UNIVERSIDADE ESTADUAL DE CAMPINAS
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"MORFOFISIOLOGIA DO SISTEMA GENITAL MASCULINO DE RATOS PÚBERES E ADULTOS APÓS PRIVAÇÃO ANDROGÊNICA DURANTE A PRÉ-PUBERDADE"

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Orientadora: Profa. Dra. Wilma De Grava Kempinas

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Se enxerguei mais longe, é porque me ergui
No ombro de gigantes

Isaac Newton

Dedico esta tese ao meu marido Rodrigo Gibin Jaldin.

Pelo imenso amor dedicado a mim e pelo respeito aos meus sonhos.

E desde então, sou porque tu és
E desde então és
sou e somos...
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Serei... Serás...Seremos...

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SUMÁRIO

Resumo

RESUMO

Os desreguladores endócrinos são agentes químicos capazes de agir como agonistas ou antagonistas dos hormônios endógenos, interferindo na homeostasia do organismo. Como o sistema endócrino tem papel crítico sobre o desenvolvimento e função do sistema genital, este pode ser considerado um alvo particularmente vulnerável a perturbações endócrinas. A literatura apresenta dados sobre a exposição aos antiandrogênicos durante a vida pré-natal e adulta e suas consequências sobre a função reprodutiva de machos. Entretanto, poucos estudos se atentaram para as consequências da privação androgênica durante a pré-puberdade sobre o sistema genital masculino. A pré-puberdade corresponde à fase em que o epidídimo, órgão reprodutor masculino responsável pela maturação e estocagem dos espermatozoides, passa por importantes mudanças morfofuncionais, além de consistir em período de maior susceptibilidade aos desreguladores endócrinos. O objetivo do presente estudo foi avaliar as possíveis consequências da privação de andrógenos durante a pré-puberdade sobre a morfofisiologia do sistema genital masculino de ratos púberes e adultos, com ênfase sobre o epidídimo e qualidade espermática. A escolha por um agente antiandrogênico foi devida à vasta exposição ambiental e ocupacional da população mundial a este grupo de contaminantes. Ratos machos da variedade Wistar foram alocados em: grupo flutamida (25mg/Kg/dia de flutamida, via oral, do dia pós natal 21 ao 44) e controle (óleo de milho, via oral, durante o mesmo período). Os animais foram avaliados aos 50 dias e 75 dias de idade. Foram analisados os níveis séricos dos hormônios sexuais (LH, FSH e testosterona), níveis de testosterona intratesticular, peso de órgãos, histologia testicular e epididimária, imunohistoquímica para marcação de receptor androgênico (AR), proteína espermática 22 (SP22), calmodulina (CALM) e Rab11A em tecido epididimário, além de marcação de CALM e Rab11A no testículo e *western blot* para AR no epidídimo. Avaliações adicionais foram realizadas nos animais de 75 dias, como comportamento sexual, fertilidade após acasalamento natural e inseminação artificial, motilidade e morfologia espermática, contagens espermáticas nos testículos e epidídimos, análise do perfil proteico de membrana espermática e contagem de células de Sertoli. No grupo tratado com flutamida, os animais púberes apresentaram redução do peso dos órgãos sexuais, relacionado à diminuição na testosterona sérica, além de alteração no padrão de

imunomarcação para AR e CALM no epidídimo. Os demais parâmetros foram comparáveis entre os grupos experimentais. Nos animais de 75 dias de idade que receberam flutamida observou-se alteração no padrão de imunomarcação para AR, CALM e Rab11A no epidídimo, diminuição do potencial de fertilidade após inseminação artificial, comprometimento da motilidade espermática, diminuição do número de espermatozoides na cabeça/corpo e cauda do epidídimo, aceleração do trânsito espermático nestas regiões epididimárias e alteração na concentração de CALM e Rab11A na membrana espermática. Os demais parâmetros foram similares entre os grupos experimentais. Os resultados obtidos mostram que a privação de andrógenos durante a pré-puberdade causou alterações na qualidade dos espermatozóides prejudicando o potencial de fertilidade dos indivíduos na idade adulta. Tais resultados parecem estar associados a mudanças no perfil proteico da membrana dos espermatozoides e na expressão de determinadas proteínas no epitélio epididimário, sugerindo que o desenvolvimento pós-natal do epidídimo pode ter sido comprometido, acarretando danos funcionais permanentes ao órgão.

Palavras-chave: Ratos Wistar, Receptores androgênicos – Antagonistas e inibidores, Epidídimo, Pré-puberdade, Genitália masculina.

Abstract

ABSTRACT

Endocrine disrupters are chemicals that can act as agonists or antagonists of endogenous hormones, interfering with the homeostasis of the organism. Since the endocrine system plays a critical role in the development and function of the male reproductive system, this is an especially vulnerable target of potential endocrine perturbations. The literature presents data on exposure to antiandrogens during the prenatal life and adulthood and its consequences on the male reproductive function. However, few studies have investigated the possible effects on the male reproductive system of rats after androgen deprivation during prepuberty. The prepubertal period comprehends the phase in which the epididymis, male reproductive organ responsible for the sperm maturation and storage, undergoes significant morphofunctional changes, besides being a period of more vulnerability to endocrine disrupters, possibly due to hormonal imprinting. The aim of this study was to evaluate the possible consequences of androgen deprivation during the prepuberty on morphophysiology of male reproductive system of pubertal and adult rats, focusing on the epididymis and sperm quality. The choice of an antiandrogen agent was due to extensive environmental and occupational exposure of the general population to this group of contaminants. For this purpose, Wistar male rats were divided into flutamide group (flutamide 25mg/Kg/day, orally, from postnatal day 21 to 44) and control group (corn oil, orally, during the same period). The animals were evaluated at 50 days and 75 days of age. At both ages it was evaluated the serum sexual hormone levels, intra-testicular testosterone levels, organ weights, testicular and epididymal histopathology, immunohistochemistry for androgen receptor (AR), sperm protein 22 (SP22), calmodulin (CALM) and Rab11A in epididymal tissue, besides immunostaining for CALM and Rab11A in the testis and Western blot for AR in the epididymis. Furthermore, additional parameters were assessed in 75-day-old animals, such as sexual behavior, fertility after natural mating and after artificial insemination, sperm motility and morphology, sperm counts in the testis and epididymis, proteomic of sperm membrane by bi-dimensional electrophoresis and Sertoli cells counts. Pubertal animals showed reduced reproductive organs weight, probably due to a decrease in serum testosterone and changes in the pattern of immunostaining for AR and CALM in the epididymis. The other parameters were comparable between the groups. In

animals at 75 days old changes in the pattern of immunostaining for AR, CALM and Rab11A in the epididymis, decreased fertility potential after artificial insemination, impaired sperm motility, decrease in the sperm numbers in the caput/corpus and cauda epididymis, acceleration of sperm transit time through these epididymal regions, and modifications in three proteins of sperm membrane were observed. Other parameters were similar between the groups. The results show that androgen deprivation during prepuberty impairs sperm quality affecting the fertility potential of the animals at adulthood. These results seem to be related to the changes in protein profile of sperm membrane and protein expression in the epididymis, suggesting that the postnatal development of the epididymis may have been compromised, causing permanent damage to the organ function.

Keywords: Wistar rats, androgen receptors - antagonists and inhibitors, epididymis, prepuberty, male reproductive system.

Introdução

1. INTRODUÇÃO

1.1. A Experimentação Animal

Animais de laboratório correspondem a quaisquer vertebrados produzidos para ou utilizados em pesquisa ou ensino. Seu uso em pesquisas pode ser considerado um privilégio concedido pela sociedade à comunidade científica, com a expectativa de que tal atividade proporcione novo e significativo conhecimento ou conduza a uma melhoria no bem-estar animal e/ou humano (McCarthy, 1999; Perry, 2007). A decisão por utilizar animais na pesquisa requer pensamento crítico e planejamento. O princípio dos "três Rs", publicado em 1959 por Russel & Burch, representa um método prático para a implementação dos princípios éticos na pesquisa experimental. Consiste em uma estratégia prática de substituição, refinamento e redução (*replacement, refinement and reduction*).

"Substituição" refere-se a métodos que visam evitar o uso de animais; trata-se de substituições absolutas (como programas computacionais) ou parciais (por exemplo, a substituição de vertebrados por animais menos derivados na escala filogenética). "Refinamento" refere-se a modificações que acarretam melhorias ao bem-estar animal e minimizam dor e estresse. "Redução" corresponde a estratégias para a obtenção do máximo de informações utilizando-se o menor número de animais possível. Para tanto, faz-se necessário uma análise cuidadosa do delineamento experimental, aplicação de novas tecnologias, uso de métodos estatísticos adequados e controle rigoroso das acomodações dos animais, atentando para temperatura, umidade relativa do ar e fotoperíodo (*Committee for the update of the guide for the care and use of laboratory animals*, 2011).

O rato é um dos animais mais comumente utilizados na pesquisa experimental, sendo o modelo de mamífero melhor caracterizado. Apresenta uma série de vantagens por prestar-se adequadamente a modelo de estudo para doenças humanas, desenvolvimento de novos agentes terapêuticos e estudo de resposta a agentes ambientais (*Committee for the update of the guide for the care and use of laboratory animals*, 2011). No campo da toxicologia e fisiologia da reprodução, o rato é o modelo experimental mais frequentemente adotado (Maeda et al., 2000). As fases do seu ciclo de vida (gestação, lactação, ciclo estral,

puberdade), relativamente mais curtas do que em outras espécies de mamíferos, são consideradas importantes vantagens deste modelo animal. Além disso, se reproduzem frequentemente e apresentam sinais físicos externos considerados indicativos do desenvolvimento sexual e de fácil monitoramento (Ojeda & Skinner, 2006).

1.2. Morfofisiologia do Sistema Genital Masculino do Rato

O sistema genital masculino do rato (Figura 1), assim como na maioria dos mamíferos, é composto por testículos (gônadas), epidídimos, ductos deferentes, glândulas sexuais e órgão copulador.

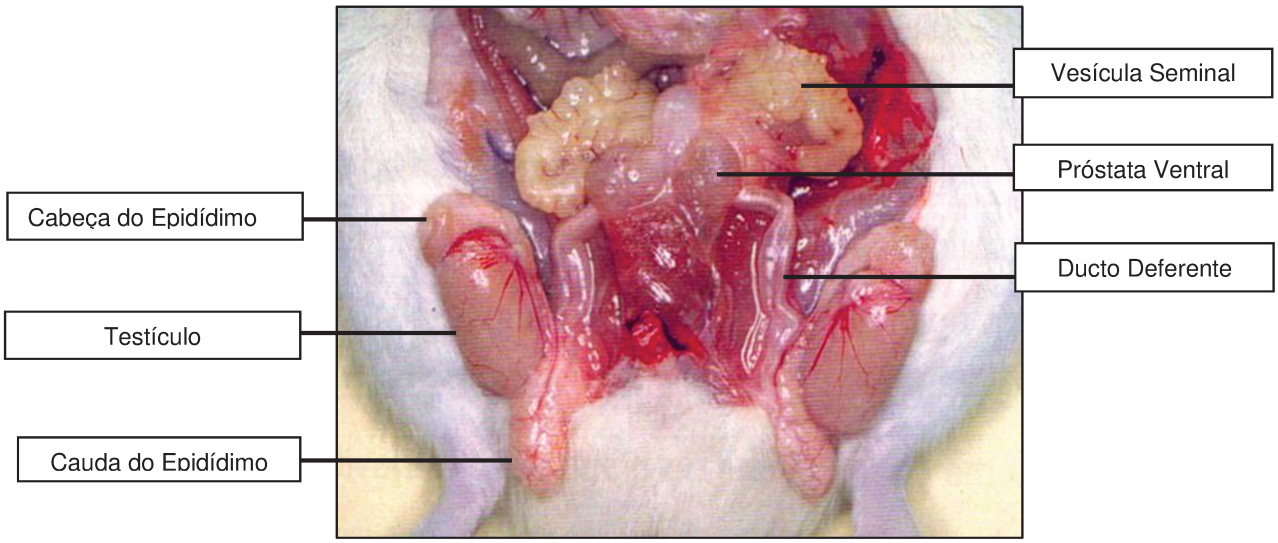

Figura 1: Aspecto macroscópico do sistema genital masculino do rato adulto.

1.2.1. Testículo e Espermatogênese

Os testículos são órgãos pares localizados no interior da bolsa escrotal e revestidos externamente por uma cápsula de tecido conjuntivo denso - a túnica albugínea. Externamente e adjacente à túnica albugínea encontra-se uma camada de peritônio visceral denominada túnica vaginal, composta por células mesoteliais, fibroblastos e fibras

colágenas entremeadas por feixes de células musculares lisas. A túnica vaginal também reveste a superfície interna da bolsa escrotal (Komárek et al., 2000). Os testículos são compostos por interstício e túbulos seminíferos, responsáveis pela esteroidogênese e espermatogênese, respectivamente (Rodriguez & Favaretto, 1999).

O interstício é composto por tecido conjuntivo, vasos sanguíneos e linfáticos, nervos, macrófagos e células de Leydig, as quais são responsáveis pela produção de andrógenos, substrato para uma variedade de outros hormônios esteroides (Russel et al., 1990). Em ratos, observa-se número reduzido de células de Leydig, que frequentemente se localizam próximo aos espaços linfáticos e se agrupam ao redor de vasos sanguíneos (Foley, 2001).

Cada testículo de rato adulto apresenta, em média, 20 túbulos seminíferos. Enquanto o parênquima testicular humano é formado por uma série de lóbulos de tecido conjuntivo que compartimentalizam os túbulos seminíferos, o testículo de rato contém quantidade escassa de tecido conjuntivo e não apresenta lóbulos. Existem pequenas áreas no final de cada túbulo seminífero que apresentam um epitélio de transição. Estas regiões são denominadas túbulos retos e conectam os túbulos seminíferos a uma rede de canais anastomosados, denominada rede testicular (Foley, 2001). Em animais adultos, os túbulos seminíferos são constituídos por epitélio germinativo, composto por células de Sertoli e células germinativas (espermatogônias, espermatócitos primários, secundários e espermátides) organizadas em camadas concêntricas.

A célula de Sertoli é uma célula somática que se estende desde a lâmina basal até a luz do túbulo seminífero. Desempenha variadas funções importantes no processo espermatogênico, dentre elas podemos destacar o suporte estrutural e nutricional às células germinativas em desenvolvimento, a formação da barreira hematotesticular através de junções intercelulares, fagocitose, e a secreção de fluido e hormônios (Foley 2001). Alterações no número, estrutura e/ou função deste tipo celular podem resultar em danos ao epitélio germinativo e comprometimento da espermatogênese (Boekelheid et al., 2005), uma vez que o número de células de Sertoli está relacionado ao tamanho testicular no indivíduo adulto e sua produção espermática (Chapin et al., 1996).

O processo de espermatogênese pode ser dividido em três fases distintas: mitótica ou proliferativa, meiótica e espermiogênica (Clermont, 1972; Russel et al., 1990). A fase mitótica é caracterizada pela proliferação das espermatogônias-tronco com a finalidade de aumentar a população espermatogonial. Em determinado momento, grupos de espermatogônias tornam-se diferenciados e formam os espermatócitos primários (Amann, 1986; Russel et al., 1990). Na fase meiótica os espermatócitos primários (diploides) sofrem meiose I originando os espermatócitos secundários (haploides), que por sua vez sofrem meiose II formando as espermátides (haploides). Durante a fase espermiogênica, as espermátides passam por um processo de citodiferenciação no qual ocorre condensação do material genético, formação do acrossoma, reposicionamento das mitocôndrias, perda de citoplasma e formação do flagelo, originando, finalmente, os espermatozoides (Clermont, 1972).

As diversas gerações de células germinativas não estão organizadas aleatoriamente no epitélio seminífero, mas formam associações celulares de composição fixa que correspondem aos estágios do ciclo espermatogênico. Um ciclo da espermatogênese de ratos dura aproximadamente 12 dias e apresenta-se 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). A duração do processo espermatogênico é regulada pelo genótipo das células germinativas, sendo geralmente constante entre indivíduos de uma mesma espécie (França et al., 2005).

O processo espermatogênico está sob o controle neuroendócrino do eixo hipotalâmico-hipofisário-gonadal e tem início a partir da puberdade, devido a um aumento na secreção de gonadotrofinas (FSH: hormônio folículo-estimulante; LH: hormônio luteinizante). O FSH age nas células de Sertoli estimulando suas funções sobre a espermatogênese, enquanto o LH estimula as células de Leydig a produzirem andrógenos (Maeda et al., 2000; Marty et al., 2003).

1.2.2. Epidídimo

1.2.2.1.Aspectos Estruturais e Desenvolvimento

Nos testículos, os túbulos seminíferos convergem formando a rede testicular, a qual se continua com os ductos eferentes. Estes ductos, por sua vez, convergem para formar um ducto único e altamente enovelado denominado epidídimo (palavra de origem grega que significa adjacente ao testículo) (Figura 2). O comprimento do ducto epididimário varia de acordo com a espécie, sendo 3 metros em ratos, 3 a 6 metros no homem e até 80 metros em cavalos (Robaire et al., 2006).

De acordo as características anatômicas e histológicas do epidídimo, diferentes formas de divisão do órgão em regiões e segmentos tem sido propostas (Nicander, 1956; Hoffer & Karnovsky, 1981; Hermo, 1995; Turner, 2003). A classificação mais utilizada divide o epidídimo de ratos em quatro regiões distinguíveis macroscopicamente (Figura 2): segmento inicial, porção mais proximal do ducto epididimário, diretamente ligada aos ductos eferentes; 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 & Hermo, 1988; Turner, 1995).

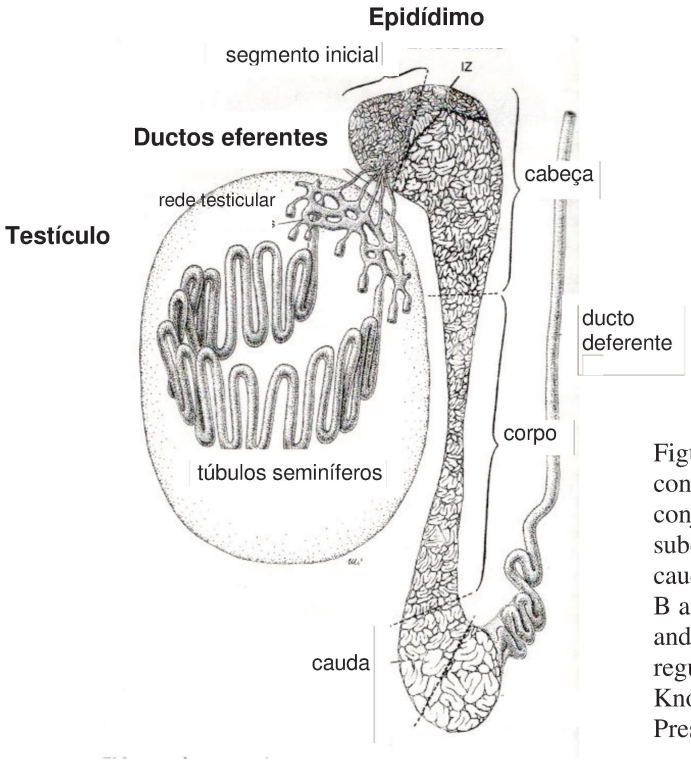

Figura 2: Diagrama representando o testículo contendo túbulos seminíferos e rede testicular, o conjunto de ductos eferentes, o epidídimo de rato subdividido em segmento inicial, cabeça, corpo e cauda, e o ducto deferente. [Adaptada de Robaire B and Hermo L, 1988. Efferent ducts, epididymis and vas deferens: structure, functions and their regulation. In *The physiology of Reproduction,* E Knobil and J D Neill, eds., pp. 999-1080. Raven Press, New York].

Utilizando os mesmos critérios, o epidídimo humano é dividido em apenas três regiões, denominadas cabeça, corpo e cauda. A ausência de segmento inicial se deve ao fato da maior parte da cabeça do epidídimo humano ser constituída por ductos eferentes (revisado por Turner, 2008).

O epidídimo de mamíferos é organizado em lóbulos separados por septos de tecido conjuntivo. Estes septos parecem não servir apenas como suporte estrutural ao órgão, mas também exerceriam papel na separação funcional das regiões epididimárias (Turner et al., 2003).

O epidídimo tem origem embrionária a partir do ducto mesonéfrico devido à ação da testosterona, que inibe a degeneração deste ducto e induz sua diferenciação em epidídimo, ducto deferente e vesícula seminal. A di-hidrotestosterona participa do processo de enovelamento do ducto epididimário e proliferação celular no órgão. A ação de ambos os hormônios no epidídimo é mediada pelo receptor de andrógeno (AR) (Robaire et al., 2006).

