposed, n=8), that received testosterone propionate (TP- DEG Ltd., CAS 57-85-2, 97% of purity) at the dose of 0.2mg/kg, subcutaneously (s.c.), from GD 12 until post-natal day – PND 21, and control group (n=8) that received corn oil (vehicle), following the same experimental protocol. Based on this experimental design, male pups were exposed to TP via placenta (during intrauterine period) and through milk (after birth). The period of the treatment coincided with the critical period of reproductive system development, which continues after birth [13]. TP was already used previously as an androgenic disruptor [6, 16-18] and it provides a model of base testing studies for environmental androgens [17].

At PND 1 litters were weighed and reduced to eight [19] and after the last day of maternal TP exposure, male pups were weaned and maintained until adulthood to perform the assessment of reproductive endpoints, described below. All the procedures were performed at 90 (adulthood) and 270 (middle age) days of age.

Organ collection

Male rats (*n*=8, one per litter) were weighed, euthanized by CO_2 inhalation followed by decapitation, between 8:00 and 10:00 a.m. The right testis and epididymis, left vas deferens, ventral prostate and seminal gland (without the coagulating gland and full of secretion) and pituitary were collected, trimmed free of fat and weighed on a precision balance. Liver and kidneys, detoxifying organs, were also weighed.

Histological evaluation and Sertoli cell numbers

The left testis and epididymis were fixed in Alfac solution (85% of which was composed of 80% alcohol, 10% of formaldehyde and 5% of glacial acetic acid) and processed for histological analysis. Organs were examined by light microscopy following specific guidelines for toxicological studies [20]. The numbers of Sertoli cell nuclei were counted in 20 cross-sections of seminiferous tubules per rat [19; with modifications].

Hormonal analysis

After decapitation, trunk blood was collected and allowed to clot (4◦C). Serum was collected after centrifugation (4°C, 20 minutes at 2,400rpm) and stored at −20◦C until analysis. Serum FSH, LH, estrogen, progesterone and testosterone concentrations were measured using a double-antibody radioimmunoassay (RIA) kit (National Institute of Arthritis, Diabetes and Kidney Diseases–NIADDK, USA). All the samples were analyzed in the same assay to avoid inter-assay variability.

Sperm motility and morphology

Sperm motility was analyzed as described by Perobelli et al. [21] on spermatozoa collected from the right vas deferens. With the aid of a syringe and needle, sperm were recovered from the left vas deferens by flushing with 1.0 ml of saline formol. To morphological analysis, 200 spermatozoa per animal were analyzed in a phase-contrast microscope [22]. Morphological abnormalities were classified into head and tail morphology [23]. Sperm were also classified as to the presence or absence of the cytoplasmic droplet.

Daily sperm production per testis, sperm number and transit time in the epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) in the testis and spermatozoa in the caput/corpus and cauda epididymis were counted as described previously [24]. To calculate the daily sperm production (DSP), the number of spermatids

at stage 19 was divided by 6.1days, which is the duration of the seminiferous cycle when these spermatids are present in the seminiferous epithelium. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by the DSP.

Sexual behavior and natural mating

Male rats (one or two per litter) were placed individually with one sexually receptive adult female rat and the following parameters were observed for 40 min: latency to the first mount, intromission, and ejaculation; numberof intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions; and total number of ejaculations [25, 26]. Males that did not mount in the initial 10 min were considered sexually inactive [27]. After completing the sexual behavior evaluation, paired animals were kept together for an additional 4 h. After this period, males and females were separated and vaginal smears collected to confirm that mating had occurred and to determine the GD 0.

Fertility test

Twenty days after mating (GD 20), naturally inseminated females were killed by CO_2 inhalation followed by decapitation. After collection of the uterus and ovaries, the numbers of corpora lutea, implants, reabsorptions, and live and dead fetuses were determined. From these results the following parameters were calculated: fertility potential (efficiency of implantation): implantation sites/corpora lutea×100; rate of pre-implantation loss: [number of corpora lutea−number of implantations/number of corpora lutea]×100; and rate of post-implantation loss: [number of implantations–numberof live fetuses]/number of implantations×100 [28].

Statistical analysis

According to the characteristics of each variable, parametric Student's *t* test (mean ± S.E.M.) or nonparametric Mann-Whitney test [median (Q_1-Q_3)] was used. Differences were considered significant when $p < 0.05$. The statistical analyses were performed by GraphPad InStat (version 3.02).

RESULTS

At PND 90, the exposure to TP provoked a diminution of body weight that was reflected in low weights of the testis, ventral prostate, pituitary and kidneys. In relation to the epididymis and liver, the male offspring from the TP-exposed group showed a significant decrease in absolute and relative weights. On the other hand, at PND 270, all the alterations found previously were no longer detectable (Table 1).

Histopathology revealed that qualitative analysis of the epididymis and qualitative/quantitative evaluation of the testis were similar between experimental groups (data not shown). Furthermore, the numbers of Sertoli cell nuclei, at both tested ages, did not differ between experimental groups (Figure 1). However, epithelial vacuoles were found in the cauda epididymis of older animals (PND 270) from both experimental groups (69% of control group and 62% of TP-exposed group), but not in 90-day-old animals (Figure 2).

Analysis of hormonal status from male offspring at PND 90 revealed alterations in the serum levels of FSH and progesterone, which were found to be elevated and diminished, respectively. Again, these changes were not detected in the 270-day-old animals (Figure 3). At 90 and 270 days of age, the levels of estrogen, LH and testosterone were similar between experimental groups.

TP exposure caused a significant decrease in type C sperm at PND 270, but this alteration was not accompanied by any disturbance in other categories in the sperm motility evaluation. Sperm morphology analysis revealed similar percentages of normal and abnormal sperm between experimental groups whereas the cytoplasmic droplet was present in the majority of spermatozoa in both the control and TP-exposed groups (Table 2).

The evaluation of sperm counts in the testis demonstrated that the number of mature testicular spermatids and DSP were diminished at PND 90. The sperm number in the cauda epididymis/gram from TP-exposed group at PND 90 was significantly reduced when compared to controls. However, the sperm counts in the testis and epididymis at PND 270 were similar between experimental groups (Table 2).

TP exposure was not able to impair the evaluated parameters in the sexual behavior test at either tested age (Table 3). Following the sexual performance assessment, the

fertility test was performed in order to analyze the ability of androgenized male offspring to produce decedents, an endpoint also unaltered by perinatal androgenization (Table 3).

DISCUSSION

In rodents, the critical period of sexual differentiation begins in the last gestational phase and continues until the first week of post-natal life [29] and this event is determined by circulating levels of androgens during this specific period [30]. Androgen is aromatized in the brain into estradiol and is responsible for causing significant sexual dimorphism, which determines endocrine patterns and male behavior [31].

Typically, chemicals that adversely affect human sex differentiation also produce predictable alterations of this process in rodents [32]. Some compounds, known as endocrine disruptors, when administered to pregnant females and/or neonates may interfere with hypothalamic sexual differentiation of offspring [33].

There is a lack of agreement in the literature regarding the effect of androgenization on the reproductive function of male rats. Some researchers have reported reduced sexual behavior and fertility [7, 18], whereas others found an absence of such alterations [17, 34]. The results presented in this experiment revealed impairment reflected in diminished weights of body and sex organs, hormone levels and sperm reserve at PND 90. These alterations, however, were not sufficient to alter the sexual behavior pattern or the capacity to produce decedents by natural mating.

Significant changes in absolute or relative male reproductive organ weights may constitute an adverse reproductive effect [26]. Wolf et al. [17] demonstrated that male body and reproductive organ weights were not affected by *in utero* TP administration. Contrary to these results, earlier experiments revealed that androgenized male rats presented smaller testes and sex accessory glands [7, 35]. In our experiment, the alteration in body weight at PND 90 may explain the low testicular, ventral prostate, kidney and pituitary weights, but this fact was not responsible for the smaller epididymis and liver, since these organs were significantly decreased even when relative weights were compared to the control group. By PND 270 these parameters had all recovered.

Previous studies showed that such gonadotropic hormones as FSH, LH, prolactin and androgens were not altered by neonatal testosterone exposure [7], but on the other hand, Rajfer and Coffey [35], Henley et al. [18] and, most recently, Cruz et al. [36] demonstrated lower levels of circulating androgens as a result of neonatal androgenization. In the present work maternal TP administration did not alter testosterone levels, but provoked a reduction in progesterone levels and increase in FSH levels in the male offspring at 90 days, but not at 270.

FSH influences Sertoli secretion of inhibin, the major factor responsible for negative feedback of FSH release [37]. The significant increase in this hormone in 90 days-old TP treated rats may suggests a dysfunction in these cells, despite normal testicular histology and Sertoli cell nuclei counts. Besides normal pituitary weight between experimental groups, we cannot also rule out the possibility of hypothalamus or pituitary dysfunction. More studies should be performed to characterize which cell type function is compromised by early androgenization.

Progesterone, which is secreted mainly by the adrenals in males, may play an important role in the normal expression of androgen-dependent sexual behavior in male mammals [38]. Thus, the decrease in progesterone levels in the current study may be due to a late functional alteration in the adrenal gland after early androgenization [39]. Androgen receptors have been characterized in the rat adrenal gland [40] and previous studies reported that neonatal sex hormones are responsible for organizational effects on the hypothalamic-pituitary-adrenal axis of male rats [41]. A more specific and profound evaluation should be performed in order to elucidate adrenal injury after perinatal TP exposure.

In aging experiments, Serre and Robaire [42] demonstrated the emergence of a localized region of the epididymal epithelium with large clear vacuoles in Brown Norway rats at 18 months of age, pointing to a signal of aging specific to the epididymis. In the present work, the vacuoles found in the cauda epididymis of the Wistar rats, at PND 270, in both experimental groups may reflect a degree of aging that accounts for our decision to characterize them as middle-aged. However, this alteration did not impair sperm function.

During most of their reproductive period, normal individuals present a uniform efficiency of sperm production. This efficiency is highly correlated with testicular weight and is influenced by age, environmental factors, hormonal status, and drugs [43]. Although our results showed, at PND 90, diminutions in testis weight, daily sperm production and in sperm reserves in the cauda epididymidis, the sperm production efficiency (number of sperm produced per day per gram of testicular parenchyma) was not altered by maternal TP treatment. These alterations did not persist on PND 270.

There is a heightened public awareness regarding the possibility that semen quality might be declining in the human population due to environmental factors [44]. Perturbation of the testicular and epididymal biochemical environments may occur after chemical exposure, altering sperm properties [26]. In the present study, androgenized male rats showed no alterations in sperm morphology or motility, suggesting that *in utero* and lactational TP exposure did not affect spermatogenesis or the post-testicular maturation process, a finding supported by the normal sperm transit time through the epididymis.

In our present experiment, we observed an increased sperm transit time in the cauda epididymis at PND 270 when compared to 90 days-old animals; but despite this result, all experimental groups were able to fertilize female rats and generate decedents. Although previous research suggested that the delay in sperm transit through the cauda of epididymis diminishes their fertility potential [45], others showed that the spermatozoa that underwent a delay in passage through the epididymis and were therefore stored for a longer period, had a normal fertility potential [46].

Previous works have shown that supra-physiological levels of testosterone would pose a great disadvantage to male rats for successful mating [7, 18]. In the current experiment, maternal TP treatment did not impair sexual behavior of the male offspring. Moreover, the fertility test showed that early TP exposure did not affect the ability to produce offspring. Corroborating our results, Lumia et al. [16] and Cruz et al. [36] demonstrated that androgenized male offspring presented normal fertility and sexual behavior.

According to Zadina et al. [6], reproductive impairment caused by TP administration is due to estradiol, as a result of testosterone conversion. However, Frick et

al. [47] demonstrated that estradiol benzoate, when administered neonatally to male rats provoked different results compared to TP exposure. Besides, Slob et al. [48] demonstrated that TP given to rat dam is metabolized by the dam and placenta into other androgens, of which androsterone (that cannot be converted in estrogens) is the most abundant in the fetus, followed by 3-α-androstanediol and epiandrosterone. Based on this theory, we can assume that the reproductive effects followed by TP exposure are caused by androgenic activity rather than estradiol conversion.

We can conclude, based on the present results, that although perinatal androgenization in the rat provokes a reduction in sperm production and reserves in adulthood, with no effects on fertility, these effects do not persist at more advanced ages, as shown by the results at PND 270. Moreover, it is interesting to emphasize that although these animals present aging signs, their sperm and fertility parameters are comparable to those of PND 90 rats. If perinatal androgenization promotes similar effects in humans of reproductive age, the results of the present work can have an impact on male reproduction health, given the less efficient spermatogenesis and lower sperm reserves in the human epididymis, compared to rodents [49].