Ao nascimento, o ducto epididimário apresenta-se revestido internamente por células epiteliais indiferenciadas e, externamente, por células musculares lisas em um interstício de tecido conjuntivo frouxo (Sun & Flickinger, 1979; 1982). A diferenciação do órgão continua durante o desenvolvimento pós-natal e, no rato, divide-se em três fases distintas: período indiferenciado, período de diferenciação e período de expansão (Figura 3).

O período indiferenciado se estende do DPN (dia pós-natal) 1 ao DPN 15 e caracteriza-se pela proliferação das células epiteliais indiferenciadas, as quais apresentam-se como células colunares sem estereocílios. Durante o período de diferenciação (DPN 16 a 44), o epitélio do ducto epididimário sofre uma série de mudanças. As células colunares indiferenciadas originam diversos tipos celulares, tais como células estreitas, claras, principais, apicais, halo e basais, as quais serão individualmente descritas a seguir. Ao final do período de diferenciação, o ducto epididimário deixa de ser um canal uniforme e passa a apresentar diferenciações regionais altamente especializadas ao longo de sua extensão, decorrentes tanto de mudanças estruturais quanto fisiológicas das células epiteliais (Dacheux et al., 2005). No período de expansão (DPN 44 até a vida adulta do animal),

ocorrem o aparecimento de espermatozoides na luz do ducto epididimário e o aumento do tamanho do órgão em decorrência ao aumento da sua extensão e peso (Rodríguez et al, 2002) (Figura 3).

Os mecanismos que regulam o desenvolvimento pós-natal do ducto epididimário ainda não são completamente conhecidos. Certamente, andrógenos luminais e circulantes exercem papel crítico neste processo, apesar de outros fatores do fluido luminal provavelmente estarem envolvidos (Rodriguéz et al., 2002). Durante o desenvolvimento pós-natal, a diferenciação histológica e funcional da cabeça do epidídimo precede à da cauda (Rajalakshmi, 1985; Limanowski et al., 2001), principalmente devido à dependência diferencial de cada região epididimária a fatores testiculares e aos níveis teciduais de andrógenos (Sun & Flickinger, 1982; Viger & Robaire, 1994). Os diversos tipos celulares que compõem o epitélio epididimário variam qualitativa e quantitativamente entre as diferentes regiões do tecido (Reid & Cleland, 1957).

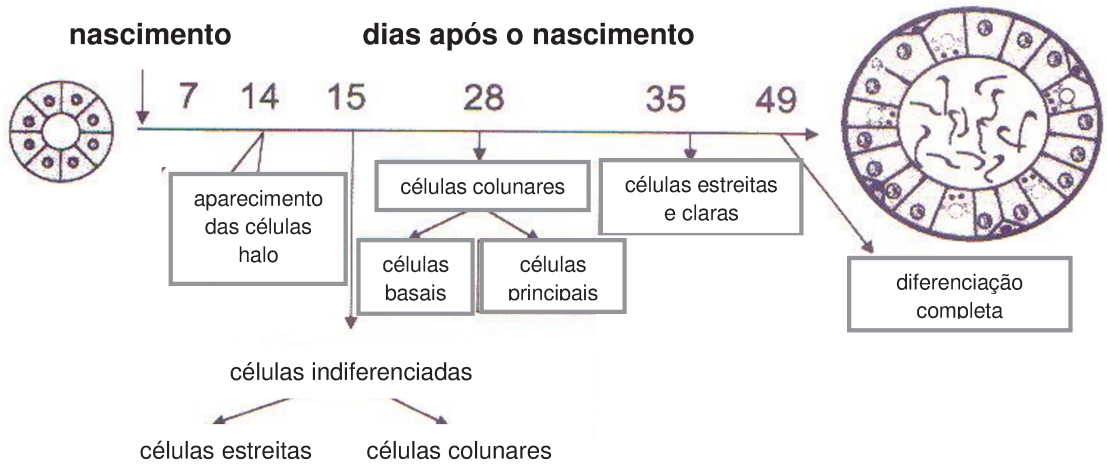

Figura 3: Diferenciação pós-natal do epitélio do ducto epididimário do rato. As células epiteliais do epidídimo são indiferenciadas até aproximadamente o dia pós natal 21, quando as células estreitas e colunares são observadas pela primeira vez. Até o dia 28 as células colunares se diferenciam em células principais e basais. A partir do dia pós-natal 36 observa-se células estreitas no segmento inicial e células claras em toda a extensão do ducto. Por volta do dia 49 o epitélio epididimário está completamente diferenciado. [Adaptada de Rodríguez, C.M., Kirby, J.L., Hinton, B.T. 2002. The Development of the Epididymis. In: Robaire, B., Hinton B. (eds.) In: *The Epididymis – from molecules to clinical practice*, Robaire B and Hermo L, eds., p. 251 – 268. Kluwer Academic/Plenum Publisher, New York].

Dados da literatura mostram evidências crescentes de que as regiões e tipos celulares do epitélio epididimário são similares entre a maioria dos mamíferos, incluindo o homem (Robaire et al., 2006). As células epiteliais do epidídimo desempenham uma série de funções, incluindo transporte de íons e pequenas moléculas orgânicas, síntese e secreção de proteínas, e absorção seletiva do conteúdo luminal, garantindo apropriada maturação e estocagem dos espermatozoides no ducto epididimário (Ezer & Robaire, 2002). Seis tipos celulares, com características morfofuncionais específicas, podem ser reconhecidos ao longo do epitélio do ducto: células principais, basais, estreitas, apicais, claras e halo.

As células principais são as mais abundantes do ducto epididimário, representando de 65% a 80% da população total de células epiteliais do epidídimo do rato, dependendo da região analisada (Trasler et al., 1988). São consideradas as células epiteliais mais importantes deste órgão e podem ser encontradas ao longo de toda a extensão do ducto. Apresentam uma maquinaria endocítica e secretora altamente desenvolvida (Robaire et al., 2006) e a estrutura e função deste tipo celular variam de acordo com a região epididimária onde se encontra. Apresentam microvilosidades e estereocílios, responsáveis pela reabsorção de fluidos e pela atividade secretora do epitélio (Cooper, 1995). A presença de *tight junctions* na porção luminal de células principais adjacentes forma a barreira hemato-epididimária, fundamental para a manutenção do microambiente luminal e proteção do espermatozoide (Hinton et al., 1995; Robaire et al., 2006).

As células apicais são encontradas apenas no epitélio do segmento inicial do epidídimo de rato, sendo ocasionalmente encontradas em outras regiões após o envelhecimento do animal (Serre & Robaire, 2002). As funções específicas das células apicais ainda são pouco conhecidas, mas sabe-se que elas são capazes de realizar endocitose de substâncias luminais (Robaire et al., 2006). As células estreitas também são restritas ao segmento inicial do epidídimo de rato. São caracterizadas pela presença de numerosas vesículas apicais, envolvidas nos processos de endocitose e secreção de íons H^+ para o lumem (Robaire et al., 2006), participando da regulação do pH luminal (Adamali & Hermo, 1996; Breton et al., 1999; Pietrement et al., 2006).

As células claras são células grandes com intensa atividade endocítica presentes na região da cabeça, corpo e cauda do epidídimo, sendo mais numerosas na cauda. São encontradas no epidídimo de diversas espécies animais, incluindo humanos (Vierula et al., 1995; Hermo & Smith, 1998; Robaire et al., 2006). Essas células atuam na acidificação do fluido luminal, evento importante para a manutenção do espermatozoide em estado quiescente durante sua estocagem na cauda do epidídimo (Adamali & Hermo, 1996; Breton et al., 1999; Pietrement et al., 2006, Robaire et al., 2006). As células basais podem ser encontradas ao longo de todo o ducto epididimário. Aparecem aderidas à lâmina basal e emitem projeções citoplasmáticas que atingem o lúmen, interagindo com outras células epiteliais através de fatores parácrinos (Shum et al., 2008). Tem sido proposto um possível papel imune das células basais (Seiler et al., 2000), bem como uma função de regulação do transporte de água e eletrólitos realizado pelas células principais (Cheung et al., 2005) e de endocitose mediada por receptores (Robaire et al., 2006). As células halo são células pequenas encontradas entre duas células principais adjacentes e estão presentes ao longo de todo ducto epididimário. Trata-se de células derivadas de monócitos, linfócitos T auxiliares ou linfócitos T citotóxicos (Serre & Robaire, 1999; Robaire et al., 2006).

1.2.2.2. Aspectos Funcionais

A função primária do epidídimo é o transporte dos espermatozoides que chegam do testículo (Orgebin-Crist, 1969; Brooks, 1983). Além de transporte, o epidídimo tem papel crucial sobre a maturação espermática, regulando o desenvolvimento da motilidade, a aquisição da capacidade de sofrer reação acrossômica e de reconhecer e fundir-se com o ovócito, além de ser sítio de importantes modificações da membrana plasmática dos espermatozoides. Em adição, o epidídimo protege os espermatozoides de espécies reativas de oxigênio (ROS) e estoca os gametas maduros na região da cauda (Cosentino & Cockett, 1986; Hermo & Robaire, 2002; Robaire et al., 2006). Estas funções são executadas dentro dos diferentes ambientes luminais presentes ao longo do ducto epididimário, que se formam durante o desenvolvimento pós-natal do órgão (Rodriguez et al., 2002).

Transporte dos Espermatozoides

Os espermatozoides chegam ao epidídimo impulsionados pelo fluido testicular e, possivelmente, pelo batimento ciliar ordenado das células epiteliais dos ductos eferentes. No entanto, as células epiteliais do epidídimo apresentam estereocílios imóveis e o fluido luminal é reabsorvido em grandes quantidades na região dos ductos eferentes e segmento inicial do epidídimo, o que sugere a existência de outros mecanismos de transporte dos espermatozoides através do ducto epididimário (Robaire et al., 2006).

O ducto epididimário é revestido externamente por uma camada de células musculares lisas que apresentam espessura e inervação crescentes da região proximal para as regiões mais distais do órgão (Baumgarten et al., 1971). Desta forma, o transporte dos espermatozoides através do epidídimo tem sido atribuído especialmente à atividade contrátil desta camada muscular externa ao ducto (Talo et al., 1979; Markkula-Viitanen et al., 1979; Jaakkola & Talo, 1982; Jaakkola, 1983; Cosentino & Cockett, 1986), a qual é influenciada por fatores hormonais e neuronais. A contratilidade do ducto epididimário é controlada pelo sistema nervoso autônomo, através de inervação adrenérgica, colinérgica e não-adrenérgica e não-colinérgica (Ricker et al., 1997), além de estar sujeita à ação de angiotensinas, vasopressinas e ocitocinas presentes no sangue (Cooper, 1998). Na cabeça e corpo do epidídimo, a inervação é mais escassa, ao passo que a cauda apresenta-se ricamente inervada por fibras do sistema nervoso simpático, as quais se mantêm presentes no ducto deferente (Kaleczyc et al., 1968; Ricker, 1998).

O tempo da passagem dos gametas pelo ducto epididimário é espécie-específico, atingindo de 3 a 15 dias dependendo da espécie (Cosentino & Cockett, 1986). No rato, o tempo de trânsito tem cerca de 8 dias (Amann et al., 1976; Robb et al., 1978; França et al., 2005). É justamente durante o trânsito dos espermatozoides pelo epidídimo que ocorre a fase final de diferenciação dos gametas conhecida por maturação espermática (Dacheux et al., 2005), responsável por tornar os espermatozoides provenientes do testículo funcionalmente maduros (Roberts, 2010).

Maturação Espermática

Quando os espermatozoides de mamíferos saem do testículo, eles possuem uma morfologia altamente especializada, porém são imóveis e incapazes de fertilizar o ovócito (Brooks, 1983; Hermo & Robaire, 2002; Gatti et al., 2004). Durante a passagem pelo ducto epididimário, muitas características morfológicas e fisiológicas dos espermatozoides são modificadas (Orgebin-Crist, 1969), resultando em desenvolvimento de capacidade móvel e aquisição de habilidade fértil (Kempinas & Klinefelter, 2010). Como consequência, os espermatozoides passam a apresentar movimento progressivo, adquirem capacidade de ascender no trato reprodutor feminino, sofrer reação acrossômica, reconhecer e ligar-se ao ovócito (Robaire et al., 2006).

O processo de maturação espermática parece depender de uma interação altamente regulada entre a lâmina própria do ducto epididimário, suas células epiteliais, o fluido luminal que banha os espermatozoides, e os gametas propriamente ditos (Bedford, 1975; Orgebin-Crist, 1975, Kempinas & Kinefelter, 2010). As etapas de maturação, determinantes para a qualidade espermática, não estão sob o controle genômico das células germinativas e ainda não são completamente compreendidas (Dacheux et al, 2005; Roberts, 2010). O microambiente intraluminal do epidídimo é rigorozamente regulado pela atividade secretora e absortiva das suas células epiteliais (Robaire et al., 2006) e sabe-se que isso é crucial para os processos de maturação dos espermatozoides (Cyr et al, 2002;. Dacheux et al, 2005).

A aquisição de potencial móvel durante o processo de maturação abrange tanto aumento na porcentagem de gametas móveis, quanto mudanças qualitativas no padrão de motilidade. Espermatozoides testiculares são imóveis e apresentam apenas uma discreta vibração no flagelo. Ao passarem pela cabeça do epidídimo, adquirem um padrão de motilidade circular; ao saírem da cauda epididimária já apresentam movimento progressivo e vigoroso (Brooks, 1983; Cosentino & Cockett, 1986; Robaire et al., 2006). No entanto, gametas no interior do epidídimo permanecem em estado quiescente até o momento da ejaculação (Brooks, 1983), provavelmente devido ao pH ácido do ambiente luminal (Adamali & Hermo, 1996; Breton et al., 1999; Pietrement et al., 2006).

A capacidade de reconhecimento e fusão com o ovócito envolve componentes e domínios específicos na membrana plasmática dos espermatozoides. Durante o processo espermatogênico, a membrana plasmática dos gametas é organizada em domínios, os quais são remodelados durante a passagem pelo epidídimo através da degradação e/ou liberação de componentes testiculares e integração de componentes secretados pelo epitélio epididimário, além da ocorrência de eventos de glicosilação e deglicosilação (Gatti et al., 2004). Essas alterações na membrana do espermatozoide permitem a exposição dos receptores de superfície necessários para o reconhecimento espécie-específico entre o espermatozoide e o ovócito, possibilitando a fecundação (Brooks, 1983).

Em todas as espécies estudadas, a capacidade móvel e o potencial de fertilização dos espermatozoides aumentam progressivamente ao longo da passagem pelo epidídimo (Orgebin-Crist, 1969; Brooks, 1983; Cosentino & Cockett, 1986; Gatti et al., 2004; Robaire et al., 2006).

Estocagem dos Espermatozoides

Nos mamíferos, o sítio mais importante de estocagem de gametas é a cauda epididimária. Apesar de o tempo normal de trânsito espermático através do epidídimo ser de 3 a 15 dias, dependendo da espécie de mamífero considerada, os gametas podem ser estocados na região da cauda por períodos em torno de 30 dias (Orgebin-Crist et al., 1975). Sabe-se que existe uma série de diferenças quanto à composição de íons, pequenas moléculas orgânicas, proteínas e glicoproteínas entre o fluido luminal da cauda e aquele encontrado nas demais regiões epididimárias. No entanto, as condições especiais que permitem a estocagem dos espermatozoides em um estado quiescente na cauda epididimária ainda não foram completamente esclarecidos. O pH luminal (Carr et al., 1985) e a presença da proteína imobilina (Usselman & Cone, 1983; Carr et al., 1985) devem ser considerados fatores importantes neste processo.

Os espermatozoides estocados na cauda do epidídimo chegaram a esta região do órgão em momentos diferentes, o que significa que apresentam idades distintas. Isso

permite que eles respondam à capacitação e sofram reação acrossômica em momentos diferentes após a entrada no trato feminino (Jones, 1999; Sullivan et al., 2005), estratégia muito importante para animais de fecundação interna, principalmente nos quais a ovulação não é sincronizada e induzida pela cópula (Sullivan et al., 2005).

Além do papel de estocagem dos gametas, outras funções têm sido atribuídas às regiões distais do epidídimo, tais como reconhecimento e eliminação de gametas anormais e/ou mortos (Robaire et al., 2006).

Proteção dos Espermatozoides

Sabe-se que a barreira hemato-epididimária possibilita a formação de um microambiente luminal especializado, fundamental para a maturação espermática. No entanto, outro papel fundamental desta barreira é proteger os gametas contra o sistema imunológico, agentes xenobióticos, espécies reativas de oxigênio, dentre outros fatores interferentes. Os mecanismos de defesa incluem a restrição dos compostos que alcançam o ambiente luminal, a síntese e secreção de proteínas específicas como as defensinas, e a síntese e secreção de compostos antioxidantes. Além disso, devido ao fato de os espermatozoides estarem em um ambiente hiperosmótico, o epidídimo deve protegê-los de mudanças acentuadas no potencial osmótico do meio (Robaire et al., 2006).

Os espermatozoides de mamíferos apresentam grandes quantidades de ácidos graxos poli-insaturados em sua membrana plasmática, o que os tornam especialmente susceptíveis à peroxidação lipídica por ROS (Poulos et al., 1973; Jones et al., 1979; Aitken & Clarkson, 1987; Vernet et al., 2004). A peroxidação lipídica da membrana dos espermatozoides tem sido relacionada a defeitos na peça intermediária, diminuição na motilidade espermática devido a defeitos no axonema, bem como diminuição na habilidade fertilizante (revisado por Vernet et al., 2004).

Interessantemente, cada região ou segmento epididimário desenvolveu seus próprios mecanismos de proteção aos gametas. Isso porque a atividade metabólica difere entre as regiões do epidídimo, produzindo diferentes tipos de ROS, além de os espermatozoides

apresentarem-se em diferentes graus de maturação, dependendo da região do ducto epididimário (Robaire et al., 2006).

1.2.2.3. Regulação das Funções Epididimárias

Regulação por Andrógenos

Nos últimos 30 anos, tem sido claramente estabelecido o papel crucial dos andrógenos na regulação de diversas funções epididimárias e na síntese e secreção de moléculas específicas associadas aos processos de maturação e estocagem de gametas (Robaire et al., 2006).

A testosterona chega ao epidídimo por duas vias principais: circulação sanguínea e fluido testicular. A testosterona que atinge o epidídimo via fluido testicular está associada à proteína ligadora de andrógeno (*androgen binding protein*, ABP), secretada pelas células de Sertoli nos túbulos seminíferos. Sugere-se que a ABP exerce importante função na manutenção de altas concentrações de andrógenos, fundamental para as regiões proximais do epidídimo (Bardin et al., 1981). No citoplasma das células epididimárias, grande parte da testosterona é metabolizada pela enzima 5α-redutase em 5-dihidrotestosterona (DHT), principal andrógeno regulador das funções epididimárias (Robaire et al., 2007; Patrão et al., 2009)

Condições de privação de andrógenos têm sido experimentalmente alcançadas através de diversas metodologias, tais como castração bilateral, tratamento com químicos antagonistas de andrógenos ou de hormônio liberador de gonadotrofina (GnRH). No estado de privação androgênica os espermatozoides tornam-se imóveis, perdem a habilidade fértil e morrem (Ezer & Robaire, 2002). Após orquiectomia, o peso, o diâmetro do lumen e a altura das células epiteliais do ducto epididimario diminuem, enquanto o volume de tecido intersticial aumenta (Delongeas et al., 1987). Mudanças morfológicas nas células principais, em contraste à manutenção da morfologia das demais células epiteliais do epidídimo, sugere que este grupo celular seja especialmente sensível aos níveis de andrógenos (Moore & Bedford, 1979). Como consequência à privação androgênica,

observa-se um comprometimento da função secretora das células principais, diminuição da atividade dos receptores androgênicos e da enzima 5α-redutase no epidídimo, sugerindo que os mecanismos de ação de andrógenos são prejudicados na ausência ou redução dos níveis de testosterona (Robaire, 1977; de Larminat et al., 1978; Pujol & Bayard, 1979; Zhu et al., 2000; Hamzeh & Robaire, 2009).

As quatro regiões epididimárias respondem de modo diferente ao reestabelecimento dos níveis normais de testosterona circulante. As alterações na cabeça, corpo e cauda do epidídimo são revertidas, o que não ocorre no segmento inicial. Estudos mostram que o segmento inicial do epidídimo depende de fatores do fluido testicular para manutenção de sua estrutura e função, além da dependência de andrógenos (Fawcett & Hoffer, 1979; Ruiz Bravo, 1988; Hamzeh &Robaire, 2009).

Regulação por Fatores Testiculares

Além de depender da presença de andrógenos circulantes, o epidídimo também depende de fatores do fluido luminal, originados a partir do testículo ou do próprio epidídimo. Em estudos experimentais nos quais a ligadura dos ductos eferentes foi utilizada para obstrução do fluxo de fluido testicular para o epidídimo, observou-se mudanças na morfologia e expressão gênica do segmento inicial do epidídimo (Robaire & Hermo, 1988; Hinton et al., 1998; Cornwall et al., 2002). Na ausência de fatores testiculares, muitas células do segmento inicial sofrem apoptose dentro de 24 horas (Nicander et al., 1983; Fan & Robaire, 1998; Turner & Riley, 1999).