REFERENCES

1 Jost A, Viger B, Prepin J, Perchellet JP. Studies on sex differentiation in mammals. Recent Prog Horm Res 1973; 29: 1-4

2 Quadros PS, Pfau JL, Goldstein AYN, De Vries GJ, Wagner CK. Sex differences in progresterone receptor expression: a potential mechanism for estradiol-mediated sexual differentiation. Endocrinology 2002; 143: 3727-3739

3 Weisz J, Ward IL. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology 1980; 106: 306-316

4 Corbier P, Kerdelhue B, Picon R, Roffi J. Changes in testicular weight and serum gonadotropin and testosterone levels before, during, and after birth in the perinatal rat. Endocrinology 1978; 103: 1985-1991

5 Bakker J. Sexual differentiation of the neuroendocrine mechanisms regulating mate recognition in mammals. J Neuroendocrinol 2003; 15: 615-621

6 Zadina JE, Dunlap JL, Gerall AA. Modifications induced by neonatal steroids in reproductive organs and behavior of male rats. J Comp Physiol Psychol 1979; 93: 314-322

7 Piacsek BE, Hostetter MW. Neonatal androgenization in the male rat: evidence for central and peripheral defects. Biol Reprod 1984; 30: 344-351

8 Gray LEJr, Ostby JS, Kelce WR. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicol Appl Pharmacol 1994; 129: 46-52

9 Radl V, Pritsch K, Munch JC, Schloter M. Structural and functional diversity of microbial communities from a lake sediment contaminated with trenbolone, an endocrine-disrupting chemical. Environ Pollut 2005; 137: 345-353

10 Swanson HE, Van der Werff ten Bosch JJ. Sex differences in growth of rats and their modification by a single injection of testosterone propionate shortly after birth. J Endocrinol 1963; 26: 197-207

11 Moguilevsky JA, Sacchi P, Rubinstein L. Effect of neonatal androgenization on luteinizing hormone, follicle stimulating hormone, prolactin and testosterone levels in male rats. J Endocrinol 1977; 74: 143-144

12 Morrison RL, Johnson DC. The effect of androgenization in male rats castrated at birth. J Endocrinol 1966; 34: 117-123

13 Wolf CJ, LeBlanc GA, Ostby JS, Gray LEJr. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 2000; 55: 152-161

14 Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001; 293(5532): 1068-1070

15 Wolf CJr, Lambright C, Mann P, Price M, Cooper RL, Ostby J, Gray LEJr. Administration of potentially antiandrogenic pesticide (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethanesulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health 1999; 15: 94–118

16 Lumia AR, Thorner KM, McGinnis MY. Effects of Chronically high doses of the anabolic androgenic steroid, testosterone, on intermale aggression and sexual behavior in male rats. Physiol Behav 1994; 55: 331-335

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

18 Henley CL, Nunez AA, Clemens LG. Exogenous androgen during development alters adult partner preference and mating behavior in gonadally intact rats. Horm Behav 2010; 57: 488-495

19 Nassr AC, Arena AC, Toledo FC, Bissacot DZ, Fernandez CD, Spinardi-Barbisan AL, Pires PW, Kempinas WG. Effects of gestational and lactational fenvalerate exposure on imune and reproductive systems of male rats. J Toxicol Environ Health A 2010; 73: 952-964

20 Foley GL. Overview of male reproductive pathology. Mechanisms of male reproductive organ toxicity. Toxicol Pathol 2001; 29: 49-63

21 Perobelli JE, Martinez MF, Franchi CAS, Fernandez CDB, Camargo JLV, Kempinas WG. Decreased sperm motility in rats orally exposed to single or mixed pesticides. J Toxicol Environ Health A 2010; 73: 991-1002

22 Seed J, Chapi RE, Clegg ED, Dostal LA, Foote RE, Hurtt ME, Klinefelter GR, Makris SL, PEerreault SD, Schrader S, Seyler D, Sprando R, Treinen KA, Veeramachaneni DN, Wise LD. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. Reprod Toxicol 1996; 10: 237–244

23 Filler R. Methods for evaluation of rats epididymal sperm morphology. In: Chapin RE, Heindel JH (eds). Male reproductive toxicology. California: Academic Press, 1993: 334–343

24 Fernandes GSA, Arena AC, Fernandez CDB, Mercadante A, Barbisan LF, Kempinas WG. Reproductive effects in male rats exposed to diuron. Reprod Toxicol 2007; 23: 106–112

25 Ågmo A. Male rat sexual behavior. Brain Res Protoc 1997; 1: 203–209

26 Clegg ED, Perreault SD, Klinefelter GR. Assessment of male reproductive toxicology. In: Hayes AW (ed). Principles and methods of toxicology. Philadelphia: Taylor & Francis, 2001: 1263–1299

27 Perobelli JE, Alves TR, Toledo FC, Fernandez CDB, Anselmo-Franci JA, Klinefelter GR, Kempinas WG. Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. Reprod Toxicol 2012; 33: 308-315

28 Damasceno DC, Kempinas WG, Volpato GT, Consonni M, Rudge MVC, Paumgartten FJR. Modelos experimentais – Laparotomia. In: Anomalias congênitas. Belo Horizonte: Coopmed, 2008: 13-21

29 MacLusky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science 1981; 211: 1294-1303

30 Hoepfner BA, Ward IL. Prenatal and neonatal androgen exposure interact to affect sexual differentiation in female rats. Behav Neurosci 1988; 102: 61-65

31 Sakuma Y. Gonadal steroid action and brain sex differentiation in the rat. J Neuroendocrinol 2009; 21: 410-414

32 Gray LE. Chemical-induced alterations of sexual differentiation: a review of effects in humans and rodents. In: Colborn T, Clement C (eds). Advances in modern environmental toxicology vol XXI. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Princeton: Princeton Scientific, 1992: 203-230

33 Gerardin DCC, Pereira OCM. Reproductive changes in male rats treated with an aromatase inhibitor. Pharmacol Biochem Behav 2002; 71: 309-313

34 Maqueo M, Kincl FS. Testicular histomorphology of young rats treated with oestradiol 17β-benzoate. Acta Endocrinol 1964; 46: 25-30

35 Rajfer J, Coffey DS. Effect of neonatal steroids on male se tissues. Invest Urol 1979; 17: 3-8

36 Cruz CD, Pereira OCM. Prenatal testosterone supplementation alters puberty onset, aggressive behavior, and partner preference in adult male rats. J Physiol Sci 2012; 62: 123-131

37 Norris DO. The Endocrinology if mammalian reproduction. In: Norris DO, ed. Vertebrate Endocrinology. San Diego: Academic Press, 1997: 357-406

38 Witt DM, Young LJ, Crews D. Progesterone and sexual behavior in males. Psychoneuroendocrinology 1994; 19: 553-562

39 Kalra PS, Kalra SP. Circadian periodicities of serum androgens, progesterone, gonadotropins and luteinizing hormone-releasing hormone in male rats: The effects of hypothalamic deafferentation, castration and adrenalectomy. Endocrinology 1977; 10: 1821-1827

40 Rifka SM, Cutler GB Jr, Sauer MA, Louriaux DL. Rat adrenal androgen receptor: a possible mediator of androgen-induced decrease in rat adrenal weight. Endocrinology 1978; 103: 1103-1110

41 McCormick CM, Furey BF, Child M, Sawyer MJ, Donohue SM. Neonatal sex hormones have 'organizational'effects on the hypothalamic-pituitary-adrenal axis of male rats. Dev Brain Res 1998; 105: 295-307

42 Serre V, Robaire B. Segment-specific morphological changes in aging brown Norway rat epididymis. Biol Reprod 1998; 58: 497-513

43 Amann RP. A Critical review of methods for evaluation of spermatogenesis from seminal characteristics. J Androl 1981; 2: 37-58

44 Andersson AM, Jørgensen N, Main KM, Toppari J, Rajpert-DeMeyts E, Leffers H, Juul A, Jensen TK, Skakkebaek NE. Adverse trends in male reproductive health: we may have reached a crucial 'tipping point'. Int J Androl 2008; 31: 74-80

45 Orgebin-Crist MC. Studies on the function of the epididymis. Biol Reprod 1969; 1: 155-175

46 Kempinas WG, Suarez JD, Roberts NL, Strader L, Ferrell J, Goldman JM, Klinefelter GR. Rat epididymal sperm quantity, quality, and transit after guanethidine-induced sympathectomy. Biol Reprod 1998; 59: 890-896

47 Frick J, Chang CC, Kincl FA. Testosterone plasma levels in adult male rats injected neonatally with estradiol benzoate or testosterone propionate. Steroids 1969; 13: 21-27

48 Slob AK, den Hamer R, Woutersen PJA, van der Werff ten Bosch JJ. Prenatal testosterone propionate and postnatal ovarian activity in the rat. Acta Endocrinol 1983; 103: 420-427

49 Amann RP. Detection of alterations in testicular and epididymal function in laboratory animals. Environ Health Perspect 1986; 70: 149-158

LEGENDS OF FIGURES

Figure 1. Number of Sertoli cell nuclei of male offspring from control and TP-exposed groups (*n*=8 animals/ group). Values are expressed as mean ± S.E.M. Student-t test.

Figure 2. Longitudinal sections of the epididymis from male rats from control and TP-exposed groups. A-D: at PND 90 (A: caput and C: cauda of control group; B: caput and D: cauda of TP-exposed group); E-H: at PND 270 (E: caput and G: cauda of control group; F: caput and H: cauda of TP-exposed group). Arrows: epithelial vacuoles. 200x. H&E.

Figure 3. Serum hormone levels of male rats from control and TP-exposed groups (*n*=8 animals/group) at both studied ages. Values are expressed as mean ± S.E.M. Student-t test. *$p<0.05$.

LEGENDS OF TABLES

Table 1.Body and organ weights of male offspring (PND 90 and 270) from control and TP-exposed groups.

Table 2.Sperm counts, motility and morphology of male offspring (PND 90 and 270) from control and TP-exposed groups.

Table 4.Sexual behavior parameters of male offspring (PND 90 and 270, one or two per litter) from control and TP-exposed groups. The numbers in parenthesis indicate the number of animals performing the behavior.

Table 5.Fertility of male offspring (PND 90 and 270, one or two per litter) from control and TP-exposed groups.

Figure 1

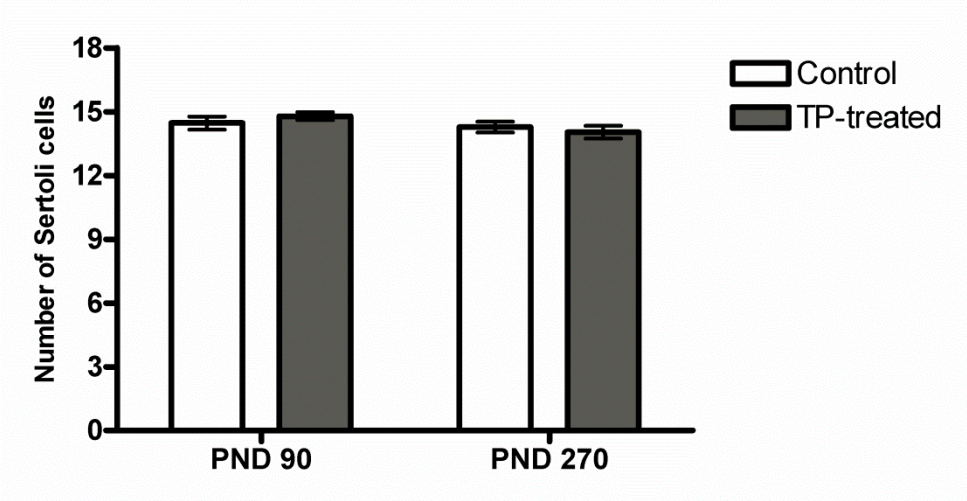
Number of Sertoli cells
18
15
12
9
6
3
0
PND 90
PND 270
Control
TP-treated

Figure 2

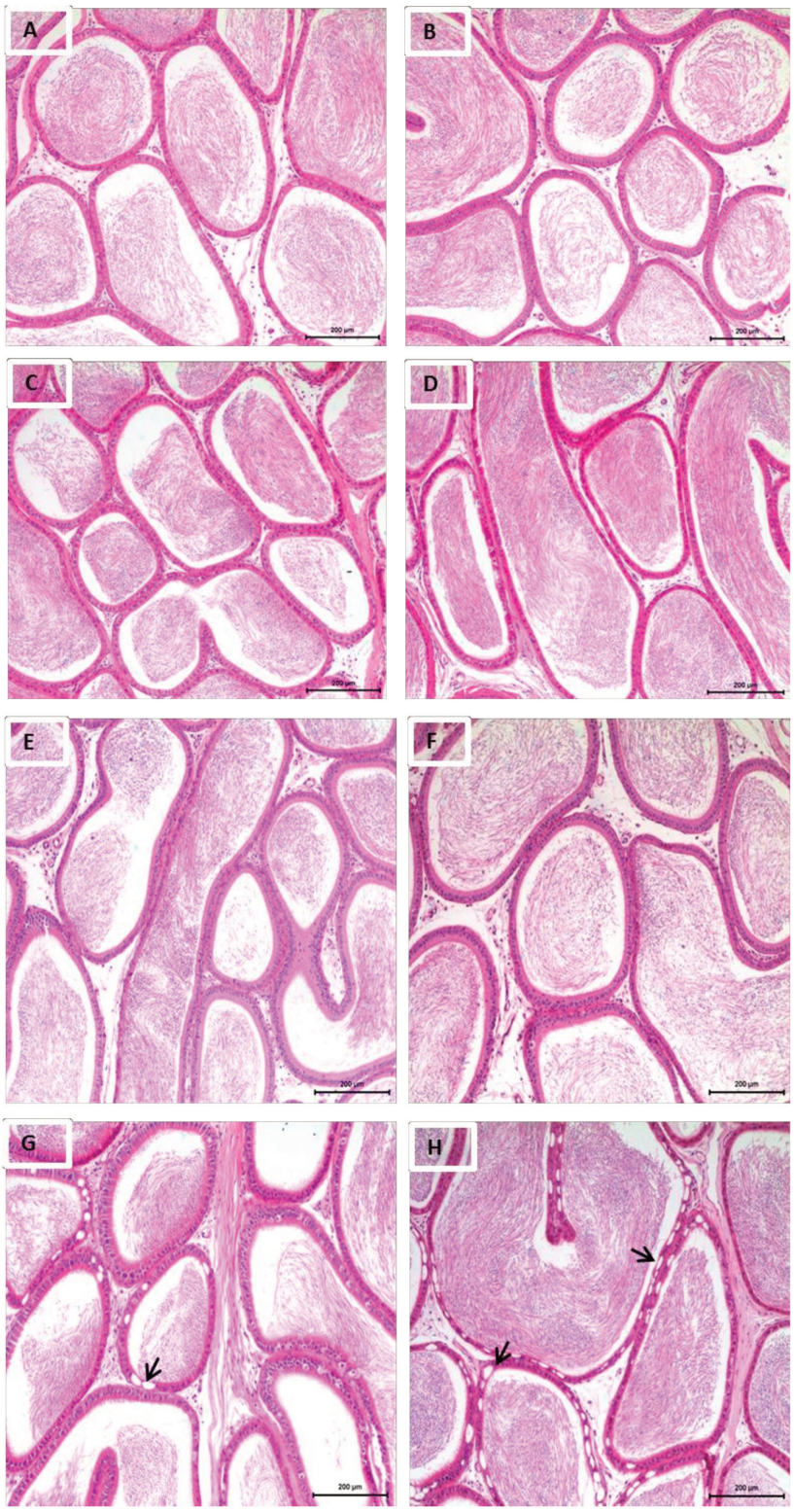

Figure 3

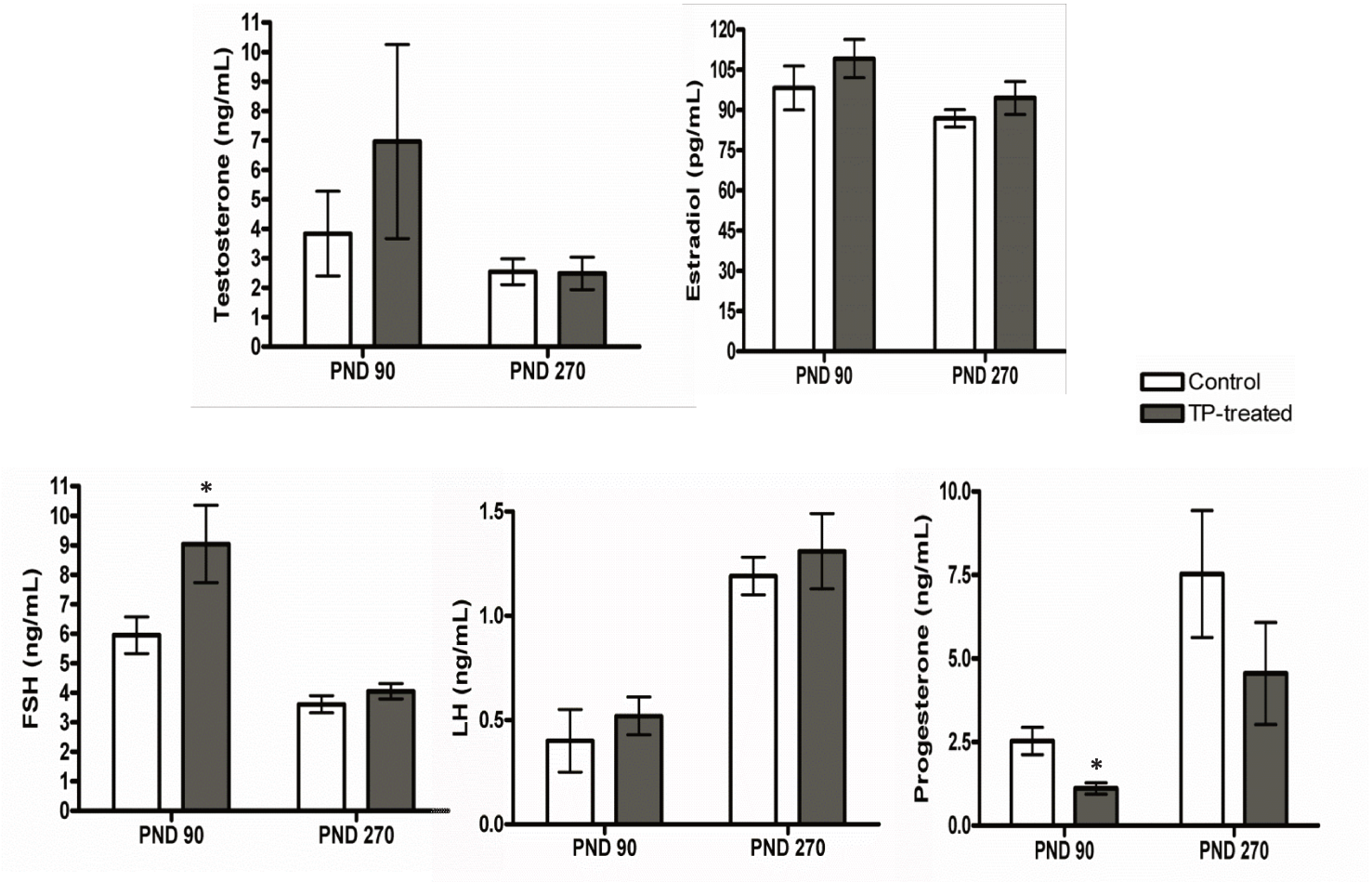
Testosterone (ng/mL)
Estradiol (pg/mL)
PND 90
PND 270
Control
TP-treated
FSH (ng/mL)
LH (ng/mL)
Progesterone (ng/mL)
*

Table 1. Body and organ weights of male offspring (PND 90 and 270) from control and TP-exposed groups.

Parameters	**PND 90**		**PND 270**	
	Control (n=8 animals)	**TP-treated (n=8 animals)**	**Control (n=8 animals)**	**TP-Treated (n=8 animals)**
Body weight (g)	386.38 ± 13.72	286.63 ± 11.85*	503.97 ± 22.98	452.19 ± 23.21
Testis (g)	1.56 ± 0.03	1.23 ± 0.05*	1.67 ± 0.04	1.53 ± 0.06
Testis (g)/100g	0.41 ± 0.01	0.43 ± 0,01	0.34 ± 0.02	0.34 ± 0.01
Epididymis (mg)	518.18±11.64	423.00± 12.78*	672.27± 14.04	639.37 ±27.63
Epididymis (mg)/100g	135.17 ± 4.52	148.34 ± 3.73*	135.70 ± 5.74	142.53 ± 4.98
Ventral prostate (mg)	386.40±13.68	291.25±23.24*	501.68±37.57	514.70 ±58.51
Ventral prostate (mg)/100g	101.58 ± 6.34	102.15 ± 7.65	99.99 ± 6.91	116.78 ± 14.79
Full seminal vesicle (g)	1.18 ± 0,05	1.06 ± 0.07	1.54 ± 0.09	1.60 ± 0.12
Vas deferens (mg)	81.06±4.23	70.79±4.09	111.67± 3.50	99.62 ±4.25
Vas deferens (mg)/100g	21.03 ± 0.95	24.88 ± 1.46	22.69 ±1.38	22.52 ± 1.75*
Pituitary (mg)	9.76 ± 0.49	7.89 ± 0.35*	9.29 ± 0.91	15.16 ± 4.54
Pituitary (mg)/00g	2.53 ± 0.10	2.76 ± 0.11	1.83 ± 0.13	3.30 ± 0.92
Kidneys (g)	2.63 + 0.10	1.95 ± 0.08*	3.25 ± 0.14	2.74 ± 0.15
Kidneys (g)/100g	0.68 ± 0.02	0.68 ± 0.01	0.65 ± 0.02	0.60 ± 0.01
Liver (g)	11.90 + 0.41	10.11 ± 0.38*	14.44 ± 0.59	12.56 ± 0.78
Liver(g)/100g	3.09 + 0.05	3.54 ± 0.07*	2.88 ± 0.08	2.77 ± 0.07

Values are expressed as mean ± S.E.M. Student-t test. *p< 0.05.

Table 2. Sperm motility and morphology of male offspring (PND 90 and 270) from control and TP-exposed groups.

	PND 90		PND 270	
Sperm motility (%)	Control (n=8 animals)	TP-treated (n=8 animals)	Control (n=8 animals)	TP-treated (n=8 animals)
Type A (motile, progressive)	83.00 (65.00 - 88.00)	77.00 (76.75 - 81.50)	86.00 (79.25 - 88.75)	83.00 (73.00 - 83.50)
Type B (motile, non-progressive)	17.00 (12.00 - 25.00)	16.00 (12.00 - 19.25)	12.50 (8.50 - 18.25)	16.00 (15.00 - 20.00)
Type C (immotile)	3.00 (1.00 - 9.00)	5.00 (2.75 - 7.50)	3.50 (2.25 - 5.75)	2.00 (1.00 - 2.00)*
Sperm morphology (%)				
Normal shaped sperm	75.00 (70.00 - 76.00)	69.25 (65.00 - 72.75)	66.50 (64.50 - 68.25)	64.00 (62.00 - 66.00)
Sperm with cytoplasmic droplet	65.00 (63.50 - 66.00)	64.00 (60.25 - 66.12)	67.77 (66.75 - 74.00)	68.00 (66.75 - 69.50)

Values are expressed as median (Q_1-Q_3). Mann-Whitney test.*$p < 0.05$.

Table 3. Sperm counts in the testis and epididymis of male offspring (PND 90 and 270) from control and TP-exposed groups.

	Experimental groups (*n*=8 animals)			
	PND 90 (n=8 animals)		**PND 270** (n=8 animals)	
	Control	TP-treated	Control	TP-treated
Mature spermatid number ($x10^6$/testis)	186.05 ± 4.93	147.25 ± 10.63*	185.74 ± 4.50	174.70 ± 11.46
Mature spermatid number ($x10^6$/g testis)	153.40 ± 9.30	143.33 ± 7.40	134.72 ± 4.17	138.90 ± 9.48
Daily sperm production ($x10^6$/testis/day)	30.50 ± 0.81	24.14 ± 1.74 *	30.45 ± 0.73	28.64 ± 1.88
Relative daily sperm production ($x10^6$/g testis/ day)	25.15 ± 1.52	23.50 ± 1.21	22.09 ± 0.68	22.77 ± 1.55
Caput/corpus epididymis sperm number ($x10^6$/organ)	105.82 ± 7.53	84.10 ± 11.08	124.16 ± 10.71	114.15 ± 13.24
Caput/corpus epididymis sperm number ($x10^6$/ g organ)	601.09 ± 47.84	586.09 ± 31.30	488.75 ± 19.44	481.41 ± 30.67
Sperm transit time in the caput/corpus epididymis (days)	3.51 ± 0.31	3.45 ± 0.31	4.08 ± 0.34	4.05 ± 0.46
Cauda epididymis sperm number ($x10^6$/organ)	209.46 ± 13.95	176.84 ± 13.43	290.31 ± 16.93	238.96 ± 24.43
Cauda epididymis sperm number ($x10^6$/g organ)	1776.90 ± 57.55	1520.60 ± 81.94*	1420.20 ± 75.38	1425.60 ± 42.38
Sperm transit time in the cauda epididymis (days)	6.91 ± 0.51	7.41 ± 0.54	9.48 ± 0.38	8.48 ± 0.86

Values are expressed as mean ± S.E.M. Student-t test. *p<0.05.

Table 4. Sexual behavior parameters of male offspring (PND 90 and 270) from control and TP-exposed groups. The numbers in parenthesis indicate the number of animals performing the behavior.