Este tipo de regulação parácrina tem sido denominada regulação lumicrina, por ocorrer em um sistema de ductos e túbulos. A regulação lumicrina não ocorre apenas entre o testículo e o epidídimo, mas também entre as diferentes regiões epididimárias e entre o epidídimo e o ducto deferente (Robaire et al., 2006).

1.2.3. Glândulas Sexuais

Em roedores, o termo glândulas sexuais refere-se à vesícula seminal, próstata, glândula bulbouretral e glândula prepucial. As principais funções das glândulas sexuais são produção de secreções que compõem o sêmen, transporte e nutrição dos espermatozoides após a ejaculação, bem como retenção do ejaculado no trato feminino por um período mais prolongado (Roberts, 2010).

A vesícula seminal encontra-se dorsolateralmente à bexiga urinária e é composta por alvéolos tubulares revestidos por epitélio que apresenta intensa atividade secretora. De fato, a secreção da vesícula seminal é responsável por aproximadamente 70% do volume do ejaculado e é rica em frutose e prostaglandinas. A vesícula seminal também produz algumas proteínas secretoras dependentes de andrógeno que participam dos processos de coagulação do ejaculado e imunoproteção dos espermatozóides (Roberts, 2010).

A próstata é a maior glândula sexual acessória do sistema genital masculino. No rato ela é constituída por três lobos: lobo dorsocranial, também denominado glândula coaguladora, encontra-se aderido à vesícula seminal, lobo ventral e lobo dorsolateral (Komárek et al., 2000). Trata-se de um conjunto de glândulas tubuloalveolares ramificadas, revestidas por epitélio colunar simples composto por células secretoras, basais e neuroendócrinas. Externamente, a próstata encontra-se revestida por uma cápsula fibroelástica rica em músculo liso (Roy-Burmam et al., 2004). A secreção da próstata forma um líquido alcalino de natureza proteica rico em zinco, ácido nítrico e colina, além de conter fosfatase ácida específica da próstata, antígeno prostático específico (PSA), amilase e fibrinolisina (Kierszenbaum, 2008, Roberts, 2010). A secreção prostática contribui com aproximadamente 25% do volume do ejaculado. No homem, a próstata é um órgão compacto e internamente dividido em três zonas ou regiões distintas, denominadas zona central, periférica e de transição (McNeal, 1981). Em termos de homologia entre a próstata de ratos e a humana, o lobo dorsocranial corresponderia à zona central, enquanto o lobo dorsolateral à zona periférica humana. O lobo ventral da próstata de roedores não apresenta qualquer homologia à próstata humana (Roy-Burman et al., 2004).

As glândulas bulbouretrais ou de Cowper são formadas por túbulos amplos bastante ramificados que contem uma única camada celular formada por células serosas e mucosas

(Komárek et al., 2000). O fluido secretado por estas glândulas neutraliza os vestígios de urina e lubrifica a uretra e a vagina, além de ser uma fonte energética para os espermatozoides (Chughtai et al., 2005). As glândulas prepuciais são glândulas sebáceas modificadas, localizadas no tecido subcutâneo da porção final do prepúcio (Komárek et al., 2000). Sua secreção rica em óleos lubrifica a glande e pode apresentar atividade antibacteriana e antiviral (Haschek & Rousseaux, 1998).

1.2.4. Ductos Eferentes e Ductos Deferentes

A rede testicular é ligada às regiões mais proximais do epidídimo pelos ductos eferentes. Estes ductos surgem separadamente a partir da rede testicular, próximo à túnica albugínea. Em sua porção inicial ou proximal são paralelos ou levemente sinuosos, apresentando maior sinuosidade em sua porção distal (Hess, 2002; Setchell & Breed, 2006). Apresentam epitélio pseudoestratificado colunar composto por células ciliadas e não ciliadas, sendo que em ratos o epitélio do ductor apresenta sulcos irregulares em toda sua extensão. O número de ductos eferentes é bastante variável entre as espécies e até mesmo entre indivíduos de uma mesma espécie. Ratos apresentam de 2 a 8 ductos eferentes, enquanto humanos apresentam de 6 a 15. Além de condução dos espermatozoides, os ductos eferentes são responsáveis pela reabsorção do fluido luminal, aumentando a concentração de espermatozoides antes da entrada no epidídimo. Atualmente, estudos têm mostrado evidências de uma possível função secretora dos ductos eferentes (Hess, 2002).

O ducto deferente liga a cauda epididimária à uretra prostática. Durante muito tempo foi considerado um simples condutor dos espermatozoides no momento da ejaculação. No entanto, novos estudos têm mostrado importantes funções do epitélio do ducto em relação aos espermatozoides. Em ratos, o ducto deferente se apresenta como um tubo muscular revestido internamente por epitélio pseudoestratificado composto por células principais, basais, claras e estreitas. Baseado em sua morfologia, pode ser dividido em região proximal, média e distal, além de apresentar uma porção terminal. Antes de chegar à próstata o ducto deferente se dilata formando uma região chamada ampola, onde as vesículas seminais desembocam (Komárek et al., 2000).

As células principais são responsáveis pela secreção de diversas substâncias, entre elas glicoproteínas, além de apresentarem função endocítica. As células epiteliais também desempenham função de proteção dos espermatozoides, reabsorção de fluido e acidificação do ambiente luminal, inibindo a motilidade espermática nesta região.

1.3. Sistema Genital Masculino como Alvo de Desreguladores Endócrinos

Desde o início dos anos 90, grande atenção tem sido dada aos agentes químicos que apresentam potencial para alterar o sistema endócrino de animais. O campo de estudo destes compostos, coletivamente denominados desreguladores endócrinos, tem crescido rapidamente e engloba áreas como imunologia, toxicologia, fisiologia da reprodução, comportamento e ecologia (Hotchkiss et al., 2002). Estas substâncias podem interferir na produção, secreção, transporte, metabolismo, interação ligante-receptor e excreção de hormônios endógenos (Wilson et al., 2008). Hormônios são moléculas naturalmente bioativas, capazes de modular diversas funções fisiológicas incluindo os processos de desenvolvimento, puberdade, comportamento, gametogênese e função sexual, estabelecendo a homeostasia do organismo (Chapin et al., 1996).

Uma vez que o sistema endócrino desempenha papel crítico sobre o desenvolvimento e função do sistema genital masculino, este pode ser considerado um alvo particularmente vulnerável a potenciais perturbações endócrinas (Johnson et al.,1997). De fato, diversos estudos experimentais têm indicado que a exposição aos desreguladores endócrinos, incluindo plastificantes, retardadores de chama, pesticidas isolados ou em misturas, dentre outros, pode prejudicar a saúde reprodutiva masculina (Mylchreest et al, 1998;. Fisher et al., 2003; Fernandes et al., 2007; Perobelli et al., 2010, Scarano et al., 2010;. Nassr et al., 2010;. Fossato Da Silva et al., 2011).

Os desreguladores endócrinos denominados antiandrogênicos são aqueles que antagonizam os efeitos dos andrógenos endógenos. Dentre estes, alguns são capazes de agir diretamente nas células-alvo competindo pelos receptores de andrógenos, como o fungicida vinclozolin, o herbicida linuron e o quimioterápico flutamida (Gray et al., 2001). Nestes

casos, podem ocorrer mudanças conformacionais no receptor e redução na transcrição de genes regulados por andrógenos (Kelce & Gray, 1999). Outros, como os ésteres de ftalatos, agem reduzindo a síntese de andrógenos (Gray et al., 2001). É muito provável que outras formas de ação também estejam envolvidas na toxicidade induzida por esta classe de compostos (Gray et al., 2001; Wilson et al., 2008). A população mundial está constantemente exposta a estes agentes químicos, os quais muitas vezes ocorrem em misturas complexas capazes de exercer efeitos cumulativos (Chapin et al., 1997; Gray et al., 2001; 2006; Blystone et al., 2009). Os efeitos de químicos antiandrogênicos sobre a reprodução masculina são dependentes da fase de exposição, sendo o período gestacional e a puberdade considerados os mais susceptíveis (Stoker et al., 2000; Sharpe, 2006).

Os seres humanos estão constantemente expostos a estes contaminantes ambientais. No entanto, as possíveis consequências da exposição aos desreguladores endócrinos sobre a função reprodutiva do homem ainda não foram completamente elucidadas, embora recentes estudos epidemiológicos sugiram associação entre exposição a produtos químicos e problemas no sistema genital masculino humano (Jensen et al., 2004; 2007; Main et al., 2004; 2006; 2007; Swan et al., 2005; Damgaard et al., 2006; 2007). Alterações no sistema genital masculino podem ser transitórias ou permanentes. Existem diversos fatores associados que poderiam interferir na fertilidade masculina humana, como exposições ocupacionais, infecções, fármacos, radiação, estresse, fatores ambientais, nicotina e álcool (Pelieger-Bruss et al., 2004). Tóxicos ambientais podem exercer seus efeitos adversos sobre a reprodução masculina em processos pré-testiculares, testiculares ou pós-testiculares (Pelieger-Bruss et al., 2004).

Atualmente, parece haver maior preocupação em saúde pública quanto à possibilidade de que a exposição aos desreguladores endócrinos prejudique a qualidade e a quantidade do sêmen da população humana. (Carlsen et al., 1992; Sharpe, 1993; Colborn et al., 1993; Auger et al., 1995; Comhaire et al., 1996; Menchini-Fabris et al., 1996; Pajarinen et al., 1997; Swan et al., 1997; Zorn et al., 1999; Gyllenborg et al., 1999). Uma vez que estudos estabeleçam este declínio na qualidade espermática e demonstrem associação entre

esta disfunção e a exposição a agentes tóxicos, o epidídimo, certamente, seria considerado um importante órgão-alvo (Klinefelter, 2002; Kempinas & Klinefelter 2010).

Nos últimos anos, alguns compostos já têm sido associados à diminuição no número de espermatozoides estocados na cauda epididimária e ao menor número de gametas disponível para ejaculação, com pouca ou nenhuma diminuição na produção espermática testicular, reafirmando a hipótese de que o epidídimo seja um alvo direto e indireto de agentes tóxicos (Klinefelter & Suarez, 1997). Compostos químicos exerceriam seus efeitos sobre o epidídimo e maturação espermática interferindo no epitélio epididimário, agindo diretamente sobre os espermatozoides em trânsito através do órgão, ou indiretamente, através de alterações no tempo de trânsito dos gametas. Neste último caso, alterações no padrão de interação entre os espermatozoides e o epitélio epididimário, a lâmina própria e o fluido luminal resultaria em prejuízo na qualidade e quantidade de espermatozoides disponíveis para ejaculação (Kempinas & Klinefelter, 2010). Demonstrar a ação de um agente químico diretamente sobre o epidídimo é um desafio, visto que se faz necessário excluir a possibilidade do efeito ter sido mediado pelo hipotálamo, hipófise ou testículos (Robaire et al., 2006). Alterações sobre a qualidade dos espermatozoides epididimários após exposição aos desreguladores endócrinos são frequentemente secundárias a injúrias testiculares (Kempinas & Klinefelter , 2010).

Sabe-se que distúrbios na função reprodutiva podem ter origem na vida fetal ou durante a infância, mesmo que seus primeiros sinais sejam detectados apenas na vida adulta, como, por exemplo, comprometimento da espermatogênese (Damgaard et al., 2002). Atualmente, o estudo de alterações durante o desenvolvimento do sistema genital após exposição a agentes químicos ambientais tem sido considerado uma importante subárea da toxicologia da reprodução (Kempinas & Klinefelter, 2010).

Estudos experimentais têm demonstrado que a exposição pré-natal a ftalatos reduz a distância anogenital, aumenta a incidência de hipospadias e malformações epididimárias (Mylchreest et al., 2000; 2002), além de agir diretamente sobre o testículo durante o período de diferenciação fetal e neonatal (Fisher et al.,2003). Essas alterações são permanentes e afetam a função testicular na vida adulta (Hoei-Hansen et al., 2003). Existem

evidências da ação dos ftalatos sobre as células de Sertoli, alterando sua capacidade funcional, e sobre as células de Leydig, causando hiperplasia focal por volta do 16º dia de gestação e, em alguns casos, acarretando formação de adenoma de células de Leydig após o nascimento (Fisher et al., 2003).

A exposição a tóxicos durante o desenvolvimento pré-natal também está associada ao aumento da incidência de agenesia e malformação do epidídimo (Foster et al., 2006; Kempinas & Klinefelter, 2010). Além disso, estudos anteriores demostraram que os antiandrógenos flutamida e vinclozolin, o TCDD (2,3,7,8-tetraclorodibenzo-p-dioxina), agentes estrogênicos como o pesticida metoxicloro, o bisfenol A, e o ftalato DBP (dibutilftalato) acarretam alterações no desenvolvimento, peso, aspecto histológico e número de espermatozoides no epidídimo após exposição durante o desenvolvimento do sistema genital masculino (Inperato-McGinley et al., 1992; Mylchreest et al., 1998; Fisher 2004; David 2006). Apesar de diversos estudos já terem demonstrado os efeitos da exposição aos desreguladores endócrinos durante o desenvolvimento pré e perinatal sobre a função reprodutiva masculina, poucos se atentaram para a exposição de animais pré-púberes e púberes.

Animais sexualmente imaturos parecem ser mais susceptíveis aos desreguladores endócrinos do que animais adultos, possivelmente devido ao *imprinting* hormonal, resultando em mudanças permanentes na vida adulta (Chapin et al., 1997). Comparadas aos adultos, crianças podem ser consideradas mais vulneráveis a agentes tóxicos por ainda estarem em processo de desenvolvimento. O desenvolvimento sexual normal depende de uma série de eventos que se iniciam na vida fetal e continuam após o nascimento. Todas as fases são reguladas por mecanismos dependentes de fatores hormonais e genéticos (Damgaard et al., 2002). O desenvolvimento pós-natal do sistema genital masculino requer sinais hormonais do eixo hipotalâmico-hipofisário, uma subsequente resposta testicular e um *feedback* do testículo sobre o eixo para modular a liberação de gonadotrofina (Marty et al., 2003).

Durante as primeiras fases do desenvolvimento pós-natal, as barreiras fisiológicas ainda não estão completamente estabelecidas, o que pode permitir que substâncias tóxicas

sejam absorvidas mais facilmente. Sabe-se que crianças absorvem 50-90% do chumbo ingerido, enquanto um adulto absorve apenas 10%. Especialmente nos primeiros anos de vida, considera-se um risco aumentado de exposição a agentes químicos através de fatores nutricionais, inicialmente através do leite materno, que pode acumular compostos lipossolúveis e, posteriormente, através do consumo de frutas e vegetais, geralmente em maiores proporções do que seria ingerido por um individuo adulto, acarretando maior exposição a químicos agrícolas. Além disso, crianças apresentam uma aréa de superfície corporal proporcionalmente maior que a de adultos e, portanto, tornam-se mais vulneráveis à exposição cutânea. Diferenças comportamentais também podem ser consideradas fatores contribuintes para maior exposição a agentes químicos, uma vez que crianças passam maior tempo em contato com o solo e com produtos escolares e de higiene pessoal, os quais são ricos em ftalatos (Damgaard et al., 2002).

O desenvolvimento sexual de ratos machos pode ser dividido em 4 fases: neonatal (DPN1 ao 7), infantil (DPN 8 ao 21), juvenil (DPN 22 a 35) e peri-puberal (DPN 36 ao 50/ 60) (Clegg, 1960; Ojeda et al., 1980). A puberdade representa um evento dinâmico e complexo que envolve um conjunto de mudanças físicas, comportamentais e hormonais, através do qual o indivíduo alcança a maturidade sexual e a capacidade reprodutiva (Golub et al., 2008). A ação de andrógenos é essencial para o sucesso do desenvolvimento do sistema genital masculino.

A pré/peripuberdade deve ser considerada um período crítico do desenvolvimento sexual uma vez que os processos de espermatogênese e esteroidogênese ainda não estão completamente estabelecidos nesta fase, tornando-a possivelmente mais susceptível a perturbações endócrinas causadas por xenôrmonios (Johnson et al., 1997). Tem sido relatado que ensaios experimentais em animais púberes são mais sensíveis e precisos para detecção de tóxicos com atividade endócrina do que estudos em indivíduos adultos (Blystone et al., 2007). Além disso, a fase pré-puberal abrange o período de diferenciação pós-natal do epidídimo, descrito anteriormente (Item 1.2.2.1.). Durante este período, o ducto epididimário apresenta importantes modificações morfológicas e funcionais, que resultam na diferenciação regional do ducto, com morfologia, expressão gênica, perfil

protéico e funções específicas (Rodriguez et al., 2002). Perturbações deste processo poderiam comprometer a função epididimária de maneira permanente, prejudicando os processos de maturação dos espermatozóides e interferindo na qualidade dos gametas. Estudos morfofuncionais sobre o desenvolvimento epididimário após insulto químico são fundamentais para as áreas de toxicologia e certamente produzirão novos conhecimentos sobre a biologia do órgão, embasando ações de impacto epidemiológico e clínico (Kempinas & Klinefelter, 2010).

Justificativa

2. JUSTIFICATIVA E RELEVÂNCIA DO TEMA

O desenvolvimento adequado do sistema genital masculino depende de uma série de eventos que se iniciam na vida fetal e continuam após o nascimento. Todas estas fases são reguladas por mecanismos dependentes de fatores hormonais e genéticos. Uma vez que os processos de gametogênese e esteroidogênese ainda não estão completamente estabelecidos durante a pré-puberdade, este deve ser considerado um período crítico do desenvolvimento, possivelmente mais susceptível a perturbações endócrinas causadas por agentes tóxicos.

Além disso, a pré-puberdade compreende o período de diferenciação pós-natal do epidídimo, momento em que o órgão passa por mudanças morfológicas e funcionais que resultam na diferenciação regional do ducto epididimário. O epidídimo é responsável pela maturação dos gametas masculinos, processo que requer a interação dos espermatozoides com os diferentes microambientes especializados estabelecidos ao longo do ducto epididimário. Esta diferenciação é impulsionada em grande parte pelo aumento de andrógenos circulantes que ocorre em torno da puberdade. No entanto, pouco se conhece a respeito das possíveis consequências reprodutivas decorrentes de perturbações no processo de desenvolvimento pós-natal do órgão.

O presente estudo visou interferir no período pré-puberal a fim de entender os possíveis efeitos adversos sobre a quantidade e a qualidade de espermatozóides na idade adulta. Para tanto, um quadro de privação androgênica foi induzido pelo uso de um potente antiandrogênico não-esteróide, a flutamida, composto químico que compete pelo receptor de andrógeno, reduzindo assim a ação fisiológica da testosterona nos tecidos-alvo. Durante a pré-puberdade os níveis de andrógenos no epidídimo estão em ascensão, porém ainda são consideravelmente mais baixos do que o observado em indivíduos adultos, o que permite a ação efetiva do agente antiandrogênico.

Escolheu-se um antagonista de andrógenos devido à ampla exposição ambiental da população em geral a este grupo de desreguladores endócrinos, que incluem pesticidas agrícolas, produtos químicos industriais, farmacêuticos, dentre outros, muitas vezes presentes como misturas complexas capazes de produzir efeitos cumulativos. Baseando-se nas crescentes evidências de que a exposição a químicos desreguladores endócrinos podem

prejudicar a competência reprodutiva masculina, considerou-se que o modelo experimental proposto é atualmente relevante para a toxicologia, bem como a biologia da reprodução

Objetivos

3. OBJETIVOS

3.1. Objetivo Geral

O objetivo do presente estudo foi avaliar as possíveis consequências da privação androgênica durante o período pré-puberal sobre a morfologia e fisiologia do sistema genital masculino de ratos púberes e adultos, com ênfase sobre aspectos epididimários e qualidade espermática.

3.2. Objetivos Específicos

Para alcançar o objetivo descrito acima, foi utilizado um químico com conhecida atividade antiandrogênica a fim de criar a condição de privação de andrógenos durante o período pré-puberal do rato. Parâmetros diretamente dependentes de andrógenos, como separação prepucial e peso de órgãos reprodutores, foram analisados para confirmar o estado de privação androgênica. Para avaliar parâmetros do sistema genital masculino de ratos púberes e adultos, os animais foram induzidos à morte no dia pós-natal 50 e 75. Em ambas as idades, foram analisados os níveis dos hormônios sexuais e aspectos histopatológicos dos testículos e epidídimos, para obtenção de informações gerais sobre a morfofisiologia do sistema genital masculino após privação androgênica durante um importante período do desenvolvimento pós-natal. Para investigar aspectos mais específicos sobre a fertilidade e a qualidade espermática dos animais experimentais, foram realizadas avaliações adicionais nos ratos adultos, tais como análise de parâmetros espermáticos (produção diária, tempo de trânsito através do epidídimo, motilidade, morfologia, perfil protéico, dentre outros) e de fertilidade (após acasalamentos naturais e inseminação artificial).

Capítulos

4. CAPÍTULOS

O presente estudo deu origem a dois manuscritos que serão apresentados a seguir.

4.1. Manuscrito I

O primeiro manuscrito foi intitulado *"Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty"* e publicado no periódico "*Reproductive Toxicology",* USA, *Elsevier,* ISSN: 0890-6238, Fator de Impacto: 3,137 (DOI 10.1016/j.reprotox.2011.12.011).

Impairment on Sperm Quality and Fertility of Adult Rats after Antiandrogen Exposure during Prepuberty

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Abbreviations:

PND – postnatal day

IUI – in utero insemination

GD – gestational day

Abstract

This study evaluated the effects of antiandrogen exposure during the prepubertal period on reproductive development and reproductive competence in adults. Male rats were divided into two groups: Flutamide, receiving 25 mg/kg/day of flutamide by oral gavage and Control, receiving vehicle daily. Dosing continued from PND21 to 44, and animals were killed on PND50 or PDN75-80. The epididymis, prostate, vas deferens and seminal vesicle weights were lower in Flutamide group on PND50, while on PND80 only seminal vesicle weight was reduced. Fertility assessed by IUI revealed a decrease in the fertility potential in the flutamide-treated adults. Flutamide accelerated sperm transit time through the epididymis, impairing sperm motility and storage. A quantitative analysis of the cauda sperm membrane proteome revealed a few significant changes in protein expression. Thus, exposure to flutamide during the prepubertal period compromises the function of the epididymis along with epididymal sperm quality at adulthood.

Keywords: antiandrogen, rat, epididymis, prepuberty, sperm quality, fertility, proteomics.

1. Introduction

The epididymis, a highly convoluted duct that connects the efferent ducts to the vas deferens, plays a crucial role in the acquisition of progressive sperm motility and ultimate fertilizing ability [1, 2]. A significant facet of this maturation process involves reorganization of the molecular architecture of the sperm plasma membrane. Membrane proteins are shed, acquired, and modified as lipid composition changes [3]. Some of these changes are directly pivotal to maturation of the sperm, but others are protective in nature [4]. The mature sperm remain protected during storage in the most distal regions of the epididymis until they are ejaculated or voided in the urine.

Specific functions occur within different regions of the epididymis with specialized microenvironments created along the interior of the epididymal duct [5]. These regional differences are established during the period of postnatal development of the epididymis, from PND 15 to 44 [6]. During this period the epididymis undergoes the requisite morphological and functional changes that resulting this regional differentiation of the duct; distinct gene and protein expression profiles can be found [7]. This differentiation of the epididymis is driven largely by the increase in circulating androgen that occurs around the time of puberty [8]. Studies are needed to elucidate damage to sperm maturation arising from disruption of the differentiation process during development.

The present study was undertaken to interfere with the prepubertal period of differentiation in order to understand the possible adverse effects on sperm quantity and quality at adulthood. We elected to attempt to alter the differentiation of the epididymis with flutamide, a potent non-steroidal antiandrogen [9] that competes for the androgen receptor and is capable of reducing the physiological action of testosterone on target tissues when endogenous tissue levels of androgen are submaximal [10,11]. Between PND21 and 44 androgen levels in the epididymis are on the rise, but far less than adult levels [8]. The choice of an antiandrogen agent was due to extensive environmental exposure of the general population to this group of endocrine disruptors, which include agricultural pesticides, industrial chemicals, pharmaceuticals, and others, many times occurring as complex mixtures of antiandrogens that need not have a common active metabolite to

produce cumulative adverse effects [12, 13, 14, 15]. Based on the notion that developmental exposure to endocrine disruptive chemicals may have adverse consequences on reproductive competence in men, we believe that this experimental model is currently relevant to toxicology as well as reproductive biology.

2. Material and Methods

2.1. Animals

Immature Wistar male rats (21 days old) and Wistar female rats (45 days old) were supplied by the Central Biotherium of UNESP – Univ Estadual Paulista. During the experiment, animals were allocated individually in polypropylene cages, with laboratory grade pine shavings as bedding. Rats were maintained under controlled temperature (± 23°C) and lighting conditions (12L/12D photoperiod). Rat chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP) and filtered tap water were provided ad libitum. Experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Biosciences Institute/UNESP Ethics Committee for Animal Research (protocol number 21/08).

2.2. Experimental design

Male rats were randomly divided into two experimental groups: control group (C, n=30) and flutamide group (F, n=30). The F group rats received daily oral gavage doses of flutamide (Sigma Aldrich – 25 mg/kg, at a volume of 3ml/kg) diluted in corn oil, while C group received only the vehicle (corn oil), at the same volume. Animals were treated from PND21 to PND44 corresponding to the prepubertal development of the epididymis. Rats were weighed daily during treatment and on alternate days after treatment. The dose of flutamide chosen in this study is effectively antiandrogenic, as described previously [16]. No clinical signs of toxicity were observed in any of the experiments herein described. The

study was conducted in two steps; Experiment 1 and Experiment 2, and described as follows.

2.3. Experiment 1:

In this experiment, male (n=40) and female (n=30) Wistar rats were used. The age at acquisition of preputial separation was evaluated starting on PND30 as an indicator of the onset of puberty. The observation criterion adopted was the age when the prepuce first separates from the glans penis, not considering the complete retraction of the prepuce [17]. On PND 50, 20 male rats (n=10/group) were killed for an evaluation of reproductive organ weights and serum hormone levels. The other 20 male rats (n=10/group) were evaluated at 70 days of age for sexual behavior and reproductive competence following natural mating with naïve females. A 10-day post-mating interval was allowed to permit recovery of sperm reserves; subsequently, at the age of 80 days these male were killed for evaluation of reproductive organ weights, sexual hormone levels, sperm motility and morphology, and the sperm membrane proteome.

2.3.1. Evaluation of Sexual Behavior and Natural Mating

At 70 days of age, male rats from each experimental group (n=10/group) were placed individually in polycarbonate crystal boxes, measuring 44×31×16 cm, 5 minutes before introduction of one sexually receptive, adult female rat (70 days old). Sexual receptivity was determined by vaginal smear and by lordosis exhibition in the presence of a sexually experienced, vasectomized male of proven sterility. These females were synchronized to achieve estrous with a single subcutaneous injection of 80μg of luteinizing releasing hormone (LHRH) agonist (Sigma Chemical Co., St Louis, Missouri) approximately 115 hours prior to the sexual behavior evaluation.

Paired animals were observed in the dark period of the cycle in a separate room under dim red light and all sexual behavior tests were performed 2-4 hours after the beginning of the dark period. The following parameters were observed for 40 min: latency to the first

mount, intromission, and ejaculation; number of intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions; and total number of ejaculations [18, 19, 20, 21,22]. Males that did not mount in the initial 10 minutes were considered sexually inactive. After the sexual behavior evaluation was complete, paired animals were kept together for an additional 4 hours. After this period, males and females were separated and vaginal smears were collected to confirm that mating occurred.

2.3.2. Fertility Evaluation

Twenty days later (GD20), naturally inseminated females were killed by decapitation. After collection of the uterus and ovaries the numbers of corpora lutea, implants, reabsorptions, 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 preimplantation loss: [number of corpora lutea – number of implantations/number of corpora lutea] × 100; and rate of postimplantation loss: [number of implantations – number of live fetuses]/number of implantations × 100.

2.3.3. Euthanasia, Body Weight and Reproductive Organ Weights of Male Rats

At 50 or 80 days of age 10 male rats per experimental group were killed by decapitation. The right testis, epididymis and vas deferens, ventral prostate and seminal vesicle (without the coagulating gland) were removed and their wet weights (absolute and relative to body weight) were recorded.

2.3.4. Serum Testosterone, FSH and LH Levels

After decapitation, blood was collected (between 9:00 and 11:30 AM) and serum was obtained by centrifugation (1 236 xg, for 20 minutes at 4ºC). The concentrations of testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were

determined by the technique of double antibody radioimmunoassay. Testosterone assay was performed using a testosterone maia® kit (Biochem Immuno System). The LH and FSH assays were done using specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK, USA). All samples were assayed in duplicate and in the same assay to avoid inter-assay errors. The intra-assay error was 3.4% for LH, 2.8% for FSH and 4% for testosterone.

2.3.5. Sperm Motility and Morphology

Immediately after euthanasia of 80-day-old animals, sperm were obtained from the left vas deferens duct and diluted in 2 ml of modified HTF medium (Human Tubular Fluid, IrvineScientific®), pre-warmed at 34ºC. A 10ul aliquot was placed in a Makler chamber (Irvine, Israel) and analyzed under a phase-contrast microscope (Leica DMLS) at 200X magnification. One hundred sperm were evaluated per animal and classified for motility into: type A: mobile, with progressive trajectory; type B: mobile, with non-progressive trajectory; type C: immotile [23].

With the aid of a syringe and needle, sperm were recovered from the right vas deferens by flushing with 1.0mL of saline formol. To analyze the sperm morphologically, smears were prepared on histological slides that were left to dry for 90 min. and 200 spermatozoa per animal were analyzed in a phase-contrast microscope (400× magnification) [24]. Morphological abnormalities were classified into two general categories: head morphology (without curvature, without characteristic curvature, pin head or isolated form, i.e., no tail attached) and tail morphology (broken or rolled into a spiral) [25]. Sperm were also classified as to the presence or absence of the cytoplasmic droplet.

2.3.6. Quantitative Evaluation of the Sperm Membrane Proteome

Sperm were obtained from the proximal cauda epididymis [26] by nicking the duct with a number 11 scalpel and allowing sperm to disperse into 2 ml of Sperm Isolation Buffer (95ml/l 10xHBSS, 0.35g/l $NaHCO_3$, 4.2g/l HEPES, 0.9g/l glucose, 10ml/l Na

pyruvate, 25 mg/l STI, pH 7.4). One ml was transferred to a microcentrifuge tube and washed twice by centrifugation (2000 rpm, 5 min, 4°C) in the same buffer, with freshly-added 0.2 mM phenylmethylsulphonyl fluoride (PMSF, Sigma, St. Louis, MO). After the final wash, sperm were extracted for 1 h at room temperature with 1 ml of 80 mM n-octyl-B-glucopyranoside in 10 mM Tris, pH 7.2 containing freshly added PMSF. Following a final centrifugation (2000 rpm, 5 min, 4°C), the supernatant was removed and frozen (−70°C).

Prior to 2-D gel electrophoresis, samples were thawed, and each extract was concentrated with 1 mM Tris buffer, pH 7.2, by three centrifugations (3,000 rpm, 30 min, 4°C) in Ultrafree-4 centrifugation filter units (Millipore, Bedford, MA). Protein concentration was determined by the Bradford method, using Bradford Reagent (Sigma Aldrich®). The absorbance was read by spectrophotometry (SPECTRO 22RS, Digital Spectrophotometer - Quimis) and protein concentrations determined by interpolation from standard curve using seven different albumin concentrations (µg/ml): 0, 60, 80, 100, 200, 400 and 600 ($R^2 = 0.99$). Sample volumes containing 30 µg of protein were lyophilized, and protein was solubilized for 30 min at room temperature in 45µl of sample buffer consisting of 5.7 g of urea, 4 ml of 10% NP-40, 0.5 ml of ampholytes (3–10 only; Serva, Heidelberg), and 0.1 g of dithiothreitol per 10 ml. Isoelectric focusing (750 V, 3.5 h) was carried out in capillary tube gels consisting of 6.24 g of urea, 1.5 ml of acrylamide solution (30% acrylamide, 1.2% bisacrylamide), 2.25 ml of 10% NP-40, and 0.65 ml of ampholytes (3–10 only) per 10 ml. Molecular weight separation was carried out in mini 14% acrylamide gels (200 V, 1h). Gels were fixed in 40% ethanol (v/v) and 10% acetic acid (v/v) in ultrapure water, fluorescence stained using Krypton Protein Stain (Pierce Biotechnology) and, finally, immersed in destaining solution (5% acetic acid in ultrapure water) for 5 min.

A Fluoro Image Analyser (FLA-5100;Fujifilm) was used to scan gels and capture high resolution images with a 532 nm laser light source. Progenesis Same Spots Software (2.0) was used for background correction, spot matching, and spot area quantification. Spots corresponding to proteins whose expression was significantly altered (upregulated or

downregulated) were punched from 2D gels using an automated Ettan spot picker. Gel punches were destained twice in an ammonium bicarbonate solution, dehydrated by acetonitrile and dried in a SpeedVac. An small volume of trypsin solution was added, the tubes placed in a Thermomixer (300rpm, 37^{o}C). Additional 20-30ul of a digestion solution was added and the incubation continued overnight; digestion was terminated with TFA. Peptides extracted directly from the solution using C18 ZipTips, were desalted and eluted onto a MALDI plate in a minimal volume of MALDI matrix solution in 80% acetonitrile-20% water. The MSMS data was processed using Protein Pilot 3.0 software (ABI, Inc). Chemical background-subtracted MALDI-MS data were processed on-line using the Aldente software. A targeted MALDI-MSMS analysis was done using an additional spot plated for each sample using a second-round of gel plug peptide extraction.

2.4. Experiment 2:

2.4.1. In Utero Insemination (IUI)

Because rats produce and ejaculate an excess of qualitatively normal sperm, the insemination in utero of a fixed, critical number of sperm has been proven to increase the sensitivity for detecting a decrease in sperm quality in the rat [27]; alterations in sexual behavior and/or sperm production and availability are not issues.

A group of adult females (n = 30) were synchronized to be in estrous on the day of insemination as described previously. Shortly after room lights were turned off on the day of proestrus, the synchronized females were paired with the sexually experienced, vasectomized males of proven sterility for 1hour. Receptive females (i.e. those that exhibited lordosis) were selected for insemination. Proximal cauda sperm were isolated and prepared for insemination as previously described [28, 29], with the following adaptations. Briefly, the sperm were released from the proximal cauda of adult animals (75 days-old) by nicking the duct with a number 11 scalpel and allowed to disperse into 2 ml of modified HTF media (Human Tubular Fluid, IrvineScientific®). After allowing 5 minutes for dispersion, a sperm aliquot was diluted 1:10 with fixative (10% formalin in PBS) and

counted using a Neubauer chamber. Within 15 minutes, each uterine horn was injected with a volume containing 5 x 10^6 sperm [24]. One female was inseminated per male. All inseminations were performed while the recipient female was in a surgical plane using a mix of ketamine and xylazine for the anesthesia. The bifurcation of the uterine horns was exposed through a low midventral incision and each horn was inseminated using a needle (13x0.45mm) attached to a 1.0-ml syringe. Each injection site was cauterized immediately upon withdrawal of the needle. When insemination was completed, the abdominal musculature was sutured. Twenty days later (GD20), the artificially inseminated females were euthanized by decapitation to enable evaluation and calculation of the fertility and reproductive performance parameters, as previously described in 2.3.3.

2.4.2. Daily Sperm Production per Testis, Sperm Number and Transit Time in the Epididymis

The right testis and epididymis of 75-day-old animals were used for sperm counts. Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) in the testis were counted as described previously [30], with adaptations adopted by Fernandes et al. [31]. Briefly, the testis, decapsulated and weighed soon after collection, was homogenized in 5 mL of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 seconds. After a 10-fold dilution, one sample was transferred to Neubauer chambers (4 fields per animal), and mature spermatids were counted. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days, of the seminiferous cycle during which these spermatids are present in the seminiferous epithelium. In the same manner, caput/corpus and cauda epididymidis portions were cut into small fragments with scissors and homogenized, and sperm counted as described for the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by DSP.

2.5. Statistical Analysis

For comparison of results between the experimental groups, parametric Student's t test or nonparametric Mann-Whitney test was performed, according to the characteristics of each variable. Differences were considered significant when $p < 0.05$. The statistical analyses were performed by GraphPad InStat (version 3.02).

3. Results

3.1. Experiment 1

Body weight gain was similar between experimental groups during the treatment as well as after this period (data not shown). There was a significant delay in the age of preputial separation in the flutamide group (Table 1), with treated males presenting with a 1 day delay in separation. At adulthood (PND 70), sexual behavior parameters were similar between the experimental groups (Table 1). Similarly, there were no differences between control and flutamide treated males with respect to fertility following natural mating. There was also no differences in other fertility indices (Table 2).

Final body weight was similar between control and flutatmide treated males at both PND 50 and PND 80 (Table 3). On PND 50, the absolute and relative weights of reproductive organs (epididymis, prostate, full and empty seminal vesicle and vas deferens) were significantly decreased in the flutamide treated males compared to controls; testis weight was similar between the groups (Table 3). On PND 80 reproductive organ weights had recovered with the exception of the seminal vesicles; full seminal vesicle weight was still reduced in flutamide treated males (Table 3).

On PND 50 there was a 41.38% reduction in serum testosterone levels in flutamide-treated males ($p=0.059$) compared to controls. Serum LH and FSH levels were unaffected by flutamide treatment in these same animals (Figure 1). On PND 80 there were no differences in serum hormone levels between the two groups (Figure 1).

Qualitative evaluations of sperm showed a statistically significant reduction in type A sperm (motile, with progressive trajectory) in adult rats exposed to flutamide compared to the control group. Consequently, the percentage of type B sperm (without progressive

motility) was significantly increased in the flutamide group (Figure 2). Sperm morphology assessments revealed that the percentages of both abnormal and normal sperm were similar between experimental groups (Table 4). In both control and flutamide treated males the cytoplasmic droplet was present in the majority of spermatozoa (Table 4).

A quantitative evaluation of the two-dimensional profile of proteins extracted from the plasma membrane of proximal cauda epididymal sperm indicated that 3 proteins changed significantly in the flutamide treated males compared to control males, i.e. spots 30, 54, 112 (Figure 3). The identification of these proteins by MALDI MSMS revealed that S112, which was down-regulated by flutamide treatment, is RB11A, while S30 and S54, both up-regulated by flutamide treatment , are cytochrome b5 type B (CYB5B) and calmodulin OS (CALM) respectively (Figure 3).

3.2. Experiment 2

The fertility of proximal cauda epididymal sperm of adult males, assessed following in utero insemination, was significantly altered by prepubertal flutamide treatment. In addition, there was a decrease in the number of fetuses (Table 2). The number of mature testicular spermatids and the DSP were similar for control and flutamide treated males. However, the number of sperm in both the proximal (caput⁄corpus) and distal (cauda) regions of the epididymis were significantly reduced in flutamide treated males (Table 4). Thus, there was a significant reduction in the sperm transit time through these regions, i.e. sperm transit was accelerated throughout the epididymis following flutamide treatment (Table 4).

4. Discussion

The final stages of sperm differentiation occur outside the gonad, in the epididymal duct [32]. The functions of the epididymis are to facilitate a transition of testicular spermatozoa to functional maturity, and store mature sperm until they are ejaculated or voided in the urine [33]. Since sperm released from the seminiferous epithelium are

transcriptionally inactive, the facets of maturation within the epididymis depend on protein and lipid alterations in the sperm that are a function of the luminal microenvironment of the epididymis [32, 33]. The intraluminal microenvironment within a given region of the epididymis reflects the secretory and absorptive activities of epididymal epithelium in that region [32, 34]. Specific secretory and absorptive activities are then pivotal to sperm maturation [32, 35]. The differentiated functions of specific epididymal regions are established during the period of postnatal development of the organ, from PND 15 to 44 under the influence of androgen [6]. Given the current speculation that chemical stressors (e.g. EDCs) are compromising male reproductive development it is important to better characterize the role in which androgen plays during this critical developmental window.

In the present study, the absence of changes in body weight during the experimental period, as well as in the final body weight, indicates that the antiandrogen exposure did not induce systemic toxicity. Thus, body weight gain was not a contributing factor in any observed reproductive effects of prepubertal antiandrogen exposure.

According to Ashby & Lefevre [36], changes in reproductive organ weights and the day of preputial separation are the two main parameters for detecting antiandrogens in peripubertal male rat assays. Thus, in the present study, the delay in the day when the prepuce first separates from the glans penis in the flutamide-treated animals is indicative of the antiandrogen activity of this chemical and suggests impaired of reproductive development.

As previously reported in a study assessing prepubertal exposure to the antiandrogen vinclozolin [37], no changes were observed in testis weight. The decreased weights of the epididymis, vas deferens, seminal vesicles and prostate in flutamide-treated rats on PND50 suggests compromised development of these androgen-dependent organs due to impaired androgen availability and action, as well as by reduced serum testostereone levels. On PND80 only the seminal vesicle weights were reduced in the flutamide-treated males indicating that during the 35-days following flutamide treatment (from PND 44 to 80) was sufficient for the growth recovery of most reproductive organs.