	PND 90		**PND 270**	
	Control	**TP-treated**	**Control**	**TP-treated**
	***n*=11 animals**	***n*=8 animals**	***n*=10 animals**	***n*=8 animals**
Number of mounts	4.00 ± 1.41 (*n*=5)	4.80 ± 1.24 (*n*=5)	2.57 ± 0.68 (*n*=7)	2.14 ± 1.32 (*n*=7)
Latency of the first intromission (s)	78.40 ± 49.01 (*n*=5)	196.83 ± 76.02 (*n*=6)	203.14 ± 83.10 (*n*=7)	64.14 ± 30.52 (*n*=7)
Number of intromissions until the first ejaculation	18.00 ± 2.70 (*n*=5)	14.17 ± 1.25 (*n*=6)	16.71 ± 2.94 (*n*=7)	16.43 ± 1.85 (*n*=7)
Latency of the first ejaculation (s)	807.40 ± 171.22 (*n*=5)	699.00 ± 203.01 (*n*=6)	628.75 ± 152.53 (*n*=4)	1008.20 ± 884.00 (*n*=5)
Latency of the first post-ejaculatory intromission (s)	269.00 ± 67.33 (*n*=5)	345.60 ± 35.60 (*n*=5)	315.50 ± 27.63 (*n*=4)	364.75 ± 32.11 (*n*=4
Number of post-ejaculatory intromissions	17.00 ± 5.13 (*n*=5)	17.40 ± 3.61 (*n*=5)	16.25 ± 3.66 (*n*=4)	8.00 ± 3.55 (*n*=6)
Number of ejaculations	2.20 ± 0.37 (*n*=5)	2.33 ± 0.42 (*n*=6)	2.00 ± 0.68 (*n*=6)	1.57 ± 0.43 (*n*=7)

Values are expressed as mean ± S.E.M. Student-t test.

Table 5. Fertility of male offspring (PND 90 and 270) from control and TP-exposed groups.

	Control (n=11 animals)	TP-treated (n=8 animals)	Control (n=10 animals)	TP-treated (n=8 animals)
	PND 90		PND 270	
Gestation rate (%)[a]	100	100	90	100
Fertility potential (%)[b]	100 (100 – 100)	100 (98.53 – 100)	100 (93.33 – 100)	100 (98.33 - 100)
Final body weight (g) [a]	379.43 ± 7.86	364.71 ± 10.22	397.20 ± 11.11	388.30 ± 9.07
Uterus + fetuses weight (g) [a]	66.77 ±3.59	68.11 ± 3.50	85.16 ± 4.33	83.09 ± 2.22
Rat weight – (uterus +fetuses) (g) [a]	312.66 ± 5.60	296.61 ± 8.24	312.04 ± 7.94	305.2 ± 8.77
Number of fetuses [a]	12.82 ± 0.70	13.63 ± 0.75	12.89 ± 0.51	12.50 ± 0.33
Number of implantations [a]	13.55 ± 0.73	14.50 ± 0.42	13.44 ± 0.44	13.00 ± 0.27
Number of corpora lutea [a]	14.18 ± 0.33	14.75 ± 0.53	13.78 ± 0.43	13.25 ± 0.37
Fetuses weights (g) [a]	3.20 ± 0.05	3.12 ± 0.09	4.91 ± 0.15	5.00 ± 0.05
Placenta weights (g) [a]	0.53 ± 0.01	0.52 ± 0.02	0.52 ± 0.02	0.53 ±0.01
Sex ratio (M:F) [a]	1.13 ± 0.14	1.81 ± 0.68	1.36 ± 0.31	1.26 ± 0.28
Pre-implantational loss (%)[b]	0 (0 - 0)	0 (0 – 1.47)	0 (0 – 6.67)	0 (0 – 1.67)
Post-implantational loss (%)[b]	0 (0 – 10.83)	3.33 (0 – 8.69)	0 (0 –7.14)	0 (0 – 7.28)

[a]Values are expressed as mean ± S.E.M. Student-t test.
[b]Values are expressed as median (Q_1-Q_3). Mann-Whitney test.

Conclusões finais

6. Conclusões finais

Os resultados provenientes da exposição ao propionato de testosterona durante os períodos inicias de desenvolvimento na prole de ratos demonstraram alterações na intensidade de marcação dos receptores esteroides uterinos em todas as doses testadas, diminuição nos índices de proliferação e morte celular no tecido uterino nas doses de 0,1 e 0,2mg/kg e anormalidades na resposta uterina a estimulação estrogênica nas doses de 0,1 e 0,2mg/kg. Em relação aos filhotes do sexo masculino, a exposição perinatal a andrógenos provocou uma redução na produção espermática e nas reservas espermáticas na idade adulta.

Estas alterações, entretanto, não foram capazes de prejudicar, tanto na prole feminina quanto masculina, a diferenciação sexual, a fisiologia do trato genital e a capacidade de gerar descendentes. Podemos concluir desta forma, que a exposição perinatal a andrógenos nas doses utilizadas neste experimento provocou o aparecimento de alterações tardias no sistema genital feminino e masculino, mas repercussões, entretanto, não foram capazes de alterar o desempenho geral de fertilidade em ambos os sexos.

Referências da introdução

6.1. Referências da introdução

Adamali HI, Hermo L. Apical and narrow cells are distinct cell types differing in their strucutre, distribution, and functions in the adult rat epididymis. J Androl 1996; 17:208-22.

Adkins-Regan E. Sex hormones and sexual orientation in animals. Psychobiology 1988; 16(4):335-47.

Adler ID. Comparision of the duration of spermatogenesis between male rodents and humans. Mutat Res 1996; 352:169-72.

Agarwal A, Hoffer AP. Ultrastructural studies on the development of the blood-epididymis barrier in immature rats. J Androl 1989; 10:425-31.

Alden RH. The oviduct and egg transport in the albino rat. Anat Rec 1942; 84: 137-69.

Amann RP, Schanbacher BD. Physiology of male reproduction. J Anim Sci 1983; 57:380-403.

Amann RP. Detection of alterations in testicular and epididymal function in laboratory animals. Environ Health Perspect 1986; 70:149-58.

Amann RP. Sperm production rates. In: Johnson AD, Gomes WR, Van-Demark NL, editores. The Testis. Volume 1. New York: Academic Press; 1970. p.433.

Andrews WW, Ojeda SR. A detailed analysis of the serum luteinizing hormone secretory profile in conscious, free-moving female rats during the time of puberty. Endocrinology 1981; 190(6):2032-9.

Baird DT. In: Austin CR, Short RV, editores. Hormonal control of Reproduction. Cambridge: Cambridge University Press; 1984. p.91-114.

Bakker J. Sexual differentiation of the neuroendocrine mechanisms regulating mate recognition in mammals. J Neuroendocrinol 2003; 15(6):615-21.

Bentvelsen FM, Brinkmann AO, van der Schoot P, van der Linder JE, van der Kwast TH, Boersma WJ et al. Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. Mol Cell Endocrinol 1995; 113: 245-53.

Biggers JD. Oogenesis. In: Gold JJ, editor. Gynecologic endocrinology. New York: Harper & Row; 1975. p.612-620.

Boué J, Boué A, Lazar P. Retrospective and prospective epidemiological studies of 1,500 karyotyped spontaneous abortions. Teratology 1975; 12:11-26.

Branham WS, Sheehan DM, Zehr DR, Medlock KL, Nelson CJ, Ridlon E. Inhibition of uterine gland genesis by tamoxifen. Endocrinology 1985 [b];117:2238-48.

Branham WS, Sheehan DM, Zehr DR, Ridlon E, Nelson CJ. The postnatal ontogeny of rat uterine glands and age-related effects of 17 beta-estradiol. Endocrinology 1985[a]; 117(5):2229-37.

Branham WS, Sheehan DM. Ovarian and adrenal contributions to postnatal growth and differentiation of the rat uterus. Biol Reprod 1995; 53:863-72.

Branham WS, Zehr DR, Chen JJ, Sheehan DM. Alterations in developing rat uterine cell populations after neonatal exposure to estrogens and antiestrogens. Teratology 1988 [b]; 38: 271-79.

Branham WS, Zehr DR, Chen JJ, Sheehan DM. Uterine abnormalities in rat exposed neonatally to diethylstilbestrol, ethynylestradiol, or clomiphene citrate. Toxicology 1988 [a]; 51: 201-12.

Brody JR, Cunha GR. Histologic, morphometric, and immunohistochemical analysis of myometrial development in rats and mice: I. Normal development. Am J Anat 1989; 186:1-20.

Brooks JE, Busch RD, Patanelli DJ, Steelman SL. A study of the effects of a new anti-androgen on the hyperplastic dog prostate. Proc Soc Exp Biol Med 1973; 143:647-55.

Burger LL, Haisenleder DJ, Wotton EM, Aylor KW, DAlkin AC, Marshall JC. The regulation of FSH beta transcription by gonadal steroids: testosterone and estradiol modulation of the activin intracellular signaling pathway. Am J Physiol Endocrinol Metab 2007; 293:E227-E285.

Cameron JL. Metabolic cues for the onset of puberty. Horm Res 1991; 36:97-103.

Carter DA, Saridaki E, Lightman SL. Sexual differentiation of oxytocin stress responsiveness: effect of neonatal androgenization, castration and luteinizing hormone releasing hormone antagonist. Acta Endocrinol 1988; 117:525-30.

Cheung KH, Leung GP, Leung MC, Shum WW, Zhou WL, Wong PY. Cell-cell interaction underlies formation of fluid in the male reproductive tract of the rat. J Gen Physiol 2005; 125(5):443-54.

Chowen-Breed JA, Steiner RA. Neropeptide gene regulation during pubertal development in the male rat. In: Delemarre-van de Waal HA, editor. Control of the onset of puberty III. New York: Elsevier Science Publishers; 1989. p.63-77.

Clark JH, Gorski J. Ontogeny of the estrogen receptor during early uterine development. Science 1970; 169: 76-8.

Clegg ED, Perreault SD, Klinefelter GR. Assessment of male reproductive toxicity. In: Hayes AW, editor. Principles and methods of toxicology. 4° edição. Philadelphia: Taylor e Francis; 2001. p.1263-4.

Clermont L, Perey B. Quantitative study of the cell population of the seminiferous tubules in immature rats. Am J Anat 1957; 100: 241-67.

Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 1972; 52:198-236.

Clulow J, Jones RC, Hensen LA, Man SY. Fluid and electrolity reabsorption in the ductuli efferentes testis. J Reprod Fertil Suppl 1998; 53:1-14.

Comhaire FH, Vermeulen A. Testosterone concentrations in the fluids of seminiferous tubules, the interstitium and the rete testis of the rat. J Endocrinol 1976; 70:229-35.

Cooper TG. The epididymis, sperm maturation and fertilization. Springer-Verlag, New York. 1986.

Corbier P, Kerdelhue B, Picon R, Roffi J. Changes in testicular weight and serum gonadotropin and testosterone levels before, during, and after birth in the perinatal rat. Endocrinology 1978; 103:1985-91.

Crisp TM, Clegg ED, Cooper RL, Morrow WP, Anderson DG, Baetcke KP et al. Environmental endocrine disruption: an effects assessment and analysis. Environ Health Perspect 1998; 106(1):11-56.

Cruz CD, Pereira OCM. Prenatal testosterone supplementation alters puberty onset, aggressive behavior, and partner preference in adult male rats. J Physiol Sci 2012; 62:123-31.

Cunha GR. Epithelial-stromal interactions in development of the urogenital tract. Int Rev Cytol 1976; 47:137-94.

Davis WP, Bortone SW. Effects of mill effluent on fish sexuality. In:Colborn T, Clements C, editors. Advances in modern environmental toxicology. Chemically induced alterations in sexual and fuctional development: the wildlife/human connection. Vol XXI. Princeton, NJ: Princeton Scientific; 1992. p.113-27.

Del Vecchio, FR. Normal development, growth, and aging of the female genital tract. In: Mohr U, Dungworth DL, Capen CC, editores. Pathobiology of the aging rat. Volume 1. Washington DC: ILSI Press; 1992. p.331-6.

Diamond M, Llacuna A, Wong CL. Sex behavior after neonatal progesterone, testosterone, estrogen, or antiandrogens. Horm Behav 1973; 4:73-88.

Diaz DR, Fleming DE, Rhees RW. The hormone-sensitive early postnatal periods for sexual differentiation of feminine behavior and luteinizing hormone secretion in male and female rats. Dev Brain Res 1995; 86: 227-32.

Dohler KD, Wuttke W. Changes with age in levels of serum gonadotropins, prolactin, and gonadal steroids in prepubertal male and female rat. Endocrinology 1975; 97:898-907.

Drews U. Local mechanisms in sex specific morphogenesis. Cytogenet Cell Genet 2000; 91: 72-80.

Dunlap KD, Sridaran R. Plasma levels of dihydrotestosterone in the cycling rat: implications for the regulation of lordosis behavior. Physiol Behav 1988; 42:199-202.

Durhan EJ, Lambright CS, Makynen EA, Lazorchak J, Hartig PC, Wilson VS et al. Identification of metabolites of trembolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect 2006; 114(1):65-8.

Dyche WJ. A comparative study of the differentiation and involution of the Mullerian duct and Wolffian duct in the male and female fetal mouse. J Morphol 1979; 162: 175-209.

Dym M. The mammalian rete testis – A morphological examination. Anat Rec 1976; 186:493-534.

Edwards TM, Moore BC, Giellette Jr LJ. Reproductive dysgenesis in wildlife: a comparative view. Int J Androl 2006; 29:109-21.

FDA - Food and Drug Administration.NADA summary #138-612, 1996. Disponível online em http://www.fda.gov/cvm/efoi/section1/138612070286.html. Acesso em maio de 2011.