The observed 41.38% reduction in serum testosterone levels in flutamide-treated males on PND50 was probably due to a direct effect of flutamide on Leydig cells in the testis rather than alteration of the hypotalamic-pituitary-gonadal axis since FSH and LH levels were unaffected by flutamide treatment. According to Blystone et al. [15], this lack of responsiveness of the hypothalamus-pituitary axis to lower serum testosterone after antiandrogenic exposure is not attributable to the immaturity of the axis, since pubertal exposure to other AR antagonists such as vinclozolin produces dramatic increases in serum LH [37].

Biologically, puberty is defined as that time when spermatogenesis first completes its entire cycle and sperm enter the epididymis, which occurs around PND 50 in Wistar rats [30]. In the rat, within a week after these sperm enter the epididymis, fertile sperm can be recovered from the proximal cauda epididymis. The sperm production/g of testis increases until PND 75, and the sperm reserves in the epididymal cauda are maximal around 100 days of age [30]. In the present study was used male rats at 75 days old and, although their sperm reserves are not maximal, the sperm that are present are fertile and, therefore, it was possible evaluate sperm quality parameters and fertility after artificial insemination.

Sexual behavior and performance are regulated by complex mechanisms that involve physiological, neurological, neuroanatomical and endocrinological aspects [38]. The sexual behavior of male rats is characterized by a series of mounts, with or without vaginal intromission, that eventually lead to an ejaculation [39]. That sexual behavior was unaltered in adults after prepubertal antiandrogen exposure is consistent with the normal serum levels of testosterone in these animals.

Evaluation of reproductive parameters after natural mating did not reveal any impairment in the fertility capacity of adult males exposed to flutamide prior to puberty. However, this is most likely attributed to the lack of sensitivity afforded by natural mating animals with inherently robust sperm production and sperm quality [20, 40]. For example, male rats and rabbits fertilize successfully even when 90% of their sperm reserves are depleted [41]. Artificial insemination has been used successfully as a strategy to evaluate sperm quality following toxicant exposure [20, 42]. In this technique only a fixed, limiting

number of sperm is inseminated to achieve 80% control fertility; sperm are not inseminated in excess and the sperm reserves in the epididymal cauda is not a interfering factor . This increases the likelihood of detecting a toxicant-induced change. Indeed, the fertility potential, the endpoint which quantifies male fertile capacity, was decreased in the flutamide group after in utero insemination. In this study, although sperm were not inseminated in excess, the fertility of control males was essentially 100%. The observed fertilization rate for flutamide-treated males was 85%. We speculate that if control fertility had been around the target, i.e. 75-80%, the resultant fertility in the treated group (i.e. 60-65%) would likely have biological significance. As it stands, it is questionable if sperm with 85% fertilizing potential are indeed compromised. Another important point to consider is the sample size used for fertility assessment (n=10). The use of a larger number of experimental animals could improve the statistical power.

Sperm motility is an important parameter to evaluate sperm quality and fertilizing potential [41]. The present study showed a decrease in the motility of sperm from adult males following flutamide-treatment. The observed decrease in sperm motility may be related to the fact that sperm transit through the epididymis was accelerated in these rats. It has been known for some time that castration with or without androgen replacement accelerates sperm transit through the epididymis [43]. More recent work using an adult castrate testosterone-implanted animal model demonstrated that exposure to a chemical that decreases serum testosterone such as chloroethylmethanesulphonate, or expsoure to the metabolite of flutamide, hydroxyflutamide, accelerates sperm transit through the epididymis [44]. Thus, reduced levels of androgen in the epididymis, as well as inhibited action of androgen in the epididymis, leads to accelerated sperm transit. It is reasonable to conclude that acceleration of sperm through the epididymis compromises sperm maturation, i.e. sperm motility and fertilizing potential. Indeed, both chloroethylmethanesulphonate and hydroxyflutamide exposures were shown to result in significantly reduced fertility following in utero insemination [44, 45]. It is therefore, reasonable to assume that the decrease in fertility observed in the present study is at least in part due to maturational compromise caused by accelerated sperm transit. That accelerated transit was observed on PND75 when organ weights and hormone levels were restored

suggests that the prepubertal exposure caused persistent alterations in the development of the epididymis. It will be important to characterize the proteome of the epididymis to determine which androgen-dependent proteins failed to become expressed, and/or persist during development.

The observed proteomic alterations in the cauda epididymal sperm plasma membrane following flutamide treatment may be related to the lower sperm quality observed in flutamide group. Unfortunately, there are currently no data in the literature relating these proteins to fertility. Calmodulin (CALM) expression, which was upregulated by the antiandrogen exposure, is a protein affected by androgens in rat prostate cells [46]. It has been suggested that androgen receptors may play a role in CALM action, which has an important association with the proliferation and differentiation of a variety of cells [47]. RB11A, which was down-regulated by following flutamide treatment is a member of GTP-binding proteins of the Rab family. These have been implicated as key regulators of membrane and protein trafficking in mammalian cells [48]. Sheach et al. [49] suggested that G-proteins are upregulated in response to androgen stimulation in ovarian cancer samples. This is therefore consistent with the observed downregulation of RB11A protein in flutamide-exposed animals at adulthood. Again, such changes in the sperm membrane proteome represent persistent alterations resulting from flutamide exposure during the prepubertal period of development and may reflect alterations induced in the testis as well as the epididymis. Previous studies using intact and castrated T-implanted adult animals exposed to hydroxyflutamide during 5 days have demonstrated direct effects of the toxicant on the epididymis [26, 44]. In the present study, while testicular effects can not be completely excluded, although we did not find alterations in DSP, sperm morphology, and testis histopatology, we can suggest that the observed effects appear to be related to the altered development of the epididymis.More studies are necessary to characterize the molecular changes which persist in both the epididiymis and epididymal sperm subsequent to impaired androgen stimulation during the prepubertal period of reproductive development.

5. Conclusion

We can conclude that, interfering with androgen action during the prepubertal period compromises reproductive competence at adulthood. Significant alterations in both the quantity and quality of sperm in the epididymis are observed at adulthood. These alterations are independent of hormone status and organ weight at adulthood indicating that specific molecular events pivotal to normal differentiation of the epididymis either fail to occur or fail to persist into adulthood.

6. Acknowledgements

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7. References

[1] Hermo L, Robaire B. Epididymal cell types and their functions. In: Robaire B, Hinton BT editors. The Epididymis – from molecules to clinical practice, New York: Kluwer Academic/ Plenum Publisher; 2002, p. 81-102.

[2] Sullivan R, Saez F, Girouard J, Frenette G. Role of exossomes in sperm maturation during the transit alongthe male reproductive tract. Blood Cells Mol Dis 2005; 35: 1–10.

[3] Saradha B, Mathur PP. Effects of environmental contaminants on male reproduction. Environ Toxicol Pharmacol 2006; 21: 34-41.

[4] Hinton BT. What does the epididymis do and how does it do it. In: The American Society of Andrology editor. Handbook of Andrology, Lawrence: Allen Press; 2010, p. 11-5.

[5] França LR, Avelar GF, Almeida FFL. Spermatogenesis and transit through the epididymis in mammalswith emphasis on pigs. Theriogenology 2005; 63: 300–18.

[6] Sun EL, Flickinger CJ. Development of cell types and regional differences of the postnatal rat epididymis. Am J Ana 1979; 154: 27.

[7] Rodríguez CM, Kirby JL, Hinton BT. The development of the epididymis. In: Robaire B, Hinton BT editors. The Epididymis – from molecules to clinical practice, New York: Kluwer Academic/ Plenum Publisher; 2002, p. 251 – 268.

[8] Marty MS, Chapin RE, Parks LG, Thorsrud BA. Development and maturation of the male reproductive system. Birth Defects Res B Dev Reprod Toxicol 2005; 68: 125-36.

[9] Mukherjee M, Chattopadhyay S, Mathur PP. Effect of flutamide on the physiological status of epididymis and epididymal sperms. Andrologia 1992; 24: 113-6.

[10] Miyata K, Yabushita S, Sukata T, Sano M, Yoshino H, Nakanishi T, Okuno Y, Mato M. Effects of perinatal exposure to flutamida on sex hormones and androgen-dependent organs in F1 male rats. J Toxicol Sci 2002; 27(1): 19-33.

[11] Foster PM, Harris MW. Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. Toxicol Sci 2005; 85: 1024-32.

[12] Chapin RE, Harris MW, Davis BJ, Ward SM, Wilson RE, Mauney MA, Lockhart AC, Smialowics RJ, Moser VC, Burka LT, Collins BJ. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. Fundam Appl Toxicol 1997; 40: 138-57.

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

[14] Gray LE Jr, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, Howdeshell K, Ankley GT, Guillettes L. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int J Androl 2006; 29: 96-104.

[15] Blystone CR, Lambright CS, Cardon MC, Furr J, Rider CV, Hartig PC, Wilso VS, Gray LE Jr. Cumulative and antagonistic effects of a mixture of the antiandrogens vinclozolin and iprodione in the pubertal male rat. Toxicol Sci 2009; 111(1): 179-88.

[16] Dhar JD, Srivastav SR, Setty BS. Flutamide as an androgen antagonist on epididymal function in the rat. Andrologia 1983; 14: 55-61.

[17] Parker RM. Testing for reproductive toxicity. In: Hood RD, editor. Developmental and reproductive toxicology: a practical approach. New York: CRC Press; 2006, p.472-4.

[18] Ahlenius S, Larsson K. Apomorphine and haloperidol-induced effects on male rat sexual behavior: no evidence for actions due to stimulation of central dopanine autoreceptors. Pharmacol Biochem Behav 1984; 21(3): 463-6.

[19] Ågmo A. Male rat sexual behavior. Brain Res Protoc 1997; 1(2): 203-9.

[20] Clegg ED, Perreault SD, Klinefelter GR. Assessment of male reproductive toxicology. In: Hayes AW editor. Principles and Methods of Toxicology, 4 Ed, Philadelphia: Taylor & Francis; 2001, p. 1263-1299.

[21] Favareto AP, Fernandez CD, da Silva DA, Anselmo-Franci JA, Kempinas WG. Persistent impairment of testicular histology and sperm motility in adult rats treated with Cisplatin at peri-puberty. Basic Clin Pharmacol Toxicol 2011, 109(2):85-96.

[22] Fernandez CDB, Bellentani FF, Fernandes GSA, Perobelli JE, Favareto APA, Nascimento AF, Cicogna AC, Kempinas WG. Diet-induced obesity in rats leads to a decrease in sperm motility. Reprod Biol Endocrinol 2011, doi: 10.1186/1477-7827-9-32.

[23] 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(13): 991-1002.

[24] Seed J, Chapi RE, Clegg ED, Dostal LA, Foote RE, Hurtt ME, Klinefelter GR, Makris SL, Perreault SD, Schrader S, Seyler D, Sprando R, Treinen KA, Rao 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(3): 237-44.

[25] Filler R. Methods for evaluation of rats epididymal sperm morphology. In: Chapin RE, Heindel JH editors. Male reproductive toxicology, California: Academic Press; 1993, p. 334-343.

[26] Klinefelter GR, Laskey JW, Ferrell J, Suarez JD, Roberts NL. Discriminant analysis indicates a single sperm protein (SP22) is predictive of fertility following exposure to epididymal toxicants. J Androl 1997; 18(2): 139-150.

[27] Klinefelter GR. Actions of toxicants on the structure and function of the epididymis. In: Robaire B, Hinton BT editors. The Epididymis – from molecules to clinical practice, New York: Kluwer Academic/ Plenum Publisher; 2002, p. 353-369.

[28] Klinefelter GR, Laskey JW, Kelce WR, Ferrell J, Roberts NL, Suarez JD, Slott V. Chloroethylmethanesulfonate-induced Effects on the Epididymis seem unrelated to altered Leydig Cell Function. Biol Reprod 1994; 51(1): 82- 91.

[29] Kempinas WG, Suarez JD, Roberts NL, Strader L, Ferrell J, Goldman JM, Narotsky MG, Perreault SD, Evenson DP, Ricker DD, Klinefelter GR. Fertility of rat epididymal sperm after chemically and surgically induced sympathectomy. Biol Reprod 1998; 59(4): 897-904.

[30] 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.

[31] Fernandes GS, Arena AC, Fernandez CDB, Mercadante A, Barbisan LF, Kempinas WG. Reproductive effects in male rats exposed to diuron. Reprod Toxicol 2007; 23(1): 106-12.

[32] Dacheux JL, Castella S, Gatti JL, Dacheux F. Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology 2005; 63: 319-41.

[33] Roberts KP. What are the components of the male reproductive system?. In: The American Society of Andrology editor. Handbook of Andrology, Lawrence: Allen Press; 2010, p. 1-5.

[34] Robaire B, Hinton B, Orgebin-Crist MC. The Epididymis. In: Neill JD editor. Physiology of Reproduction, Elsevier; 2006, p. 1071-148.

[35] Cyr DG, Finnson KW, Dufresne J, Gregory M. Cellular interactions and the blood-epididymal barrier. In: Robaire B, Hinton BT editors. The Epididymis – from molecules to clinical practice, New York: Kluwer Academic/ Plenum Publisher; 2002, p. 103-18.

[36] Ashby J, Lefevre A. The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of antiandrogens, oestrogens and metabolic modulators. J Appl Toxicol 2000; 20: 35-47.

[37] Monosson E, Kelce WR, Lambright C, Ostby J, Gray LE Jr. Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. Toxicol Ind Health 1999; 15(1-2): 65-79.

[38]Chan JSW, Olivier B, Jong TR, Snoeren EMS, Kooijman E, van Hasselt FN, Limpens JHW, Kas MJH, Waldinger MD, Oosting RS. Translation research into sexual disorders: Pharmacology and genomics. Eur J Pharmacol 2008; 585: 426-35.

[39] Hull EM, Dominguez JM. Sexual behavior in male rodents. Horm Behav 2007; 52: 45-55.

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

[41] Mangelsdorf I, Buschmann J, Orthen B. Some aspects relating to the evaluation of the effects of chemicals on male fertility. Regul Toxicol Pharmacol 2003; 37: 356-69.

[42] Kempinas WG, Klinefelter GR. The epididymis as a target for toxicants. In: McQueen C A editor. Comprehensive Toxicology, Oxford: Academic Press; 2010, p. 149-66.

[43] Dyson AL, Orgebin Crist MC. Effect of hypophysectomy, castration, and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. Endocrinology 1973; 93:391-402.

[44] Klinefelter G R, Suarez J D. Toxicant-induced acceleration of epididymal sperm transit: androgen-dependent proteins may be involved. Reprod Toxicol 1997; 11(4): 511-19.

[45] Fernandez CDB, Porto EM, Arena AC, Kempinas WG. Effects of altered epididymal sperm transit time on sperm quality. Int J Androl 2008; 31: 427–37

[46] Furuya Y, Lundmo P, Short A D, Gill D L, Isaacs J T. The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. Cancer Res 1994; 54: 6167–75.

[47] Cifuentes E, Mataraza JM, Yoshida BA, Menon M, Sacks DB, Barrack ER, Prem-Veer Reddy G. Physical and functional interaction of androgen receptor with calmodulin in prostate cancer cells. PNAS 2004; 101(2): 464-69.

[48] Ren JC, Zhu Q, Lapaglia N, Emanuele NV, Emanuele MA. Ethanol-induced alterations in Rab proteins: possible implications for pituitary dysfunction. Alcohol 2005; 35(2):103-12.

[49] Sheach LA, Adeney EM, Kucukmetin A, Wilkinson SJ, Fisher AD, Elattar A, Robson CN, Edmondson RJ. Androgen-related expression of G-proteins in ovarian cancer. Br J Cancer. 2009; 101(3):498-503.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Figure legends

Figure 1. Serum hormone levels (ng/mL) in rats from control and flutamide groups at 50 and 80 days of age. Values expressed as mean ± SEM. Mann-Whitney test.

Figure 2. Sperm motility of rats from control (n=10) and flutamide (n=10) groups. Values expressed as median. Mann-Whitney test. *$p < 0.05$.

Figure 3. Two dimensional proteome of proximal cauda sperm plasma membrane proteins of control (A) and flutamide group (B). Numbered spots indicate proteins differentially expressed following flutamide treatment. (C) Graph showing the treatment-related changes in spot area after background correction (IOD or integrated optical density units) of CALM, RB11A and CYB5B proteins (n= 7/group).

Table 1: Age of preputial separation and sexual behavior parameters of male rats at 70 days old.

	Experimental Groups	
	Control (n=10)	Flutamide (n=10)
[a]Preputial separation (days)	32.55 ± 0.34	33.70 ± 0.38*
[b]Latency to the first intromission (s)	118.20 ± 24.66 (10)	205.27 ± 46.35 (10)
[b]Number of intromissions until the first ejaculation	18.30 ± 2.40 (10)	15.36 ± 1.59 (10)
[b]Latency to the first ejaculation (s)	794.67 ± 58.92 (9)	790.82 ± 94.52 (10)
[b]First post-ejaculatory intromission (s)	1108.1 ± 66.32 (9)	1110.3 ± 102.41 (10)
[b]Latency to the first post-ejaculatory intromission (s)	313.44 ± 11.90 (9)	319.45 ± 14.32 (10)
[b]Number of post-ejaculatory intromissions	20.22 ± 3.74 (9)	18.91 ± 1.94 (10)
[b]Number of ejaculations	2.33 ± 0.16 (9)	2.45 ± 0.21 (10)

[a]Values expressed as mean ± SEM. Student's t test. *$p<0.05$. [b]Values expressed as mean ± SEM. Mann-Whitney Test. Times are expressed in seconds. The number of animals that presented the behavior is indicated in parentheses.

Table 2: Fertility and reproductive performance of male rats after natural mating and artificial insemination.

	Natural Mating		Artificial Insemination	
	Control (n=10)	Flutamide (n=10)	Control (n=10)	Flutamide (n=9)
[a]Body weight of dams (g)	336.91 ± 6.01	329.26 ± 8.88	336.18 ± 7.35	328.92 ± 5.80
[a]Uterus weight with fetuses (g)	58.67 ± 2.35	60.34 ± 3.40	58.89 ± 3.42	43.79 ± 5.50*
[a]Number of corpora lutea	12.58 ± 0.42	12.91 ± 0.53	12.80 ± 0.59	12.22 ± 0.52
[a]Implant number	12.08 ± 0.54	12.45 ± 0.49	12.10 ± 0.62	9.56 ± 0.80*
[a]Reapsortions number	0.92 ± 0.40	1.09 ± 0.55	0.30 ± 0.15	0.33 ± 0.24
[a]Number of fetuses	11.17 ± 0.56	11.36 ± 0.65	11.80 ± 0.63	8.89 ± 0.99*
[a]Fetus weight (g)	3.45 ± 0.07	3.50 ± 0.10	3.04 ± 0.05	3.13 ± 0.06
[b]Fertility potential (%)	100.00 (92.15 – 100.00)	100.00 (96.67 – 100.00)	100.00 (92.45 – 100.00)	84.62 (61.54 – 91.67)*
[b]Pre-implantation loss (%)	0.00 (0.00 – 7.85)	0.00 (0.00 – 3.33)	0.00 (0.00 – 7.56)	15.39 (8.33 – 38.46)*
[b]Post-implantation loss (%)	7.69 (0.00 – 8.52)	0.00 (0.00 – 12.14)	0.00 (0.00 – 5.00)	0.00 (0.00 – 0.00)

[a]Values expressed as mean ± SEM. Student's t test. *$p<0.05$. [b]Values expressed as median and interquartile intervals. Mann-Whitney test.*$p<0.05$.

Table 3: Final body weight and absolute and relative reproductive organ weights of male rats from control group and futamide group at 50 and 80 days old.

	Experimental Groups			
	50 days old		80 days old	
	Control (n=10)	Flutamide (n=10)	Control (n=10)	Flutamide (n=10)
Final body weight (g)	219.52 ± 7.82	234.89 ± 7.77	356.03 ± 8.04	363.54 ± 9.68
Testes (g)	1.08 ± 0.03	1.09 ± 0.05	1.54 ± 0.05	1.62 ± 0.05
Testes (mg/100 g)	495.09 ± 15.57	465.85 ± 22.62	434.56 ± 16.34	449.33 ± 17.99
Epididymis (mg)	164.23 ± 7.21	101.63 ± 5.35**	486.32 ± 7.91	467.52 ± 11.72
Epididymis (mg/100 g)	74.95 ± 2.65	43.72 ± 2.80**	136.96 ± 2.57	129.12 ± 3.73
Ventral prostate (mg)	133.54 ± 11.31	78.22 ± 7.59*	311.94 ± 15.18	310.11 ± 28.94
Ventral prostate (mg/100 g)	60.93 ± 4.51	33.78 ± 3.68*	87.79 ± 4.37	84.93 ± 7.64
Vas deferens (mg)	50.35 ± 2.18	37.58 ± 1.88*	96.82 ± 1.90	89.70 ± 2.41
Vas deferens (mg/100 g)	23.05 ± 1.03	16.11 ± 0.89**	27.25 ± 0.55	24.75 ± 1.15
Seminal Vesicle full (mg)	241.27 ± 27.12	83.99 ± 8.76*	946.85 ± 24.68	735.84 ± 41.05**
Seminal Vesicle empty (mg)	106.78 ± 8.51	53.07 ± 4.94*	294.23 ± 16.00	259.44 ± 12.28

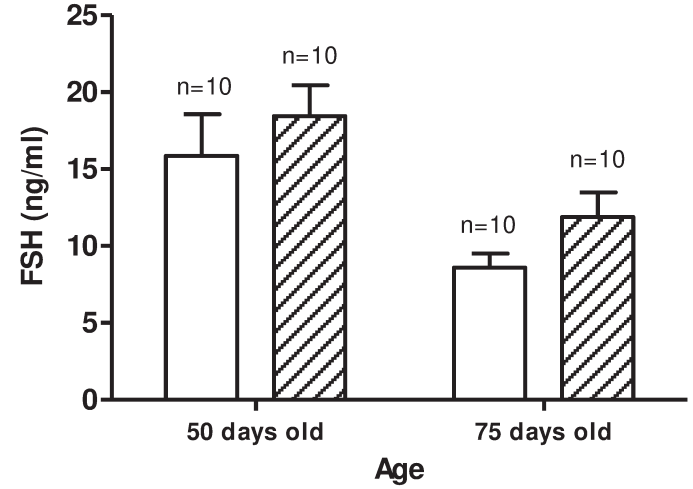
Values expressed as mean ± SEM. Mann-Whitney test. *$p \leq 0.001$;**$p \leq 0.0001$

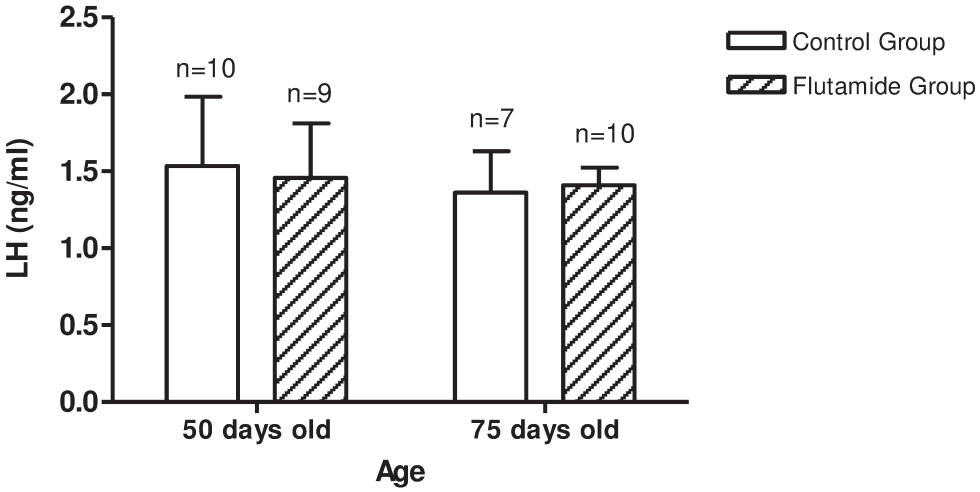
Table 4: Sperm count parameters (75 day-old animals) and sperm morphology (80 day-old animals) of male rats from control and flutamide groups.

	Control (n=9)	Flutamide (n=10)
[a]Spermatid number (x 10^6/testis)	249.36 ± 6.94	250.91 ± 6.92
[a]Spermatid number (x 10^6/g testis)	171.29 ± 5.15	172.32 ± 4.85
[a]Daily sperm production (x10^6/testis/day)	40.88 ± 1.14	41.13 ± 1.13
[a]Relative sperm production (x10^6/g testis/day)	28.08 ± 0.84	28.25 ± 0.79
[a]Caput/corpus epididymis sperm number (x10^6/organ)	113.72 ± 4.57	90.51 ± 6.66*
[a]Caput/corpus epididymis sperm number (x 10^6/g organ)	625.99 ± 37.86	539.81 ± 44.96
[a]Sperm transit time in the caput/corpus epididymis (days)	2.80 ± 0.14	2.21 ± 0.17*
[a]Cauda epididymis sperm number (10^6⁄organ)	144.61 ± 10.10	113.74 ± 9.39*
[a]Cauda epididymis sperm number (10^6⁄g organ)	1 360.0 ± 101.37	1 415.3 ± 120.14
[a]Sperm transit time in the cauda epididymis (days)	3.54 ± 0.22	2.76 ± 0.21*
[b]Normal shaped sperm (%)	90.25 (85.75 – 91.50)	92.50 (84.37 – 95.37)
[b]Sperm with cytoplasmic droplet (%)	77.00 (65.37 – 82.87)	72.00 (65.37 – 81.87)

[a]Values expressed as mean ± SEM. Mann-Whitney Test. *$p<0.05$. [b]Values expessed as median and interquartile intervals. Mann-Whitney Test.

Figure 1


25
20
15
10
5
0
FSH (ng/ml)
n=10
n=10
n=10
n=10
50 days old
75 days old
Age


2.5
2.0
1.5
1.0
0.5
0.0
LH (ng/ml)
n=10
n=9
n=7
n=10
50 days old
75 days old
Age
Control Group
Flutamide Group

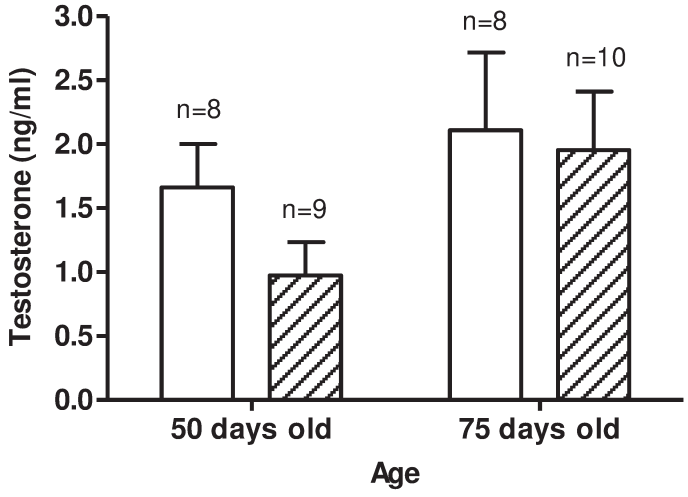

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Testosterone (ng/ml)
n=8
n=9
n=8
n=10
50 days old
75 days old
Age

Figure 2

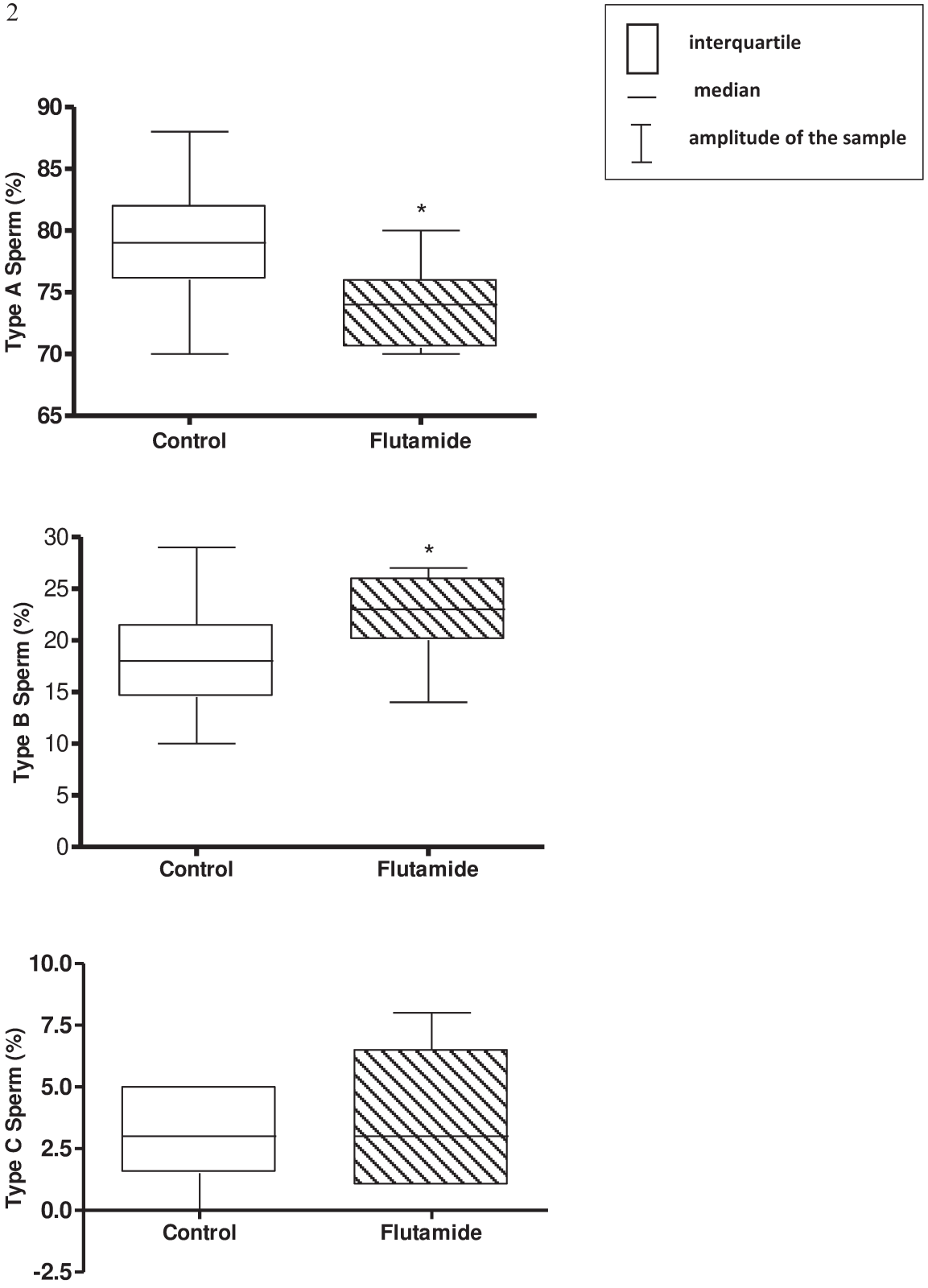
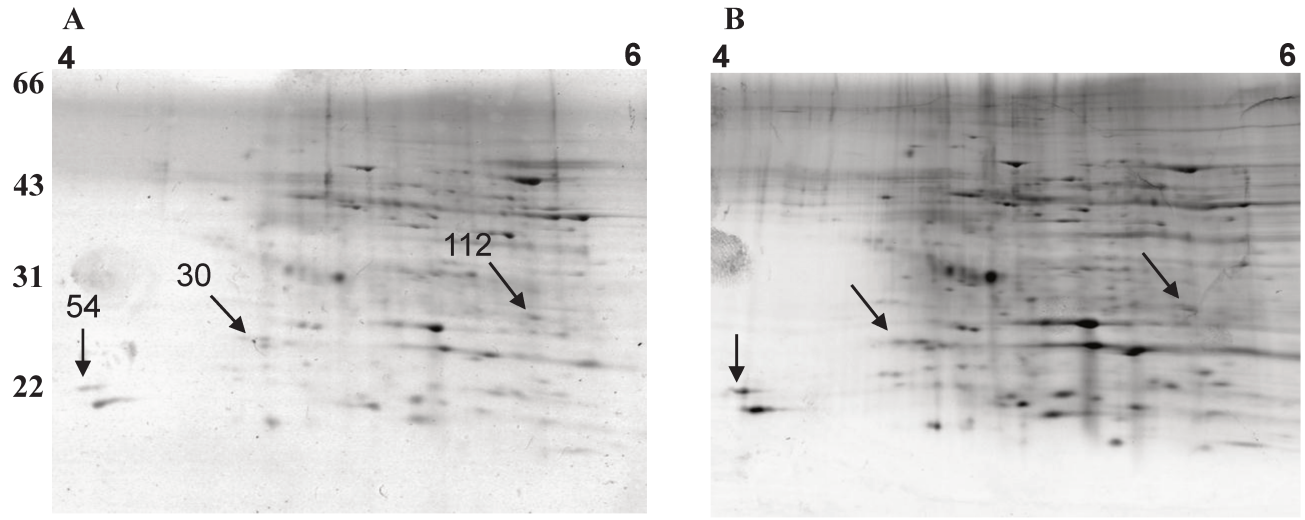
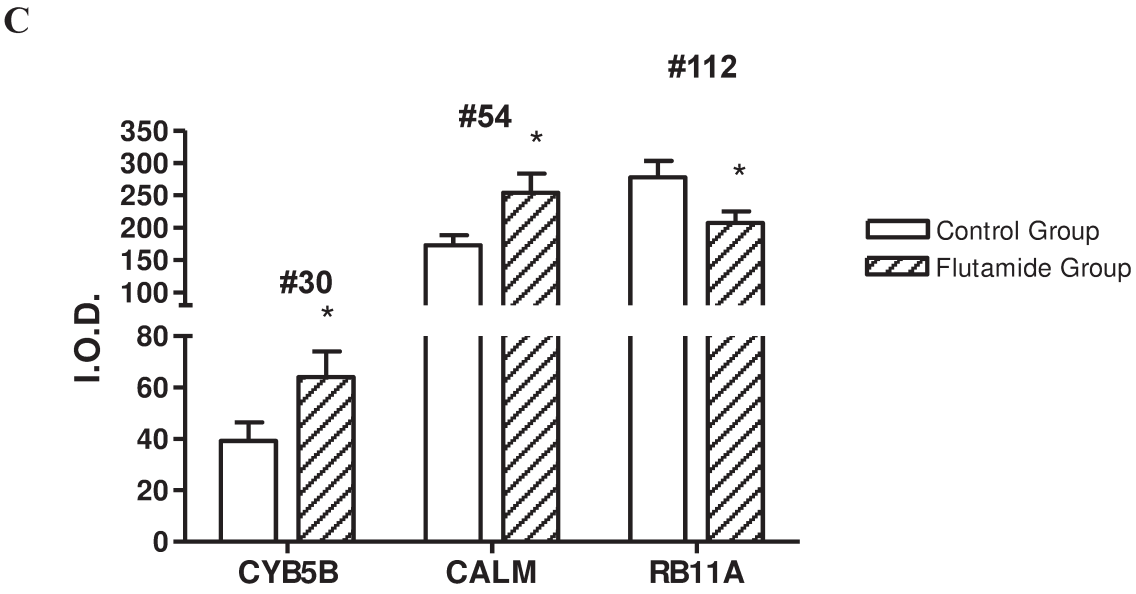

interquartile
median
amplitude of the sample
Type A Sperm (%)
90
85
80
75
70
65
*
Control
Flutamide
Type B Sperm (%)
30
25
20
15
10
5
0
*
Control
Flutamide
Type C Sperm (%)
10.0
7.5
5.0
2.5
0.0
-2.5
Control
Flutamide

Figure 3


A
4
6
66
43
31
22
54
30
112
B
4
6


C
#112
#54
#30
*
*
*
350
300
250
200
150
100
80
60
40
20
0
I.O.D.
CYB5B
CALM
RB11A
Control Group
Flutamide Group

4.2. Manuscrito II

O segundo manuscrito foi intitulado “Androgen deprivation during prepuberty interferes in proteins expression in pubertal and adult rat epididymis” e será submetido para publicação no periódico “*International Journal of Andrology”,* USA, *Wiley-blackwell,* Online ISSN: 1365-2605, Fator de Impacto: 3.601.

Androgen deprivation during prepuberty interferes in proteins expression in pubertal and adult rat epididymis.

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Abstract

Sperm maturation in the epididymis includes the acquisition of progressive and sustained motility of spermatozoids and the development of fertility capacity. Regional differentiation of the epididymal duct, crucial to the sperm maturation processes, occurs during its postnatal development**.** Although the experimental administration of androgen antagonists to achieve an androgen deprivation state in the epididymis is well established, few studies have focused on experimental testosterone deprivation in immature animals during the postnatal epididymal differentiation**.** Animals in this development phase seem more affected by hormonal injuries than older animals, possibly due to hormonal imprinting and the rapid and interactive endocrine and morphological changes. A previous study showed a decrease in sperm quality and fertility potential of male rats exposed to antiandrogen during prepuberty, suggesting persistent impairment of male reproductive competence in adulthood. The present study aimed to evaluate the histological organization of the testes and epididymis after premature androgen deprivation and to investigate possible changes in the testicular and epididymal epithelium focusing on the expression pattern of androgen receptor-AR, sperm protein 22-SP22, and two proteins (calmodulin and Rab11A) possibly related to previously observed fertility impairment. Although androgen deprivation did not impair the testicular and epididymal morphology of pubertal and adult animals, the AR immunostaining in the epididymis was decreased by antiandrogen exposure. The immunostaining for Calm and Rab11A in the epididymis was, respectively, increased and decreased in the flutamide-exposed animals, while SP22 immunostaining was similar in both the experimental groups. These changes in the pattern of proteins expression in the epididymis may be related to impaired epididymal function, sperm quality and fertility capacity, as previously observed. More studies are necessary to better investigate the molecular changes in epididymis after hormonal injury during prepuberty and the permanent consequences on sperm quality and fertility.

Introduction

Sperm maturation in the epididymis includes the acquisition of progressive and sustained motility of spermatozoids and the development of fertility capacity (Yeung and Cooper, 2002; Roberts, 2010). In addition, the plasma sperm membrane undergoes constant remodeling, with a sequential attachment and shedding of molecules that require precise timing and order of epididymal gene expression in the different segments of the organ (Sipilä et al., 2011). The rodent epididymis can be divided into 4 distinct regions (initial segment, caput, corpus and cauda), which can be further subdivided into several segments, each one presenting a unique environment that contributes to the sequential process of sperm maturation (Turner, 2003; Sipilä P et al., 2011).

These segments of the epididymal duct are established during the postnatal development of the organ, which includes the undifferentiated period, differentiation phase and expansion period (Sun and Flickinger, 1979). The differentiation stage is especially important because the epididymis undergoes important changes in its morphology, function and gene and protein expression resulting in the segmentation of the duct (Rodríguez et al., 2002). The complete mechanisms that regulate the growth and differentiation of the epididymis are unknown, although the critical role of luminal and circulating androgens should be considered (Robaire et al., 2006).

Endogenous androgens exert most of their effects by binding to androgen receptor (AR), a ligand-activated transcription factor, resulting in the control of androgen-responsive gene transcription in target cells (Patrão et al., 2009). Expression of AR itself is hormonally regulated by androgens (Zhun et al., 2000). Lumicrine interaction between androgens and AR involves transport of testicular testosterone bound to androgen binding protein (ABP) in the proximal epididymis. The testosterone-ABP complexes are taken up to the cytoplasm of the epithelial cells, where the enzyme 5α-reductase converts this hormone into dihydrotestosterone, the main androgen in the regulation of epididymal functions (Robaire et al., 2007, Patrão et al., 2009).

The experimental administration of androgen antagonists to achieve androgen deprivation of the epididymis is well established (Kaur et al 1992, Dhar et al., 1983). Data

in the literature have shown that epididymal androgen receptors and 5α-reductase activity are both decreased in the androgen-deficient state and the testosterone replacement appears to be sufficient for the recovery of caput, corpus and cauda epididymis, but does not completely reverse the effects on the initial segment (Robaire et al., 1977; Moniem et al., 1978; Fawcett and Hoffer, 1979; Holland et al., 1992). On the other hand, few studies have focused on experimental testosterone deprivation in immature animals during the postnatal differentiation of the epididymis. Animals in this developmental phase seem to be more affected by hormonal injuries than older animals, possibly due to hormonal imprinting and the rapid and interactive endocrine and morphological changes (Chapin et al., 1997; Stoker et al., 2000).

A previous study showed a decrease in sperm quality and fertility potential of male rats exposed to antiandrogen during prepuberty, suggesting persistent impairment of epididymal function and reproductive competence during adulthood. In addition, these adverse effects appear to be associated with some changes in the protein profile of the sperm membrane (Perobelli et al., 2012). The aim of the present study was to better investigate the role of testicular and post-testicular events in this compromising of sperm quality by evaluating the structural organization of the testis and epididymis and investigating possible changes in the proteins expression of these reproductive organs after premature androgen deprivation, focusing on the expression pattern of androgen receptor-AR, sperm protein 22-SP22, and two proteins (calmodulin and Rab11A) possibly related to previously observed fertility impairment, as shown by the evaluation sperm membrane proteomic.

Material and Methods

Animals

Immature Wistar male rats (21 days old) were supplied by the Central Biotherium of UNESP – Univ Estadual Paulista. During the experiment, animals were allocated individually into polypropylene cages, with laboratory grade pine shavings as bedding. Rats

were maintained under controlled temperature (± 23°C) and lighting conditions (12L/12D photoperiod). Rat chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*. Experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Biosciences Institute/UNESP Ethics Committee for Animal Research (protocol number 21/08).