Foley GL. Overview of male reproductive pathology. Toxicol Pathol 2001; 29(1):49-63.

Ford JJ. Testicular control of defeminization in male pigs. Biol Reprod 1982; 27:425.

Forsberg JG. Histogenesis of irreversible changes in the female genital tract after perinatal exposure to hormones and related substances. In: Mori T, Nagasawa H, editores. Toxicity of hormones in perinatal life. Boca Raton: CRC Press; 1988. p. 39-62.

Frawley LS, Hendricks DM. Model of gonadotropin secretion in infantile female rats and the role of estrogen in feedback regulation. Endocrinology 1979; 108: 1064-72.

Frick J, Chang CC, Kincl FA. Testosterone plasma levels in adult male rats injected neonatally with estradiol benzoate or testosterone propionate. Steroids 1969; 13(1):21-7.

George FW, Wilson JD. Gonads and ducts in mammals. In: Knobil E, Neil JD, editores. The physiology of reproduction. 2° edição. New York: Raven Press; 1994. p.3-28.

Ginsburg M, Snow MH, McLaren A. Primordial germ cells in the mouse embryo during gastrulation. Development 1990; 110:521-8.

Gore-Langton RE, Armstrong DT. In: Knobil E, Neill JD, editores. The physiology of reproduction. New York: Raven Press; 1994. p. 571-627.

Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF et al. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod 2001; 64: 1608-13.

Gray, L.E.,Jr.; Wilson, V.S.; Stoker, T.; Lambright, C.; Furr, J.; Noriega, N.; Howdeshell, K.; Ankley, G.T.; Guillette, L. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int J Androl., v.29, p.96-104,2006.

Greene, R.; Burrill, M.; Ivy, A. The effect of antenatal androgens on sexual development in female rats. Am J Anat., v.65, p.415-469, 1939.

Guerra MT, de Toledo FC, Kempinas WG. In utero and lactational exposure to fenvalerate disrupts reproductive function in female rats. Reprod Toxicol 2011; 32(3):298-303.

Guyton AC, Hall JE. Fisiologia Feminina antes da Gravidez e os Hormônios Femininos. In: Guyton AC, Hall JE, editores. Tratado de fisiologia médica. Rio de Janeiro: Guanabara Koogan; 2002 [a]. p. 869–82.

Guyton AC, Hall JE. Funções reprodutoras e hormonais masculinas (e função da glândula pineal). In: Guyton AC, Hall JE, editores. Tratado de Fisiologia Médica. 10° edição. Rio de Janeiro: Guanabara-Koogan; 2002[b]. p.857-68.

Habert R, Picon R. Testosterone, dihydrotestosterone and estradiol-17β levels in maternal and fetal plasma and in fetal testes in the rat. J Steroid Biochem 1984; 21:193-8.

Haisenleder DJ, Yasin M, Marshall JC. Gonadotropin subunit and gonadotropin-releasing hormone receptor gene expression are regulated by alterations in the frequency of calcium pulsatile signals. Endocrinology 1997; 138: 5227-30.

Hall PF. In: Knobil E, Neill JD, editores. The physiology of reproduction, New York:Raven Press; 1994. p. 1335-62.

Hamilton DW. Structure and function of the epithelium lining the ductuli efferentes, ductus epididymidis and ductus deferents in the rat. In: Greep RO, Astwood EB, editores. Handbook of physiology. Sec 7, vol 5. Washington DC: American Physiological Society; 1975. p.259-301.

Hamilton DW. The mammalian epididymis. In: Balin H, Glassner S, editores. Reproductive Biology. Amsterdam: Excerpta Medica; 1972. p. 268-337.

Haschek W, Rousseaux CG. Male reproductive system. In: Haschek W, Rousseaux CG, editores. Fundamentals of toxicologic pathology. San Diego: Academic Press, 1998. p.443-84.

Hebel R, Stromberg MW. Anatomy and embryology of the laboratory rat. Worthsee: BioMed Verlag. 1986.

Heindel JJ. In: Daston G, Kimmel C, editores. An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington DC: ILSI Press; 1998. p.57-74.

Henley CL, Nunez AA, Clemens LG. Exogenous androgen during development alters adult partner preference and mating behavior in gonadally intact male rats. Horm Behav 2010; 57:488-95.

Hermo L, Adamali HI, Andonian S. Immunolocalization of CA II and H+ V-ATPase in the epithelial cells of the mouse and rat epididymis. J Androl 2000; 21: 376-91.

Hermo L, Barin K, Robaire B. Structural differentiation of the epithelial cells of the testicular excurrente duct system of rats during postnatal development. Anat Rec 1992; 233:205-28.

Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J et al. Estrogen receptor (alpha e beta) expression in the excurrent ducts of the adult male rat reproductive tract. J Androl 1997; 18:602-11.

Hirobe S, He WW, Lee MM, Donahoe PK. Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 1992; 131: 854-62.

Hochereau-de Reviers MT, Courot M. Sertoli cells and development of seminiferous epithelium. Ann Biol Anim Biochem Biophys 1978; 18:573.

Hoepfner BA, Ward IL. Prenatal and neonatal androgen exposure interact to affect sexual differentiation in female rats. Behav Neurosci 1988; 102:61-5.

Hom YK, Young P, Thomson AA, Cunha GR. Keratinocyte growth factor injected into female mouse neonates stimulates uterine and vaginal epithelial growth. Endocrinology 1998; 139:3772-9.

Hooker CW. The intertubular tissue of the testis. In: Johnson AD, Gomes WR, VanDemark NL, editores. The Testis. Vol 1. New York: Academic Press; 1970. p. 483.

Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenbergh JG, Gray Jr LE. Prenatal testosterone exposure permanently masculinizes anogenital distance nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol Sci 2007; 96(2):335-45.

Hu J, Gray CA, Spercer TE. Gene expression. Profiling of neonatal mouse uterine development. Biol Reprod 2004; 70:1870-6.

Huckins C, Clermont Y. Evolution of gonocytes in the rat testis during late embryonic and early post-natal life. Arch Anat Histol Embriol 1968; 51: 341-54.

Huffman L, Hendricks SE. Prenatally injected testosterone proprionate and sexual behavior of female rats. Physiol Behav 1981; 26:773-8.

Huhtaniemi I. Fetal testis – a very special endocrine organ. Eur J Endocrinol 1994; 130: 25-31.

Inglis JK. Introduction to laboratory animal science and technology. Pergamon, Oxford. 1980.

Jiang FX, Temple-Smith P, Wreford NG. Postnatal differentiation and development of the rat epididymis: a stereologial study. Anat Rec 1994; 238:191-8.

Josso N, Picard JY, Dacheux JL, Courot M. Detection of anti-Mullerian activity in boar rete testis fluid. J Reprod Fertil 1979; 57:397.

Jost A, Vigier B, Prepin J, Perchellet JP. Studies on sex differentiation in mammals. Rec Prog Horm Res 1973; 29:1.

Jost A, Vigier B, Prepin J. Freemartins in cattle: the first steps of sexual organogenesis. J Reprod Fertil 1972; 29: 349.

Junqueira LC, Carneiro J. Aparelho reprodutor feminino. In: Histologia básica. Rio de Janeiro: Guanabara-Koogan; 2008[a]. p. 431-51.

Junqueira LC, Carneiro J. Aparelho reprodutor masculino. In: Histologia básica. Rio de Janeiro: Guanabara-Koogan; 2008[b]. p. 414-28.

Karsch FJ. In: Austin CR, Short RV, editores. Hormonal control of Reproduction. Cambridge: Cambridge University Press; 1984. p.1-20.

Kavlock RJ. Overview of endocrine disruptor research activity in the United States. Chemosphere 1999; 39:1227-36.

Komárek V, Gembardt C, Krinke A, Mahrous T, Schaetti P. Synopsis of the organ anatomy. In: Krinke GJ editor. The laboratory rat - the handbook of experimental animals. San Diego: Academic Press; 2000. p.302-16.

Korenbrott CC, Huhtaniemi IT, Weiner RL. Preputial separation as an external sign of pubertal development in the male rat. Biol Reprod 1977; 17:298-303.

Kurilo LF. Oogenesis in antenatal development in man. Hum Genet 1981; 57:86-92.

Kurita T, Young P, Brody JR, Lydon JP, O'Malley BW, Cunha GR. Stromal progesterone receptors mediate the inhibitory effects of progesterone on estrogen-induced uterine epithelial cell deoxyribonucleicnacid systhesis. Endocrinology 1998; 139:4708-13.

Lino BF. Cell count correction factors for the quantitative histological analysis of the germinal epithelium of the ram. Anat Rec 1971; 170:413.

Lucas, A. Prorammimg by early nutrition in man. In: The childhood environment and adult disease. Bock, G.R.; Whelan, J., editors. CIBA Foundation Symposium 156. Chichester, UK: Wiley; 1991, p.38-55.

MacLuscky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science 1981; 211: 1294-302.

Maddocks S, Sharpe RM. The route of secretion of inhibin from the rat testis. J Endocrinol 1989; 120:R5-R8.

Maeda K, Ohkura S, Tsukamura H. Physiology of reproduction. In: Krinke GJ, editor. The laboratory rat – the handbook of experimental animals. San Diego: Academic Press; 2000. p.145-76.

Maekawa M, Kamimura K, Nagano T. Peritubular myoid cells in the testis: Their structure and function. Arch Histol Cytol 1996; 59:1-13.

Marty MS, Chapin RE, Parks LG, Thorsrud BA. Development and maturation of the male reproductive system. Birth Defects Res B 2003; 68:125-36.

McCarrey JR. Development of the germ cell. In: Desjardins C, Ewing LL, editores. Cell and Molecular Biology of the Testis. New York: Oxford University Press; 1993. p.58-89.

McEwen BS, Lieberburg I, Chaptal C, Krey L. Aromatization: important for sexual differentiation of the neonatal rat brain. Horm Behav 1977; 9:249-63.

McLaren A. Mammalian germ cells: birth, sex, and immortality. Cell Struct Funct 2001; 26:119-22.

Medlock KL, Sheehan DM, Branham WS. The postnatal ontogeny of the rat uterine estrogen receptor. J Steroid Biochem 1981; 15:285-8.

Medlock KL, Sheehan DM, Nelson CJ, Branham WS. Effect of postnatal DES treatment on uterine growth, development and estrogen receptor levels. J Steroid Biochem 1988; 29:527-32.

Meijs-Roelofs HMA, Uilenbrock JTJ, De Jong FH, Welschen R. Plasma estradiol-17β and its relationship to serum follicle-stimulating hormone in immature female rats. J Endocrinol 1973; 59:295-304.

Meisel RL, Sachs BD. In: Knobil E, Neill JD, editores. The physiology of reproduction. New York:Raven Press; 1994. p. 3-105.

Mena MA, Arriaza CA, Tchernitchin AN. Early postnatal androgenization imprints selective changes in the action of estrogens in the rat uterus. Biol Reprod 1992; 46:1080- 5.

Miller WL. Molecular biology of steroid synthesis. Endocr Rev 1988; 9:295-318.

Moore KL, Persaud TVN. O início do desenvolvimento humano: primeira semana. In: Embriologia Clínica. 8° edição. Rio de Janeiro: Elsevier; 2008. p.16-43.

Morton B, Harrigan-Lum J, Albagi L, Jooss T. The activation of motility in quiescent hamster sperm from the epididymis by calcium and cyclin nucleotides . Biochem Biophys Res Commun 1974; 56:372-9.

Mutai M. National and international guidelines for the conduct of chemical safety studies: choice of strains. In: Krinke GJ, editor. The laboratory rat – the handbook of experimental animals. San Diego: Academic Press; 2000. p.17-8.

Naftolin F, Ryan KJ, Davies IJ, Reddy VV, Flores F, Petro Z et al. The formation of estrogens by central neuroendrocrine tissues. Rec Prog Horm Res 1975; 31:295-319.

National Academic of Science. Hormonally active agents in the environment. National Academic Press, Washington DC. 1999.

Negro-Vilar A, Ojeda SR, McCann SM. Evidence for changes in sensitivity to testosterone negative feedback on gonadotropin release during sexual development in the male rat. Endocrinology 1973; 729-35.

Newcombe N, Clulow J, Man SY, Jones RC. pH and bicarbonate in the ductuli efferentes testis of the rat. Int J Androl 2000; 23:46-50.

Norris DO. Vertebrate Endocrinology. San Diego: Academic Press; 1996.

O'Malley BW. Steroid hormone action in eukaryotic cells. J Clin Invest 1984; 74: 307-12.

O'Rahilly R. Prenatal human development. In: Wynn RM, Jollie WP, editores. Biology of the uterus. 2° edição. New York: Plenum Medical Book Company; 1989: p.35-55.

Ogasawara Y, Okamoto S, Kitamura Y, Matsumoto K. Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or adrenalectomized mice, assayed by incorporation by [^{125}I] iododeoxyuridine. Endocrinology 1983; 113: 582-7.

Ohkura S, Tsukamura H, Maeda K. In: Krinke GJ, editor. The laboratory rat – the handbook of experimental animals. San Diego: Academic Press; 2000. p.406-7.