Experimental design

Male rats were randomly divided into two experimental groups: control group (C, n=14) and flutamide group (F, n=16). The F group received daily oral doses of flutamide (Sigma Aldrich – 25 mg/kg, at a volume of 3ml/kg) diluted in corn oil, while the C group received only the vehicle (corn oil), at the same volume. Animals were treated from post natal day (PND) 21 to PND44 corresponding to the prepubertal development of the epididymis. Rats were weighed daily during treatment and on alternate days after this period. The dose of flutamide chosen in this study is effectively antiandrogenic, as described previously (Dhar et al., 1983; Klinefelter et al., 1997a; Klinefelter et al., 1997b).

The animals were killed on PND 50 (n= 7-8 rats/group) and PND 75 (n=7-8 rats/group) for the evaluation of total body weight, weight of organs, intratesticular testosterone levels, histopathology of the testis and the epididymis, immunohistochemistry in the testis for calmodulin and Rab11A, and immunohistochemistry in the epididymis for androgen receptor (AR), sperm protein 22 (SP22), calmodulin and Rab11A. Besides, it was performed Western blot assay for AR in epididymal extract.

Euthanasia, body weight and organ weights of male rats

At 50 and 75 days of age, 7-8 male rats per experimental group were killed by decapitation. The right testis, epididymis and vas deferens, ventral prostate, seminal vesicle (without the coagulating gland), liver and kidneys were removed and their wet weights (absolute and relative to body weight) were recorded.

Ex vivo testosterone production

The right testis of each animal was removed, decapsulated, and the parenchyma was sliced into approximately 50 mg pieces. Each piece was weighed and placed into a 1.5-ml microfuge tube containing 1.0 ml Medium 199 (M199). The M199 was buffered with 0.71 g/L sodium bicarbonate (NaHCO3) and 2.1 g/L Hepes, and contained 0.1% BSA (bovine serum albumine) and 25 mg/L soybean trypsin inhibitor, pH 7.4. The parenchyma was incubated, in duplicate, for 2 hours at 34°C [44]. After centrifugation (5 min., 10,000 × *g*), the medium was frozen at −70°C until the determination of testosterone levels by the technique of double antibody radioimmunoassay. Testosterone assay was performed using a *testosterone maia® kit* (Biochem Immuno System). All samples were assayed in duplicate and in the same assay to avoid inter-assay errors. The intra-assay error was 4%.

Histological procedures

The left testis and epididymis were collected and fixed in Bouin's fluid for 24 h (25% formaldehyde, 70% saturated solution of picric acid and 5% glacial acetic acid). The pieces were embedded in paraffin wax and sectioned at 5µm (cross-sections of the testis and longitudinal sections of the epididymis). Sections used for histological evaluation were stained with hematoxylin and eosin (HE), examined and photographed by light microscopy. Sections used for immunohistochemistry were prepared in silanized slices. The evaluations were performed in blind assays.

Histopathology Evaluation

Testis

Seminiferous tubule cross-sections were randomly chosen in three non-serial sections per animal, totaling 100 tubules/animal. They were classified as normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, few germ cell layers, vacuole formation). The interstitium was qualitatively

evaluated. In addition, the numbers of Sertoli cell nuclei were counted in 20 cross-sections of seminiferous tubules per rat at stage VII of spermatogenesis (Clermont, 1972) under a light microscope (Zeiss, Axiostar plus), at 400x magnification. Only Sertoli cells exhibiting typical morphological nuclear features were quantified.

Epididymis

The histopathological analysis of the epididymis was qualitative, analyzing the entirety of each epididymal histological section. The aspects of the epithelium, lumen and interstitial tissue were evaluated.

Immunohistochemistry Assay

Testes

- *Calmodulin (CALM) and Rab11A*

Testes cross-sections (n=4 animals/experimental group) were deparaffinized using xylene (Sigma Chemical Co., St. Louis, MO, USA) and hydrated using decreasing concentrations of ethanol. The slides were immersed in 0.01M sodium citrate buffer, 0.05% Tween 20 and submitted to antigen recovery in a microwave for 60 sec. Next, the sections were washed twice in Delbecco`s Phosphate Buffered Saline (DPBS), 5 min each, and incubated with blocking solution for 30 min at 34º (1% BSA and 3% Tween 20 in DPBS buffer). Incubation with 3% hydrogen peroxide in water for 15 min at 34º was used to block the endogenous peroxidase. Subsequently, the slides were incubated with the following primary antibodies diluted in blocking solution (1:200), overnight at 4º C: the polyclonal Calm Ig (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal Rab11A Ig (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Following two 5 min washes in DPBS, the sections were incubated for 1h at room temperature with appropriate secondary antibody (Vector Biotinylated Universal Antibody) diluted in blocking solution (1:100). The Standard Vectastain ABC system (Santa Cruz Biotechnologies) was used to evidence the immunocomplex formed by secondary antibody linked to the primary antibody, according to the manufacturer's instructions. After two 5

min washes in DPBS, peroxidase activity was revealed using 3,3'-Diaminobenzidine (Impact DAB) for 20 sec. The sections were washed in distilled water, counterstained with hematoxylin, dehydrated using increasing concentrations of ethanol, cleared by xylene and coverslipped with Vectamount. Sections were analyzed under a light microscope, and the images were captured by a digital camera coupled to the microscope.

Epididymis

- *Androgen Receptor (AR)*

The immunohistochemistry studies were performed as previously described by Silva et al., 2010. Briefly, epididymal longitudinal sections (n=3 animals/experimental group) were deparaffinized using xylene (Sigma Chemical Co., St. Louis, MO, USA) and hydrated using decreasing concentrations of ethanol. The blockage of endogenous peroxidase was performed in 30% H_2O_2 solution for 30 min at room temperature. The sections were washed with TBS buffer (20 mM Tris, 150 mM NaCl, 0.1% BSA) and incubated with 300 mM glycine in TBS buffer for 5 min. After a TBS wash, the slides were incubated with blocking solution (5% BSA in TBS buffer) for 15 min at room temperature. The Avidin/Biotin blocking kit (Vector laboratories, Burlingame, CA, USA) was used to block endogenous biotin following the manufacturer's instructions. Then, sections were incubated overnight at 4°C with primary antibody against Androgen Receptor (AR, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking solution (1: 250). Following three 2 min washes in TBS and 15min incubation with blocking solution, the sections were incubated for 30 min at room temperature with appropriate secondary antibody (anti-rabbit) conjugated to biotin diluted in blocking solution (1:200). The Standard Vectastain ABC system (Santa Cruz Biotechnologies) was used to evidence the immunocomplex formed by secondary antibody linked to the primary antibody, according to the manufacturer's instructions. The slides were incubated for 90 min at room temperature with avidin-biotin. After three 2 min washes in TBS, peroxidase activity was revealed using a solution of TBS, 0.1 M imidazole, DAB (3,3 '-diaminobenzidine) 0.075% and 0.002% H2O2 for 10 min at room temperature. The sections were washed in TBS and counterstained with methylene blue, dehydrated by applying increasing concentrations of ethanol, cleared by xylene and

coverslipped with Permount. Sections were analyzed under light microscope, based on the subdivision of the epididymis of rats into six distinct regions (proximal and distal initial segment, caput, corpus, proximal and distal cauda), as indicated in the results. The images were captured by a digital camera attached to the microscope.

- *CALM, Rab 11A, Sperm Protein 22 (SP22)*

Epididymal longitudinal sections (n=4 animals/experimental group) were deparaffinized using xylene (Sigma Chemical Co., St. Louis, MO, USA) and hydrated using decreasing concentrations of ethanol. The slides were immersed in 0.01M sodium citrate buffer, 0.05% Tween 20 and submitted to antigen recovery in a microwave for 60 sec. Next, the sections were washed twice in Delbecco`s Phosphate Buffered Saline (DPBS), 5 min each, and incubated with blocking solution for 30 min at 34º (1% BSA and 3% Tween 20 in DPBS buffer). Incubation with 3% hydrogen peroxide in water for 15 min at 34º was used to block the endogenous peroxidase. Subsequently, the slides were incubated with the following primary antibodies diluted in blocking solution, overnight at 4º C: the polyclonal Calm Ig (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), the monoclonal Rab11A Ig (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or the polyclonal SP22 (1:250, Welch et al. 1998).

Following two 5 min washes in DPBS, the sections were incubated for 1h at room temperature with appropriate secondary antibody (Vector Biotinylated Universal Antibody) conjugated to biotin diluted in blocking solution (1:100). The Standard Vectastain ABC system (Santa Cruz Biotechnologies) was used to evidence the immunocomplex formed by secondary antibody linked to the primary antibody, according to the manufacturer's instructions. After two 5 min washes in DPBS, peroxidase activity was revealed using 3,3'-Diaminobenzidine (Impact DAB for 20 sec). The sections were washed in distilled water, counterstained with hematoxylin, dehydrated using increasing concentrations of ethanol, cleared by xylene and coverslipped with Vectamount. Sections were analyzed under a light microscope, based on the subdivision of the rat epididymis into six distinct segments

(proximal and distal initial segment, caput, corpus, proximal and distal cauda), as indicated in the results. The images were captured by a digital camera coupled to the microscope.

Western Blot Assay

A Western blot experiment was performed on epididymal total protein extracts, as described previously by Silva et al., 2010. Briefly, the epididymis (n= 3-4 animals/experimental group) was collected, dissected, divided into SI/caput, corpus and cauda and immediately frozen in liquid nitrogen and stored at -80ºC. The tissues (approximately 100mg per epididymal region) were pulverized in liquid nitrogen and dounce-homogenized in 3 volumes of RIPA buffer (1%NP-40, v/v, 0.5% sodium deoxicolate, v/v, 0.1% sodium dodecyl sulfate, w/v), in Phosphate Buffered Saline (PBS) buffer, containing protease inhibitors (1mM phenylmethylsulfonil fluoride, 0.5mM pefabloc, 10µg/ml aprotinin, 20µg/ml leupeptin and 10µg/ml pepstatin). After 30min of incubation at 4°C, samples were centrifuged twice (15,000 × g, 4°C, 10 min) and the supernatant (total protein extract) was aliquoted. Protein concentration was determined with BioRad protein assay reagent (Invitrogen, San Diego, CA, USA), using bovine serum albumin as the standard.

Total protein extracts (100µg) were size-separated on 8% acrylamide/bis-acrylamide gels (37.5:1). Pre-stained protein molecular weight standards (New England BioLabs, Beverly, MA, USA) were used as a reference. The proteins were transferred onto a PVDF membrane (Immobilon-P; Millipore, Billerica, MA, USA) according to standard protocol. Transfer and equal protein loading were verified by Comassie blue staining. Membranes were incubated for 1.5h at room temperature with blocking solution containing 5% non-fat milk (w/v) in TBS-T buffer (100mM Tris-Cl (pH 8.0), 150 mM NaCl, and 0.05% Tween-20, v/v). After 5 min wash with TBS-T, membranes were incubated with primary antibody against AR (1:600, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or against β actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1h at room temperature. The β-actin was used as internal control. The membranes were washed with TBS-T buffer and subsequently incubated for 30 min at room temperature with appropriate secondary

antibody conjugated to peroxidase conjugated secondary antibody (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Results were visualized by a chemiluminescent detection (ECL Luminol, New England Nuclear Lifew Sciwences Products, Boston, MA, USA) using autoradiography films.

Statistical Analysis

For comparison of results between the experimental groups, parametric Student's *t* test or nonparametric Mann-Whitney test was performed, according to the characteristics of each variable. Differences were considered significant when $p < 0.05$. The statistical analyses were performed by GraphPad InStat (version 3.02).

Results

Body weight gain was similar between experimental groups during the treatment as well as after this period (data not shown). In the same way, final body weight was comparable between control and flutamide-treated rats at both PND 50 and PND 75. On PND 50, the absolute and relative weights of reproductive organs (epididymis, prostate, full seminal vesicle and vas deferens) were significantly decreased in the flutamide group compared to control. There was no difference in the testis absolute weight between the groups while testis relative weight was increased in flutamide-exposed animals. On PND 75 reproductive organ weights had recovered and were similar between experimental groups. The absolute and relative weights of the liver and the paired kidneys were comparable between control and flutamide group at both studied ages (Table 1).

The intratesticular testosterone levels did not present a significant difference between control and flutamide-dosed animals at 50 days old. In 75-day-old animals, this parameter had shown a 94% increase in flutamide group compared to control, although this alteration was not statistically significant ($p=0.189$) (Fig. 1).

Quantitative histopathological analysis of the testes (Table 2) and qualitative histopathological analysis of the epididymidis (data not shown) did not reveal alterations in

flutamide-exposed rats in relation to control rats at either of the studied ages. The number of Sertoli cell nuclei per seminiferous tubule (stage IX of the spermatogenesis) was similar between the experimental groups (Table 2).

There was no modification in the immunostaining pattern for CALM and Rab11A in the testis of dosed rats when compared to control rats at both studied ages (data not shown). In epididymal tissue, positive immunostaining for (AR) was observed in epithelial, interstitial and smooth muscle cells from the initial segment, caput, corpus and cauda epididymis, in an age- and region-specific pattern (Figure 2 and 3). Changes in the AR immunostaining pattern were detected in the epididymis of flutamide-exposed animals on PND50. In the epithelial cells, diminutions in the number and intensity of nuclear staining were observed in the proximal and distal initial segment and caput, whereas the corpus and cauda epididymis showed a slighter reduction in the nuclear staining intensity. The cytoplasmic staining was similar between control and flutamide groups in all epididymal regions. The AR immunostaining in the smooth muscle cells showed a reduction in the number and intensity of nuclear staining only in the proximal and distal initial segment. Decreases in the immunostaining intensity were noted in the interstitial cells of all epididymal regions (Fig. 2).

In the epididymis of flutamide-exposed animals on PND75, the number of epithelial cells with nuclear staining for AR were lower in flutamide group when compared to control, only in the distal initial segment and caput. In the other epididymal regions the AR immunostaining pattern in epithelial cells was similar between experimental groups. The AR immunostaining pattern in interstitial and smooth muscle cells was comparable between the control and flutamide groups in all epididymal regions (Fig. 3). The Western blot assay detected the AR as a single band, with the expected molecular weight of approximately 110KDa in rat epididymal tissue at 50 and 75 days of age (Fig. 4A and 4B, respectively).

The immunostaining pattern for SP22 along the epididymis was similar between control and flutamide-dosed animals at either of the studied ages (data not shown). The immunostaining for CALM in the epididymis epithelium was increased in flutamide group

both at 50 and 75 days old, specifically in the cauda region (Fig. 5 and 6, respectively). The immunostaining for Rab11A in the epididymis epithelium was similar between the experimental groups at 50 days of age (data not shown). On the other hand, there was a reduction in the immunostaining for Rab11A in the epididymal tissue of flutamide-exposed animals at PND 75, only in the cauda epididymis (Fig. 7).

Discussion

The present study aimed to investigate whether chemically-induced androgen deprivation during a critical period of male reproductive development interferes in the histological organization and expression of specific proteins in testicular and epididymal tissue. The present study was accomplished to better comprehend the possible changes in the testis and epididymis involved in the previously observed impairment on sperm quality and fertility ability of male rats exposed to flutamide during prepuberty (Perobelli et al., 2012).

The absence of alterations in body weight evolution during the experimental period, as well as in the final body weight and liver and kidney weights, indicates that the antiandrogen exposure did not induce systemic toxicity in the experimental animals. Corroborating previous studies (Monosson et al. 1999, Perobelli et al., 2012), no changes were observed in absolute testis weight. The decreased weights of the epididymis, vas deferens, seminal vesicle and prostate in flutamide-treated rats on PND50 suggest compromised development of these androgen-dependent organs and support the efficiency of such chemically-induced androgen deprivation. On PND75 all reproductive organs presented a growth recovery.

The testicular histopathology was evaluated to detect some impairment of spermatogenesis, since this parameter is considered sensitive for evaluating testicular toxicity (Takayama et al., 1995; Anahara et al., 2008). No alterations were found either in the morphology of seminiferous tubules or in the testicular interstitium of flutamide-exposed animals. Adding to this result, the number of Sertoli cells per seminiferous tubule was similar between the groups. These somatic cells are responsible for creating an

appropriate environment for germ cell proliferation and maturation (Boekelheid et al., 2005). These results suggest that flutamide exposure did not interfere in the spermatogenesis process.

Some studies have documented elevation in plasma testosterone levels after antiandrogen exposure (Friry-Santini et al., 2007; Rouquie´ et al., 2009, Svechnikov et al., 2010). The experimental design adopted in the present study failed to show this effect (Perobelli et al., 2012). Therefore, *ex vivo* intratesticular testosterone production was assessed to be a more precise measure of the androgenic status of the male when compared to serum dosages. The 94% increase in the intratesticular testosterone levels of flutamide-exposed animals at 75 days old may be due to an impairment of the hormonal control of the hypothalamic-pituitary-testis axis. The lesser ability of testosterone to bind AR in the androgen-deprivation situation suppresses the inhibitory feedback effect of testosterone on LH production by preventing the hypothalamus and anterior pituitary from recognizing the presence of testosterone. This can result in hyperstimulation of Leydig cells and a consequent increase of testosterone levels (Svechnikov et al., 2010). This hyperstimulation of Leydig cells during the antiandrogen exposure period may provoke desensitization of these cells and, consequently, permanent impairment of hormonal axis control.

Although the androgen deprivation did not impair the epididymal duct morphology of pubertal and adult animals, the AR immunostaining in the epididymis was decreased by antiandrogen exposure in both the studied ages. Epididymal androgen receptors and 5α-reductase activity are both decreased in the androgen-deficient state, suggesting that the mechanisms of androgen action are compromised by androgen withdrawal (Ezer and Robaire, 2002). The quantity of AR-positive cells in the epididymis increases during the postnatal development of the duct, confirming the importance of these receptors in the normal development of the organ (You, 1998). Thus, the observed changes in the AR expression pattern in the epididymis of immature and adult rats suggest an impairment of the postnatal development of the organ and could be related to the compromising of epididymal function and the lower sperm quality and fertility capacity previously observed (Perobelli et al., 2012).

Although SP22 is described as an important molecular biomarker of male fertility (to review see Kempinas and Klinefelter, 2010), no alteration was observed in the pattern of SP22 immunostaining in the epididymis tissue of flutamide-exposed animals, when compared to controls. This result complements the results obtained previously (Perobelli et al., 2012), in which SP22 levels were found to be unaltered in the sperm membrane protein extracts from rats dosed with flutamide during prepuberty.

The immunostaining for CALM and Rab11A in the testis and epididymis was investigated, since a previous study revealed modifications in these proteins by evaluating the sperm membrane protein profile (Perobelli et al., 2012). The CALM is localized in the acrosomal region and flagellum of the sperm. The roles of this protein in sperm physiology are diverse and include the support of capacitation-related changes in plasma membrane properties (Ignotz and Suarez, 2005). The Rab11 is a small GTP binding protein involved in vesicular trafficking. Although it has been described as an essential protein in the fertilization process in drosophila (Tiwari et al., 2008), there are no data in the literature about its involvement in mammals fertilization. The immunostaining for CALM and Rab11A in the epididymis tissue was difuse, occuring in the epithelium, spermatozoa in the lumen and in the interstitium. The increase in CALM expression and the decrease in Rab11A expression observed in the epididymal cauda of flutamide-exposed rats may indicate changes in the physiology of the epididymis and in the sperm maturation processes, impairing the ability of sperm suffer the capacitation process and, consequently, the fertility competence of the animals at adulthood. More studies are needed to better evaluate the role of these proteins in the mammal sperm maturation and fertility.

In the present study, although the androgen deprivation during prepuberty did not alter the histological organization of the testes and epididymis either at puberty or at adulthood, the treatment impaired the expression of specific proteins in epididymal tissue at puberty and adulthood, possibly interfering in processes of post-testicular sperm maturation and compromising sperm quality and fertility in a persistent manner. Further studies are necessary to better investigate the molecular mechanisms involved in the impairment on reproductive competence of male rats after hormonal injury during prepuberty.

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References

Anahara R, Toyama Y & Mori C. (2008). Review of the histological effects of the anti-androgen, flutamide, on mouse testis. *Reprod Toxicol* 25(2), 139-143.

Boekelheid K, Johnson KJ & Richburg JH. Sertoli cell toxicants. In: Skinner M.K. & Griswold M.D. (Eds). Sertoli cell Biology. San Diego: Elsevier Academic Press, 2005: 345-382p.

Chapin RE, Harris MW, Davis BJ, Ward SM, Wilson RE, Mauney MA, Lockhart AC, Smialowicz RJ, Moser VC, Burka LT & Collins BJ. (1997). The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam Appl Toxicol* 40(1), 138-157.