Ojeda SR, Skkiner MK. Puberty in the rat. In: Knobil E, Neill JD, editores. The physiology of reproduction. New York: Elsevier, 2006: p.2061-126.

Ojeda SR, Urbasnki HF. In: Knobil E, Neill JD, editores. The physiology of reproduction. 2° edição. New York: Raven Press; 1994. p.363-409.

Orlando EF, Kolok AS, Binzcik GA, Gates JL, Horton MK, Lambrigth CS et al. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel sensitive species, the fathead minnow. Environ Health Perspect 2004; 112:353-8.

Orth J. Proliferation of Sertoli cells in fetal and postnatal rats: A quantitative autoradiographic study. Anat Rec 1982; 203:485-92.

Parks LG, Lambrigth CS, Orlando EF, Guillette LJJr, Ankley GT, Gray LEJr. Masculinization of female mosquitofish in Kraft mill effluent-contamined Fenholloway River water is associated with androgen receptor agonist activity. Toxicol Sci 2001; 62:257-67.

Peters H. Migration of gonocytes into the mammalian gonad and their differentiation. Phil Trans Roy Soc Lond B 1970; 259:91-101.

Pfaff DW, Schwartz-Giblin S, McCarthy MM, Kow LM. In: Knobil E, Neill JD, editores. The physiology of reproduction. New York: Raven Press; 1994. p. 107-220.

Pholpramool C, Chaturapanich G. Effect of sodium and potassium concentrations and pH in the maintenance of motility of rabbit and rat epididymial spermatozoa. J Reprod Fertil 1979; 57:245-51.

Piacsek BE, Hostetter MW. Neonatal androgenization in the male rat: evidence for central and peripheral defects. BiolReprod 1984; 30:344-51.

Piffer RC, Garcia PC, Pereira OCM. Adult partner preference and sexual behavior of male rats exposed prenatally to betamethasone. Physiol Behav 2009; 98:163-7.

Plowchalk DR, Smith BJ, Mattison DR. In: Hendel JJ, Chapin RE, editores. Methods in toxicology, volume 3, part B. Female reproductive toxicology. San Diego: Academic Press. 1993. p.57-68.

Pollak EI, Sachs BD. Masculine sexual behavior and morphology: paradoxical effects of perinatal androgen treatment in male and female rats. Behav Biol 1975; 13:401-11.

Pryor JL, Hughes C, Foster W, Hales BF, Robaire B. Critical windows of exposure for children's health: the reproductive system in animal and humas. Environ Health Perspect 2000; 108(3):491-503.

Quadros PS, Pfau JL, Goldstein AYN, De Vries GJ, Wagner CK. Sex differences in progresterone receptor expression: a potential mechanism for estradiol-mediated sexual differentiation. Endocrinology 2002; 143:3727-39.

Radl V, Pritsch K, Munch JC, Schloter M. Structural and functional diversity of microbial communities from a lake sediment contaminated with trenbolone, an endocrine-disrupting chemical. Environ Pollut 2005; 137(2):345-53.

Rang HP, Dale MM, Ritter JM, Moore PK. O Sistema Reprodutor. In: Farmacologia. 5° edição. Rio de Janeiro: Elsevier. 2004; p.488-507.

Reddy VVR, Naftolin F, Ryan KJ. Conversion of androstenedione to estrone by neural tissues from fetal and neonatal rats. Endocrinology 1974; 117-21.

Rhees RW, Kirk BA, Sephton S, Lephart ED. Effects of prenatal testosterone on sexual behavior, reproductive morphology and LH secretion in the female rat. Dev Neurosci 1997; 19:430-7.

Robaire B, Hermo L. Efferent ducts, epididymis, and vas deferents: structure, functions, and their regulation. In: Knobil F, editor. The physiology of reproduction. New York: Raven Press; 1988. p.999-1080.

Robaire B, Hinton BT, Orgebin-Crist MC. The epididymis. In: Knobil E, Neill JD, editores. The physiology of reproduction. 3 edição. New York: Raven Press; 2006. p. 1071-147.

Robb GW, Amman RP, Killian GJ. Daily sperm production and epididymal sperm reserves of puberal and adult rats. J Reprod Fertil 1978; 54:103–7.

Romieu I, Hernandez-Avila M, Lazcano-Ponce E, Weber JP, Dewailly E. Breast cancer, lactation history, and serum organochlorines. Am J Epidemiol 2000; 152:363–70.

Rosner W. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. Endocr Rev 1990; 11:80-91.

Rush ME, Blake CA. Serum testosterone concentrarion during the 4-day estrous cycle in normal and adrenalectomized rats. Proc Soc Exp Biol Med 1982; 169:216-21.

Russel LD, Ettlin RA, Sinhahikin AT, Clegg ED. Mammalian spermatogenesis. In: Russel LD, Ettlin R, Hikim APS, Clegg ED, editores. Histologycal and histopathologycal evaluation of the testis. Claerwater: Cache River Press; 1990. p.1-40.

Russel LD. In: Mohr U, Dungworth DL, Capen CC, editores. Pathobiology on the aging rat. Washington DC:ILSI Press; 1992. p.395-405.

Ryan BC, Vandenbergh JG. Intrauterine position effects. Neurosci Biobehav Rev 2002; 26:665-78.

Saitou M, Barton SC, Surani MA. A molecular programme for the specification of germ cell fate in mice. Nature 2002; 418:293-300.

Sakuma Y. Gonadal steroid action and brain sex differentiation in the rat. J Neuroendocrinol 2009; 21:410-4.

Sandberg AA, Karr JP, Müntzing J. The prostates of dog, baboon and rat. In: Spring-Mills E, Hafez ESE, editores. Male accessory sex glands, biology and pathology. New York: Elsevier/North Holland Biomedical Press; 1980. p.565-608.

Schoenwolf GC, Bleyl SB, Brauer PR, Francis-West PH. Desenvolvimento do sistema urogenital. In: Larsen Embriologia humana. 4 edição. Rio de Janeiro: Guanabara Koogan; 2009. p.447-504.

Schwartz, Z.; Guest, J.F.; Elder, M.G.; White, J.O. Enzyme activities in the androgenized rat uterus refractory to oestrogenic stimulation. J Steroid Biochem 1986; 25:491-6.

Seiler P, Cooper TG, Nieschlag E. Sperm number and condition affect the number of basal cells and their expression of macrophage antigen in the murine epididymis. Int J Androl 2000; 23:65-76.

Setchell BP, Voglmayr JK, Waites GMH. A blood-testis barrier restricting passage from blood into rete testis fluid but not into lymph. J Physiol 1969; 200: 73-85.

Sheehan DM, Branham WS, Medlock KL, Olson ME, Zehr DR. Uterine response to estradiol in the neonatal rat. Endocrinology 1981; 109: 76-82.

Shiina H et al. Premature ovarian failure in androgen receptor-deficient mice. Proc Natl Acad Sci U.S.A. 2006; 103:224-9.

Slob, A.K.; den Hamer, R.; Woutersen, P.J.; van der Werff ten Bosch, J.J. Prenatal testosterone proprionate and postnatal ovarian activity in the rat. Acta Endocrinol., v.103, p.420-427, 1983.

Steinberger A, Steinberger E. Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. Biol Reprod 1971; 4:84-7.

Steinberger A. The Sertoli cell and its role in spermatogenesis. In: Frajese G, Hafez ESE, Conti C, Fabbrini A, editores. Oligozoospermia: Recent progress in andrology. New York: Raven Press; 1981. p. 35.

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F et al. Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. Nature 1992; 359: 76-9.

Sun EL, Flickinger CJ. Development of cell types and of regional differences in the postnatal rat epididymis. Am J Anat 1979; 154:27-56.

Sun EL, Flickinger CJ. Morphological characteristics of cells with apical nuclei in the initial segment of the adult rat epididymis. Anat Rec 1980; 196: 285-93.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 2005; 113:1056-61.

Taguchi O, Bigsby RM, Cunha GR. Estrogen responsiveness and the estrogen receptor during development of the murine female reproductive tract. Dev Growth Differ 1988; 30: 301-13.

Terasawa E, Fernandez DL. Neurobiological mechanisms of the onset of puberty in primates. Endocr Rev 2001; 22:111–51.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ et al. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996; 104(4):741-803.

Toran-Allerand CD. On the genesis of sexual differentiation of the general nervous system: morphogenetic consequences of steroidal exposure and possible role of alpha-fetoprotein. Prog Brain Res 1984; 61: 63-98.

Trasler JM, Hermo L, Robaire B. Morphological changes in the testis and epididymis of rats treated with cyclophosphamide: a quantitative approach. Biol Reprod 1988; 38:463-79.

Turner TT, Eomgardner D, Jacobs JP, Nguyen QA. Association of segmentation of the epididymis interstitium with segmented tubule function in rat and mice. Reproduction 2003; 125:871-8.

Turner TT, Howards SS. Factors involved in the initiation of sperm motility. Biol Reprod 1978; 18:571-8.

Turner TT. On the epididymis and its role in the development of the fertile ejaculate. J Androl 1995; 16(4):292-8.

U.S. Environmental Protection Agency. 1997. *Special report on environmental endocrine disruption: An effects assessment and analysis*. Washington, DC: Office of Research and Development. EPA/630/R-96/012.

US – EPA - U.S Environmental Protection Agency. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. 1996. Disponível online em: http://www.epa.gov/opptendo/finalrpt.htm. Acesso em 04 de janeiro de 2013.

Van den Dungen HM, van Dieten JAMJ, van Rees GP, Schomaker J. Testicular weight, tubular diameter and number of Sertoli cells in rats are decreased after early prepubertal administration of an LHRH-antagonist; the quality of spermatozoa is not impaired. Life Sci 1990; 46:1081-9.

Veri JP, Hermo L, Robaire B. Immunocytochemical localization of the Yf subunit of glutathione S-transferase P shows regional variation in the staining of epithelial

cells of the testis, efferent ducts, and epididymis of the male rat. J Androl 1993; 14: 23-44.

Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV et al. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit Rev Toxicol 2000; 30:71-133.

Weiss J, Taylor GR, Zimmermann F, Nebendahl K. Collection of body fluids. In: Krinke GJ, editor. The laboratory rat – the handbook of experimental animals. San Diego: Academic Press; 2000. p.503.

Weisz J, Ward IL. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology 1980; 106:306-16.

Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB et al. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. J Clin Invest 2008; 118:1479-90.

Welsh M, Saunders PT, Marchetti NJ, Sharpe RM. Androgen-dependent mechanisms of wolffian duct development and their perturbation by flutamide. Endocrinology 2006; 147:4820-30.

Weniger JP. Estrogen production by fetal rat gonads. J Steroid Biochem Mol Biol 1993; 44:459-62.

Wensing CJ. Testicular descent in the rat and comparison of this process in the rat with that in the pig. Zoology 1986; 423-34.

Westwood FR. The female rat reproductive cycle: a practical histological guide to staging. Toxicol Pathol 2008; 36(3):375-84.

Wiesner BP. The post-natal development of the genital organs in the albino rat. J Obstet Gynaecol Br Emp 1934; 41:867-922.

Wilson JD, Siiteri PK. Developmental pattern of testosterone synthesis in the fetal gonad of the rabbit. Endocrinology 1973; 92:1182.

Wilson JG. Reproductive capacity of adult female rats treated prepubertally with estrogenic hormone. Anat Rec 1943; 86: 341-59.

Witschi E. Prenatal vertebrate development. In: Altman PL, Dittmer DS, editores. Growth. Federation of American Societies for Experimental Biology. Washington; 1962. p.304-29.

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

Wong PYD, Lee WM, Tsang AYF. The effects of sodium and amiloride on the motility of caudal epididymal spermatozoa of the rat. Experientia 1981; 37:69-71.

Yamashita S, Newbold RR, McLachlan JA, Korach KS. The role of the estrogen receptor in uterine epithelial proliferation and cytodifferentiation in neonatal mice. Endocrinology 1990; 127:2456-63.

Yuan YD, Carlson RG. Structure, cyclic change, and function, vagina and vulva, rat. In: Jones TC, Mohr U, Hunt RD, editores. Genital system. 1987.Springer, Berlin Heidelberg New York (Monographs on pathology of laboratory animals).

Zadina JE, Dunlap JL, Gerall AA. Modifications induced by neonatal steroids in reproductive organs and behavior of male rats. J Comp Physiol Psychol 1979; 93(2):314-22.

Zhu LJ, Bagchi MK, Bagchi IC. Attenuation of calcitonin gene expression in pregnant rat uterus leads to a block in embryonic implantation. Endocrinology 1998; 193:330-9.

Zirkin BR, Ewing LL, Kromann N, Cochran RC. Testosterone secretion by rat, rabbit, guinea pig, dog, and hamster testes perfused in vitro: correlation with Leydig cell ultrastructure. Endocrinology 1980; 106:1867.

Anexo

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

CERTIFICADO

Certificamos que o Protocolo nº **104/2009-CEEA**, sobre "Estrutura do trato genital e função reprodutiva de ratas expostas a propionato detestosterona in útero e durante a lactação", sob a responsabilidade de **WILMA DE GRAVA KEMPINAS** , está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de **06/03/2009**.

Botucatu, 6 de março de 2009.

Profa. Dra. PATRÍCIA FERNANDA F. PINHEIRO
Presidente - CEEA

SÉRGIO PRIMO VICENTINI
Secretário – CEEA

Instituto de Biociências – Diretoria Técnica Acadêmica
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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada "**Estrutura do trato genital e função reprodutiva da prole masculina e feminina de ratos expostos ao propionato de testosterona *in utero* e durante a lactaçã**":

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, 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 104/2009-CEEA, Instituição: Comitê de ética para a experimentação animal do instituto de Biociências da UNESP de Botucatu.

( ) 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(a): Marina Trevizan Guerra

Orientador(a): Dra. Wilma De Grava Kempinas

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

Carimbo e assinatura

Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
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Carimbo e assinatura

Apêndices

9. Apêndices

9.1. Receptores esteroides no tecido uterino de ratas

9.1.1. Receptores estrogênicos

O termo "estrógeno" deriva do latim "*oestrus*" e do grego "*oistros*", palavras que fazem referência à fase na qual fêmeas são sexualmente receptivas (Morani et al., 2008). Os estrógenos desempenham um papel importante no crescimento, diferenciação e função dos tecios reprodutivos masculinos e femininos (Sharpe, 1998). Em fêmeas, este hormônio induz a replicação de DNA e a proliferação celular no útero de mamíferos (Marcus, 1974) por modificar a expressão de diversos genes como receptores de progesterona (Savouret et al., 1994) e proto-oncogenes como *c-fos* (Nephew et al., 1995).

Na década de 70, Jensen e colaboradores (1972) concluíram que os efeitos biológicos decorrentes do estrógeno ocorrem através da ativação de receptores estrogênicos (ERs). Vinte anos depois, o ER foi clonado e caracterizado como um membro da superfamília de receptores nucleares (Walter et al., 1985). Os genes codificadores para ERs são altamente conservados entre humanos e roedores, contendo estruturas gênicas e protéicas similares (Wilson e Westberry, 2009)

Os ERs, membros da superfamília de receptores hormonais esteroides/ tireoideos (Mangelsdorf et al., 1995), são considerados fatores de transcrição ligante-dependentes (Hiroi et al., 1999) e existem na forma inativa no núcleo da célula (Cato et al., 2002). Eles ligam-se especificamente ao estrógeno e regulam a transcrição gênica via elementos estrógeno-responsivos (estrogen-responsive elements – EREs) localizados em regiões específicas de genes-alvo. Esta ligação recruta cofatores que reorganizam estruturas cromossômicas e ativam a transcrição gênica (Watanabe et al., 2003).

Durante a década de 80 acreditava-se na existência de somente um tipo de ER, denominado ERα (Green et al., 1986), até que um novo tipo, denominado ERβ foi isolado em roedores (Kuiper et al., 1996; Tremblay et al., 1997) e humanos (Mosselman et al., 1996). Os ERα e ERβ possuem uma distribuição tecidual distinta (Kuiper et al., 1997).

Em ratas em desenvolvimento, o momento de aparição dos receptores estrogênicos localizados nuclearmente está correlacionado com o desaparecimento da proteína plasmática ligadora de estrógeno – α-fetoproteina (AFP) da circulação e com o aumento de estradiol livre e ativo no plasma (Greenstein et al., 1977). ERα e ERβ são altamente expressos no estroma e no epitélio uterino de roedores imaturas. Aparentemente, o ERβ mantém o útero quiescente antes que o ovário começe a secretar estradiol (Morani et al., 2008). Já em útero de roedores maduras, o ERβ está envolvido no amadurecimento cervical essencial no momento do parto (Wu et al., 2000) e na decidualização (Tessier et al., 2000). Em ratas adultas, o ERα é o subtipo predominante em células epiteliais glandulares e luminais em tecido uterino (Wang et al., 1999).

Mowa e Iwanaga (2000) avaliaram a expressão de RNA mensageiro (RNAm) para ERs em tecidos reprodutivos de ratas neonatais. RNAm de ERα aparece pela primeira vez em células mesenquimais do ducto Mülleriano ainda no feto, no DG 14. Com o desenvolvimento uterino, no DG 17, esta marcação é encontrada no estroma deste órgão, mas não no epitélio. Evidências de RNAm de ERα no epitélio uterino é somente encontrada após o DPN4-6; no epitélio glandular, ele é encontrado somente após o DPN 14. Em relação ao ERβ, o RNAm para este subtipo de receptor é encontrado somente após o nascimento, a partir do DPN 7, no estroma uterino.

Mendoza-Rodriguez et al. (2003) demonstraram por técnicas de imunohistoquímicas que ERβ não foi encontrado em ambos os epitélios uterinos durante todas as fases do ciclo estral, mas foi detectado principalmente no citoplasma das células estromais, com imunomarcações mais intensas nas fases de metaestro e diestro. Contrariamente, Pelletier et al. (2000) não encontraram nenhuma marcação para ERβ em nenhum compartimento uterino.

O ERα foi encontrado no núcleo de células estromais durante todos os estágios do ciclo estral e, nos epitélios uterinos luminal e glandular, um maior número de células imunomarcadas foi observado durante as fases de metaestro e diestro (Mendoza-Rodriguez et al., 2003). Este subtipo também foi encontrado em núcleo de células uterinas musculares (Pelletier et al., 2000).

Estes resultados sugerem que os subtipos de ERs medeiam mecanismos de ação distintos para a ação estrogênica no epitélio e no estroma de útero de ratas durante o ciclo estral e que o ERα é o subtipo envolvido na mediação dos principais efeitos estrogênicos no útero (Pelletier et al., 2000; Mendoza-Rodriguez et al., 2003).

9.1.2. Receptores androgênicos

Os hormônios androgênicos estão envolvidos no desenvolvimento e na função fisiológica de órgãos sexuais acessórios masculinos assim como no funcionamento de diversos outros órgãos e tecidos, incluindo o sistema genital feminino (Kowalski et al., 2004). A ação androgênica é mediada por receptores androgênicos (ARs) que pertencem à superfamília de reguladores de transcrição ligante-responsivos (Carson-Jurica et al., 1990) e que estão presentes nas células-alvo como complexos inativos no citoplasma na ausência do hormônio ligante (Cato et al., 2002).

Através da técnica de imunohistoquímica, ARs foram localizados numa variedade de tecidos humanos, incluindo tecidos reprodutivos em ambos os sexos (Kimura et al., 1993). Em ratos, ARs já foram localizados tanto no sistema genital masculino quanto no feminino (Tetsuka et al., 1995). O útero de ratas contém ARs citoplasmáticos que são translocados para o núcleo após ligação com a testosterona (Schmidt e Katzenellenbogen, 1979; Takeda et al., 1989).

Embora os andrógenos tenham a capacidade de aumentar significativamente o peso uterino (Armstrong et al., 1976), pouco se sabe sobre os mecanismos de ação destes hormônios no útero (Hirai et al., 1994). Os ARs possuem um papel importante na proliferação celular do epitélio uterino induzida por estrógeno. A presença deste receptor no estroma uterino amplifica a sinalização do ERα por induzir fatores de crescimento específicos e a proliferação celular epitelial de um modo parácrino (Okada et al., 2005).

Os ARs são expressos já no sistema genital feminino fetal de roedores. Embora no dia pós-coito 17 os níveis de AR no tubérculo genital estejam similares entre fêmeas e machos, a quantidade deste receptor diminui após o dia 18 pós-coito no tecido urogenital feminino (Bentvelsen et al., 1994). O tratamento de ratas imaturas com andrógenos resulta

no aparecimento de um efeito uterotrófico, indicando que os ARs nesta idade, já estão funcionalmente ativos (Lerner et al., 1966).

Em ratas adultas, o número de ARs oscila durante o ciclo estral e os níveis de estradiol, progesterona e testosterona podem estar envolvidos na regulação desta flutuação; a concentração de AR diminui concomitantemente com a queda de estradiol (Büchi e Weber, 1983). Hirai et al. (1994) demonstraram, através de técnicas de hibridização *in situ*, uma forte marcação para RNAm de AR no endométrio e nas glândulas endometriais em ratas adultas e marcações moderadas foram encontradas no miométrio uterino.

Estes resultados demonstrando a presença de ARs em útero de ratas indicam que os andrógenos desempenham um papel essencial no desenvolvimento e função do útero (Pelletier et al., 2000) e age também como mediador das funções reprodutivas feminina.

9.1.3. Receptores progesterônicos

O hormônio esteroide progesterona é um componente chave na regulação complexa da função reprodutiva feminina (Graham e Clarke, 1997), incluindo ovulação, desenvolvimento mamário e uterino e expressão neuro-comportamental associado à resposta sexual (Lydon et al., 1995). A progesterona também possui um efeito anti-inflamatório no útero e este papel parece ser fundamental para a geração de um sítio imunologicamente privilegiado para facilitar a implantação do embrião e prevenir a rejeição embrionária (Conneely e Lydon, 2000).

Juntamente com o estrógeno, a progesterona promove a base para as mudanças cíclicas que ocorrem no tecido uterino durante o ciclo estral. As ações destes hormônios são primariamente mediadas por ligação a receptores intracelulares específicos presentes nas células-alvo (Sahlin et al., 2006).

Os efeitos fisiológicos da progesterona são mediados pela interação com proteínas intracelulares específicas denominadas receptores progesterônicos (PRs) (Conneely e Lydon, 2000). Os PRs pertencem a uma grande família de reguladores de transcrição ativados por ligantes, conhecida como superfamília de receptores nucleares (Mangelsdorf et al., 1995; Graham e Clarke, 1997). Estes receptores são compostos por duas isoformas,

denominadas A e B; ambas são resultado da expressão de um único gene em roedores e humanos (Kraus et al., 1994).

A ligação da progesterona ao PR induz uma mudança conformacional significativa que resulta em ligação a regiões promotoras específicas de genes-alvo (Tsai e O'Malley, 1994) e interação com proteínas coativadoras para formar um produto de transcrição (Kamei et al., 1996).

PRs são expressos no epitélio, estroma e miométrio uterinos, e sua expressão espaço-temporal nestes compartimentos é controlada por estrógenos (Clarke e Sutherland, 1990; Graham e Clarke, 1997). Estudos prévios demonstraram que o PR e os sítios de ligação da progesterona são regulados, via ERα, por estrógenos no trato reprodutivo feminino (Kurita et al., 2000; Okada et al., 2002).Em geral, PR é um bom marcador para ação estrogênica na maioria dos tecidos devido aos múltiplos EREs presentes na região 5' de seu gene (Kraus et al., 1994).

A expressão imunohistoquímica uterina para PR já foi descrita para ratas e a presença deste receptor foi detectada pela primeira vez em células epiteliais uterinas no DPN 5. Em relação às células do estroma e células musculares, o PR foi primeiramente localizado no DPN 12 e 15, respectivamente. Em ratas adultas, PRs estão localizados predominantemente no núcleo de células epiteliais, estromais e musculares uterinas durante o ciclo estral, embora em diferentes fases do ciclo, o PR demonstre variações nos níveis de expressão. Os núcleos das células epiteliais apresentam um maior nível de PR no diestro, enquanto as células do estroma e musculares, no proestro (Ohta et al., 1993).

9.1.4. Referências bibliográficas

Armstrong DT, Papkoff H. Stimulation of aromatization of exogenous and endogenous androgens in ovaries of hypophysectomized rats in vivo by follicle-stimulating hormone. Endocrinology 1976; 99:987-91.

Bentvelsen FM, McPhaul MJ, Wilson JD, George FW. The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen. Mol Cell Endocrinol 1994; 105: 21-6.

Büchi KA, Weber E. Variation of cytoplasmic rat uterine androgen receptors. Mol Cell Endocr 1983; 29: 295-307.

Carson-Jurica MA, Schrader WT, O'Malley BW. Steroid receptor superfamily: structure and functions. Endocr Rev 1990; 11: 201-22.

Cato ACB, Nestl A, Mink S. Rapid actions of steroid receptors in cellular signaling pathways. Sci STKE 2002; 138:re9.

Clarke CL, Sutherland RL. Progestin regulation of cellular proliferation. Endocr Rev 1990; 11: 266-300.

Conneely OM, Lydon JP. Progesterone receptors in reproduction: functional impact of the A and B isoforms. Steroids 2000; 65: 571-77.

Graham JD, Clarke CL. Physiological action of progesterone in target tissues. Endocr Rev 1997; 18(4): 502-19.

Green S, Walter P, Kumar V, Krust A, Bornet JM, Argos P et al. Human oestrogen receptor cDNA: sequence and homology to v-erb-A. Nature 1986; 320: 134-9.

Greenstein BD, Puig-Duran E, MacKinnon PCB. Measurement of the undound oestradiol-17β fraction in sera of developing female rats by a miniature method of steady-state gel filtration. J Endocrinol 1977; 72: 56.

Hirai M, Hirata S, Osada T, Hagihara K, Kato J. Androgen receptor mRNA in the rat ovary and uterus. J Steroid Biochem Mol Biol 1994; 49:1-7.

Hiroi H, Inoue S, Watanabe T, Goto W, Orimo A, Momoeda M et al. Differential immunolocalization of strogen receptor α and β rat ovary and uterus. J Mol Endocrinol 1999; 22: 37-44.

Jensen EV, Jacobson HI, Smith S, Jungblut PW, De Sombre ER. The use of estrogen antagonist in hormone receptor studies. Gynecol Invest 1972; 3: 108-23.

Kamei Y, Xu L, Heinzel T . A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 1996; 85: 403-14.

Kimura F, Mizokami A, Oconuma T, Sasano H, Nagura H. Immunocystochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. J Histochem Cytochem 1993; 4:671-8.

Kowalski AA, Vale-Cruz DS, Simmen FA, Simmen RCM. Uterine androgen receptors: roles in estrogen-mediated gene expression and DNA synthesis. Biol Reprod 2004; 70:1349-57.

Kraus WL, Montano MM, Katzenellenbogen BS. Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distinct promoters. Mol Endocrinol 1994; 7: 1603-16.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138: 863-70.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JÅ. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996; 93: 5925-30.

Kurita T, Lee K, Cooke PS, Taylor JA, Lubahn DB, Cunha GR. Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract. Biol Reprod 2000; 62: 821-30.

Lerner LJ, Hilf R, Turkheimer AR, Michel I, Engel SL. Effects of hormone antagonists on morphological and biochemical changes induced by hormone steroids in the immature rat uterus. Endocrinology 1966; 78(1): 111-24.

Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 1995; 9: 2266-78.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Shulz G, Umesono K et al. The nuclear receptor superfamily: the second decade. Cell 1995; 83:835-9.

Marcus GJ. Mitosis in the rat uterus during the estrous cycle, early pregnancy, and early pseudopregnancy. Biol Reprod 1974; 10: 447-52.

Mendoza-Rodriguez CA; Merchant-Larios H, Segura-Valdez ML, Moreno-Mendoza N, Cruz ME, Arteaga-Lopez P et al. c-fos and estrogen receptor gene expression. Pattern in the rat uterine epithelium during the estrous cycle. Mol Reprod Dev 2003; 64:379-88.

Morani A, Warner M, Gustafsson J-Å. Biological functions and clinical implications of oestrogen receptors alfa and beta in epithelial tissues. J Intern Med 2008; 264: 128-42.

Mosselman S, Polman J, Dijkema R. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett 1996; 392: 49-53.

Mowa CN, Iwanaga T. Developmenta changes of the oestrogen receptor-α and –β mRNAs in the female reproductive organ of the rat – an analysis by in situ hybridization. J Endocrinol 2000; 167: 363-9.

Nephew KP, Peters GA, Khan SA. Cellular localization of estradiol induced c-fos messenger ribonucleic acid in the rat uterus: c-fos expression and uterine cell proliferation do not correlate strictly. Endocrinology 1995; 136: 3007-15.

Ohta Y, Sato T, Iguchi T. Immunocytochemical localication of progesterone receptor in the reproductive tract of adult female rats. Biol Reprod 1993; 48: 205-213.

Okada A, Ohta Y, Buchanan DL, Sato T, Iguchi T. Effects of estrogen on ontogenetic expression of progesterone receptor in the fetal female rat reproductive tract. Mol Cell Endocrinol 2002; 195: 55-64.

Okada A, Sato T, Ohta Y, Iguchi T. Sex steroid hormone receptors in developing female reproductive tract of laboratory rodents. J Toxicol Sci 2005; 30(2): 75-89.

Pelletier G, Labrie C, Labrie F. Localization of oestrogen receptor α, oestrogen receptor β and androgen receptors in the rat reproductive organs. J Endocrinol 2000; 165:359-370.

Savouret JF, Rauch M, Redeuilh G, Sar S, Chauchereau A, Woodruff K et al. Interplay between estrogens, progestins, retinoic acid, and AP-1 on a single regularoty site in the progesterone receptor gene. J Biol Chem 1994; 269: 28955-62.

Schmidt WN, Katzenellenbogen BS. Androgen-uterine interactions: an assessment of androgen interaction with the testosterone and estrogen receptor systems and stimulation of uterine growth and progesterone –receptor synthesis. Mol Cell Endocrinol 1979; 15(2): 91-108.

Sharpe RM. The roles of oestrogen in the male. Trends in Endocrinol Metab 1998; 9:371-8.

Takeda H, Chodak G, Mutchnik S, Nakamoto T, Chang C. Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor. J Endocrinol 1989; 126: 17-25.

Tessier C, Deb S, Prigent-Tessier A, Ferguson-Gottschall S, Gibori GB, Shiu RP et al. Estrogen receptors alpha and beta in rat decidua cells: cell-specific expression and differential regulation by steroid hormones and prolactin. Endocrinology 2000; 141: 3842-51.

Tetsuka M, Whitelaw PF, Bremmer WJ, Millar MR, Smyth CD, Hillier JG. Developmental regulation of androgen in rat ovary. J Endocrinol 1995; 145:535-43.

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F et al. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 1997; 11: 353-65.

Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994; 63:451-86.

Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM et al. Cloning of the human estrogen receptor cDNA. Proc Natl Acad Sci USA 1985; 82: 7889-93.

Wang H, Masironi B, Eriksson H, Sahlin L. A comparative study of estrogen receptors α and β in the rat uterus. Biol Reprod 1999; 61: 955-64.

Watanabe H, Suzuki A, Kobayashi M, Takahashi E, Itamoto M, Lubahn DB et al. Analysis of temporal changes in the expression. Of estrogen-regulated genes in the uterus. J Mol Endocrinol 2003; 30: 347-58.

Wilson ME, Westberry JM. Regulation of oestrogen receptor gene expression: new insights and novel mechanisms. J Neuroendocrinol 2009; 21: 238-42.

Wu JJ, Geimonen E, Andersen J. Increased expression of estrogen receptor beta in human uterine smooth muscle at term. Eur J Endocrinol 2000; 142: 92-9.

9.2. Resultados não apresentados no manuscrito "LONG-TERM EFFECTS OF PERINATAL ANDROGENIZATION ON REPRODUCTIVE PARAMETERS OF MALE RAT OFFSPRING"

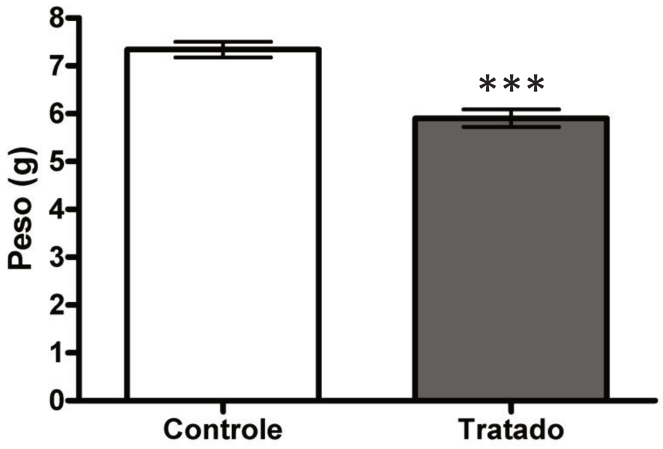

Figura 1. Peso corpóreo de ratos no DPN 1 pertencentes ao grupo controle (*n*=8 ninhadas) e tratado (*n*=8 ninhadas), cujas mães foram expostas ao óleo de milho ou propionato de testosterona, respectivamente, do DG 12 ao DPN 21. Teste "t" de Student. p<0,001.

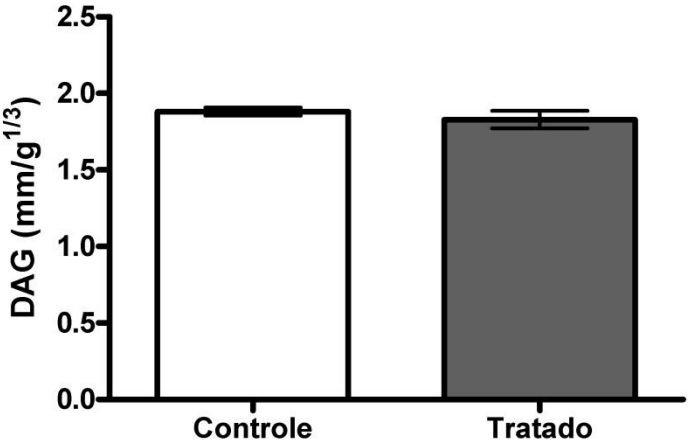

Figura 2. Distância anogenital (DAG) relativa (DAG/$\sqrt[3]{}$peso corpóreo) de ratos no DPN 1 pertencentes ao grupo controle (*n*=8 ninhadas) e tratado (*n*=8 ninhadas), cujas mães foram expostas ao óleo de milho ou propionato de testosterona, respectivamente, do DG 12 ao DPN 21. Teste "t" de Student.

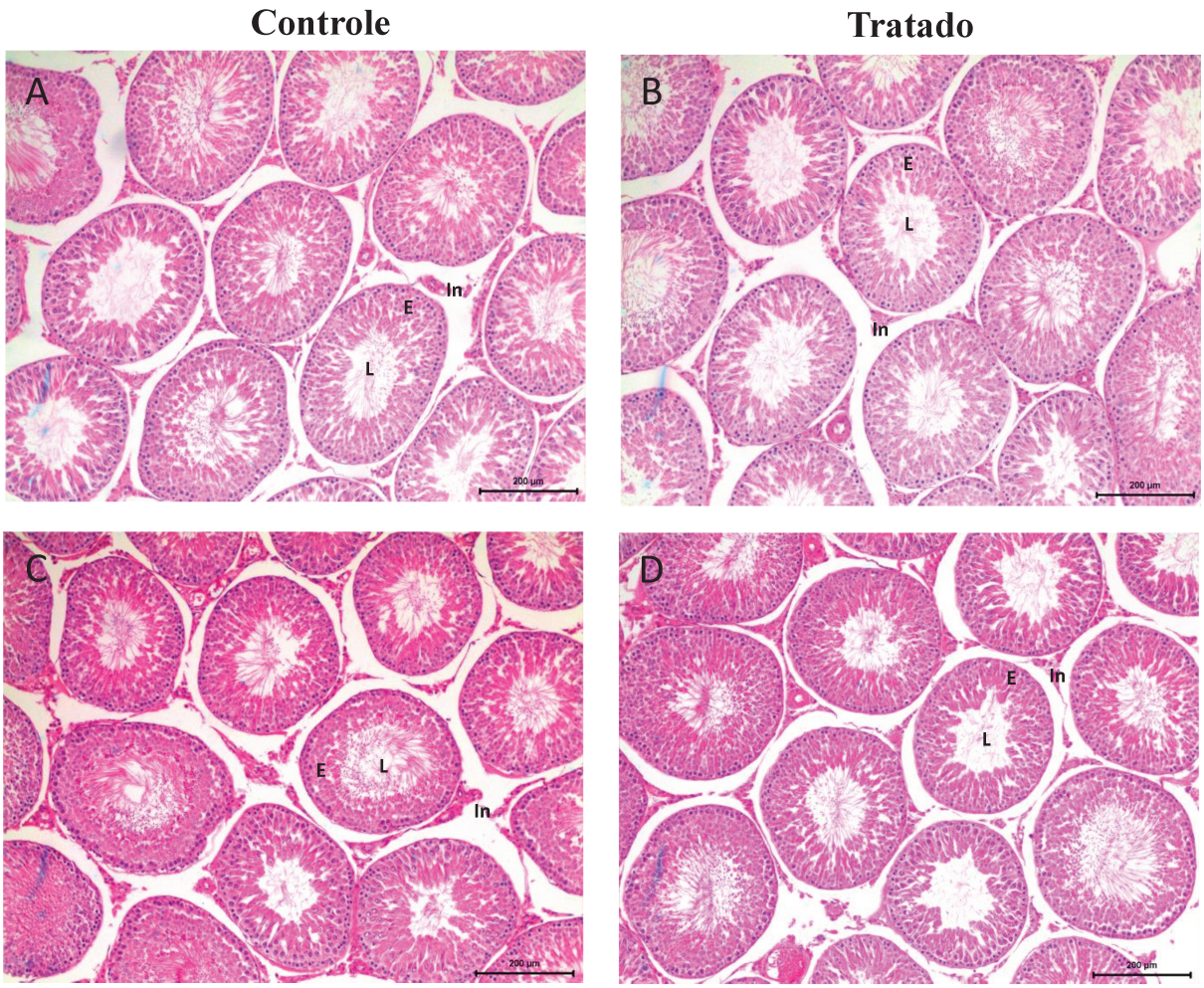

Figura 3. Fotomicrografia transversal de testículo de ratos pertencentes ao grupo controle e tratado com TP. A: grupo controle aos 90 dias de idade; B: grupo tratado aos 90 dias de idade; C: grupo controle aos 270 dias; D: grupo tratado aos 270 dias de idade. E: epitélio seminífero; L: lúmen; In: interstício. 100X. H.E.