Clermont Y. (1972) Kintics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* 52, 198-236.

Dhar JD, Srivastav SR & Setty BS. (1983). Flutamide as an androgen antagonist on epididymal function in the rat. *Andrologia* 14, 55-61.

Ezer N & Robaire B. Androgenic Regulation of the structure and function of the epididymis. In: Robaire B. & Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 297-316p.

Fawcett DW & Hoffer AP (1979). Failure of exogenous androgen to prevent regression of the initial segments of the rat epididymis after efferent duct ligation or orchidectomy. *Biol Reprod* 20(2), 162-181.

Friry-Santini C, Rouquie´ D, Kennel P, Tinwell H, Benahmed M, & Bars R. (2007). Correlation between protein accumulation profiles andconventional toxicological findings using a model antiandrogenic compound,flutamide. *Toxicol. Sci* 97, 81–93.

Hamzeh M & Robaire B. (2009). Effect of testosterone on epithelial cell proliferation in the regressed rat epididymis. *J androl* 30, 200-212.

Holland MK, Vreeburg JT & Orgebin-Crist MC. (1992). Testicular regulation of epididymal protein secretion. *J Androl* 13(3),266-273.

Ignotz GG & Suarez SS. (2005). Calcium/calmodulin and calmodulin kinase II stimulate hyperactivation in demembranated bovine sperm. *Biol Reprod* 73, 519-526.

Kaur J, Ramakrishnan PR & Rajalakshmi M. (1992). Effect of cyproterone acetate on structure and function of rhesus monkey reproductive organs. *Anat Rec* 234(1), 62-72.

Kempinas WDG & Klinefelter GR. The epididymis as a target for toxicants. In Charlene A. & McQueen (Ed.). Comprehensive Toxicology, Oxford: Academic press, 2010: 149-66p.

Klinefelter G R & Suarez J D. (1997). Toxicant-induced acceleration of epididymal sperm transit: androgen-dependent proteins may be involved. *Reprod Toxicol* 11(4), 511-519.

Klinefelter GR, Laskey JW, Ferrell J, Suarez JD & Roberts NL. (1997). Discriminant analysis indicates a single sperm protein (SP22) is predictive of fertility following exposure to epididymal toxicants. *J Androl* 18(2), 139-150.

Moniem KA, Glover TD & Lubicz-Nawrocki CW (1978). Effects of duct ligation and orchidectomy on histochemical reactions in the hamster epididymis. *J Reprod Fertil* 54(1),173-176.

Monosson E, Kelce WR, Lambright C, Ostby J & Gray LE Jr. (1999). Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. *Toxicol Ind Health* 15(1-2), 65-79.

Patrão MTCC, Silva EJR & Avellar MCW (2009). Androgens and the male reproductive tract: an overview of classical roles and current perspectives. *Arq Bras Endocrinol Metab* 53, 934-945.

Perobelli JE, Alves TR, Toledo FC, Fernandez CDB, Anselmo-Franci JA, Klinefelter GR & Kempinas WDG. (2012). Impairment on Sperm Quality and Fertility of Adult Rats after Antiandrogen Exposure during Prepuberty. *Reprod Toxicol* dx.doi.org/10.1016/j.reprotox.2011.12.011.

Robaire B, Ewing LL, Zirkin BR & Irby DC. (1977). Steroid delta4-5alpha-reductase and 3alpha-hydroxysteroid dehydrogenase in the rat epididymis. *Endocrinology* 101(5), 1379-1390.

Robaire B., Hinton B., Orgebin-Crist M.C. The Epididymis. In: Knobil E., Neil JD. (Eds.). The physiology of reproduction. New York: Elsevier, 2006: 1071-148p.

Robaire B, Seenundun S, Hamzeh M & Lamour SA. (2007). Androgenic regulation of novel genes in the epididymis. *Asian J Androl* 9(4), 545-553.

Roberts KP. What are the components of the male reproductive system?. In: The American Society of Andrology (Ed). Handbook of Andrology. Lawrence: Allen Press, 2010: 1-5p.

Rodríguez CM, Kirby JL & Hinton BT (2002). The development of the epididymis. In: Robaire B. & Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 251 – 268p.

Rouquie´ D, Friry-Santini C, Schorsch F, Tinwell H & Bars R. (2009). Standard and Molecular NOAELs for Rat Testicular Toxicity Induced by Flutamide. *Toxicol Sci* 109(1), 59–65.

Silva EJ, Queiróz DB, Honda L & Avellar MC. (2010). Glucocorticoid receptor in the rat epididymis: expression, cellular distribution and regulation by steroid hormones. *Mol Cell Endocrinol* 25(1-2), 64-77.

Sipilä P, Krutskikh A, Pujianto DA, Poutanem M & Huhtaniemi I. (1997). Regional expression of androgen receptor coregulators and androgen action in the mouse epididymis. *J Androl* 32(6), 711-717.

Stoker TE, Parks LG, Gray LE & Cooper RL. (2000). Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. A focus on the EDSTAC recommendations. Endocrine Disrupter Screening and Testing Advisory Committee. *Crit Rev Toxicol* 30(2),197-252.

Sun EL & Flickinger CJ. (1979). Development of cell types and regional differences of the postnatal rat epididymis. *Am J Ana* 154, 27.

Svechnikov K, IzzoG, Landreh L, Weisser J & S¨oder O. (2010). Endocrine Disruptors and Leydig Cell Function. *J Biomed and Biotechnol* doi:10.1155/2010/684504.

Takayama S, Akaike M, Kawashima K, Takahashi M & Kurokawa K. (1995). A collaborative study in Japan on optimal treatment period and parameters for detection of male fertility disordes induced by drugs in rats. *J Am Coll Toxicol* 14, 266-292.

Tiwari AK, Alone DP & Roy JK. (2008). Rab11 is essential for fertility in Drosophila. *Cell Biol Int* 32(9), 1158-1168.

Turner TT, Bomgardner D, Jacobs JP & Nguyen QA. (2003). Association of segmentation of the epididymal interstitium with segmented tubule function in rats and mice. *Reproduction* 125(6), 871-878.

Yeung CH & Cooper TG. Acquisition and development of sperm motility upon maturation in the epididymis. In: Robaire B. & Hinton B.T. (Eds). The epididymis – from

molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 417-434p.

You L & Sar M. (1998). Androgen receptor expression in the testes and epiddidymis in pre-natal and post-natal SD rats. *Endocrinology* 9(3), 253-261.

Zhu LJ, Hardy MP, Inigo IV, Huhtaniemi I, Bardin CW & Moo-Young AJ. (2000). Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. *Biol Reprod* 63, 368-376.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Figure legends

Figure 1: Intratesticular testosterone levels (ng/g testis). Rats from control and flutamide groups at 50 and 75 days old. Values expressed as mean ± SEM. Mann Whitney test.

Figure 2: Immunostaining of androgen receptor (AR) in rat epididymis at 50 days old. A, B: proximal initial segment, C, D: distal initial segment, E, F: caput, G, H: corpus, I, J: proximal cauda, K, L: distal cauda. Ep: epithelium, M: smooth muscle cells, In: interstitial tissue, L: lumen. Observe nuclear staining (arrow). Left column: control group, right column: flutamide group (control n=3, flutamide n=3). Final magnification, 400X.

Figure 3: Immunostaining for androgen receptor (AR) in rat epididymis at 75 days old. A, B: proximal initial segment, C, D: distal initial segment, E, F: caput, G, H: corpus, I, J: proximal cauda, K, L: distal cauda. Ep: epithelium, M: smooth muscle cells, In: interstitial tissue, L: lumen. Observe nuclear staining (arrow). Left column: control group, right column: flutamide group (control n=3, flutamide n=3). Final magnification, 400X.

Figure 4: Androgen receptor (AR) detection by Western blot analysis using initial segment/caput (CP), corpus (CO) and cauda (CD) epididymis total protein extracts from control and flutamide group (n=3-4 animals/experimental group). A: animals at 50 days old, B: animals at 75 days old. Internal control: β actin.

Figure 5: Immunostaining for calmodulin (CALM) in rat epididymis at 50 days old. A, B: proximal initial segment, C, D: distal initial segment, E, F: caput, G, H: corpus, I, J: proximal cauda, K, L: distal cauda. Ep: epithelium, M: smooth muscle cells, In: interstitial tissue, L: lumen. Left column: control group, right column: flutamide group, (control n=4, flutamide n=4). Final magnification, 400X.

Figure 6: Immunostaining for calmodulin (CALM) in rat epididymis at 75 days old. A, B: proximal initial segment, C, D: distal initial segment, E, F: caput, G, H: corpus, I, J: proximal cauda, K, L: distal cauda. Ep: epithelium, M: smooth muscle cells, In: interstitial tissue, L: lumen. Left column: control group, right column: flutamide group, (control n=4, flutamide n=4). Final magnification, 400X.

Figure 7: Immunostaining for Rab11A in rat epididymis at 75 days old. A, B: proximal initial segment, C, D: distal initial segment, E, F: caput, G, H: corpus, I, J: proximal cauda, K, L: distal cauda. Ep: epithelium, M: smooth muscle cells, In: interstitial tissue, L: lumen. Left column: control group, right column: flutamide group, (control n=4, flutamide n=4). Final magnification, 400X.

Table 1: Final body weight and absolute and relative reproductive organ weights of male rats from control and futamide groups at 50 and 75 days old.

	50 days-old		*75 days-old*	
	Control (n=7)	*Flutamide (n=8)*	*Control (n=7)*	*Flutamide (n=8)*
Final body weight (g)	263.40±7.68	248.81±10.21	371.09±14.15	373.94±15.59
Testis (g)	1.27±0.06	1.33±0.04	1.60±0.04	1.64±0.04
Testis (mg/100 g BW)	481.36±14.25	536.72±15.85*	436.97±23.28	441.73±13.32
Epididymis (mg)	228.26±11.80	147.46±9.57****	502.97±9.35	500.07±16.39
Epididymis (mg/100 g BW)	86.57±3.40	59.27±3.04****	136.33±3.62	134.37±3.59
Ventral prostate (mg)	174.07±11.84	101.02±11.75***	354.07±42	335.8±39.44
Ventral prostate (mg/100 g BW)	66.32±4.54	40.81±4.87**	93.75±8.64	89.43±9.96
Vas deferens (mg)	58.33±2.10	42.89±1.86****	75.81±3.50	77.05±4.07
Vas deferens (mg/100 frg BW)	22.17±0.67	17.30±0.68***	20.52±0.92	20.59±0.62
Seminal Vesicle full (mg)	397.91±25.65	171.94±17.59****	1.078.11±71.21	908.55±71.20
Seminal Vesicle full (mg/100g BW)	150.66±7.27	68.67±6.00****	242.26±21.04	243.84±19.73
Liver (g)	`12.35±0.45	11.89±0.43	15.09±0.79	15.09±0.49
Liver (g/100g BW)	4.69±0.12	4.79±0.12	4.06±0.09	4.05±0.05
Paired kidneys (g)	2.06±0.08	1.95±0.06	2.67±0.11	2.63±0.12
Paired kidneys (g/100g BW)	0.78±0.02	0.79±0.02	0.72±0.01	0.71±0.02

Values expressed as mean ± SEM. Student's t test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. BW-body weight

Table 2: Testicular histological analysis (50- and 75-day-old animals) and Sertoli cell nuclei number (75-day-old animals) in rats from control and flutamide groups

	50 days old		*75 days old*	
	Control (n=7)	*Flutamide (n=7)*	*Control (n=7)*	*Flutamide (n=8)*
Normal seminiferous tubules[1]	95.00 (94.50-97.00)	97.00 (94.50-98.00)	95.00 (93.25-96.00)	95.00 (94.50-96.25)
Anormal seminiferous tubules[1]	5.00 (3.00-5.50)	3.00 (2.00-5.50)	5.00 (4.00-6.75)	5.00 (5.00-6.50)
Sertoli cell nuclei number[2]			17.31±0.89	19.26±0.29

[1]Values expressed as median and interquartile intervals. [2]Values expressed as Mean ±SEM. Mann Whitney test.

Figure 1

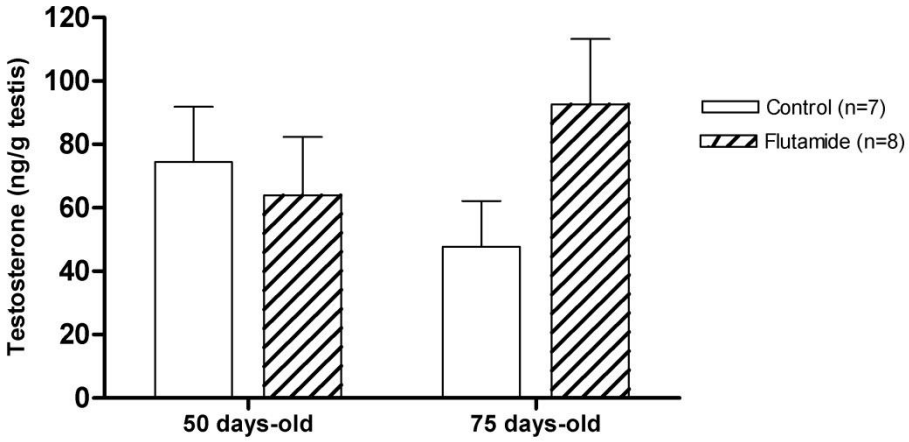
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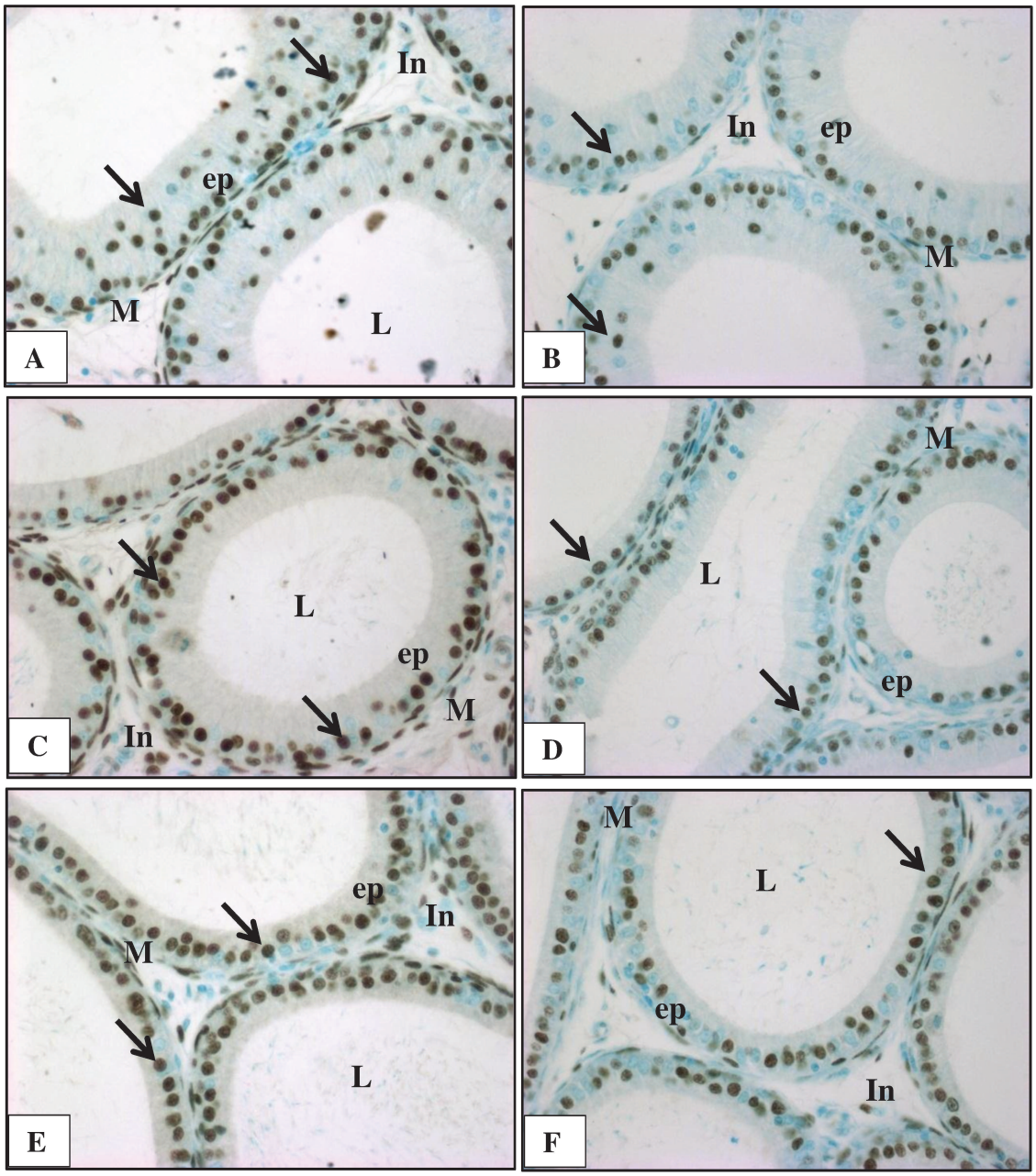
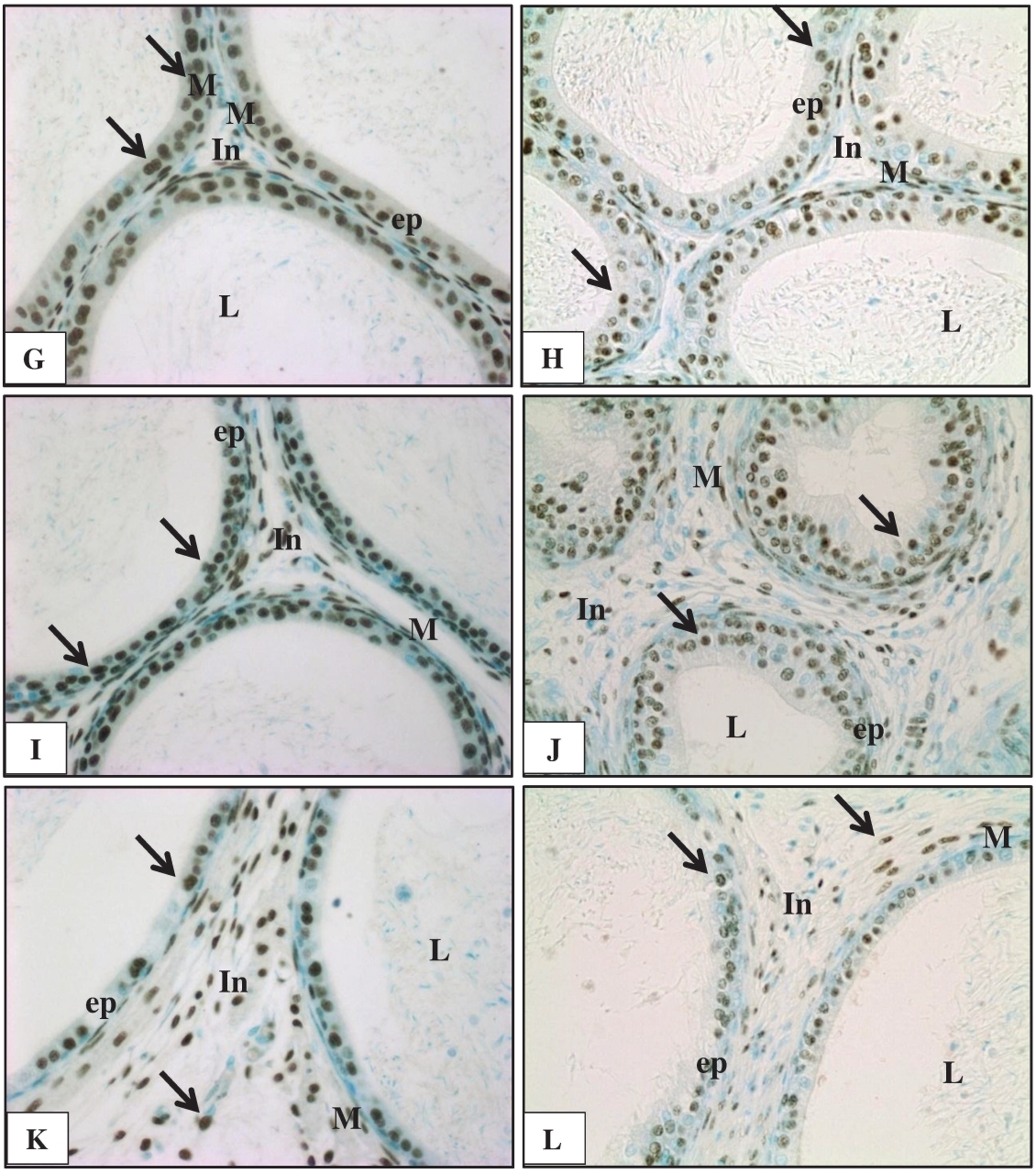
Figure 2

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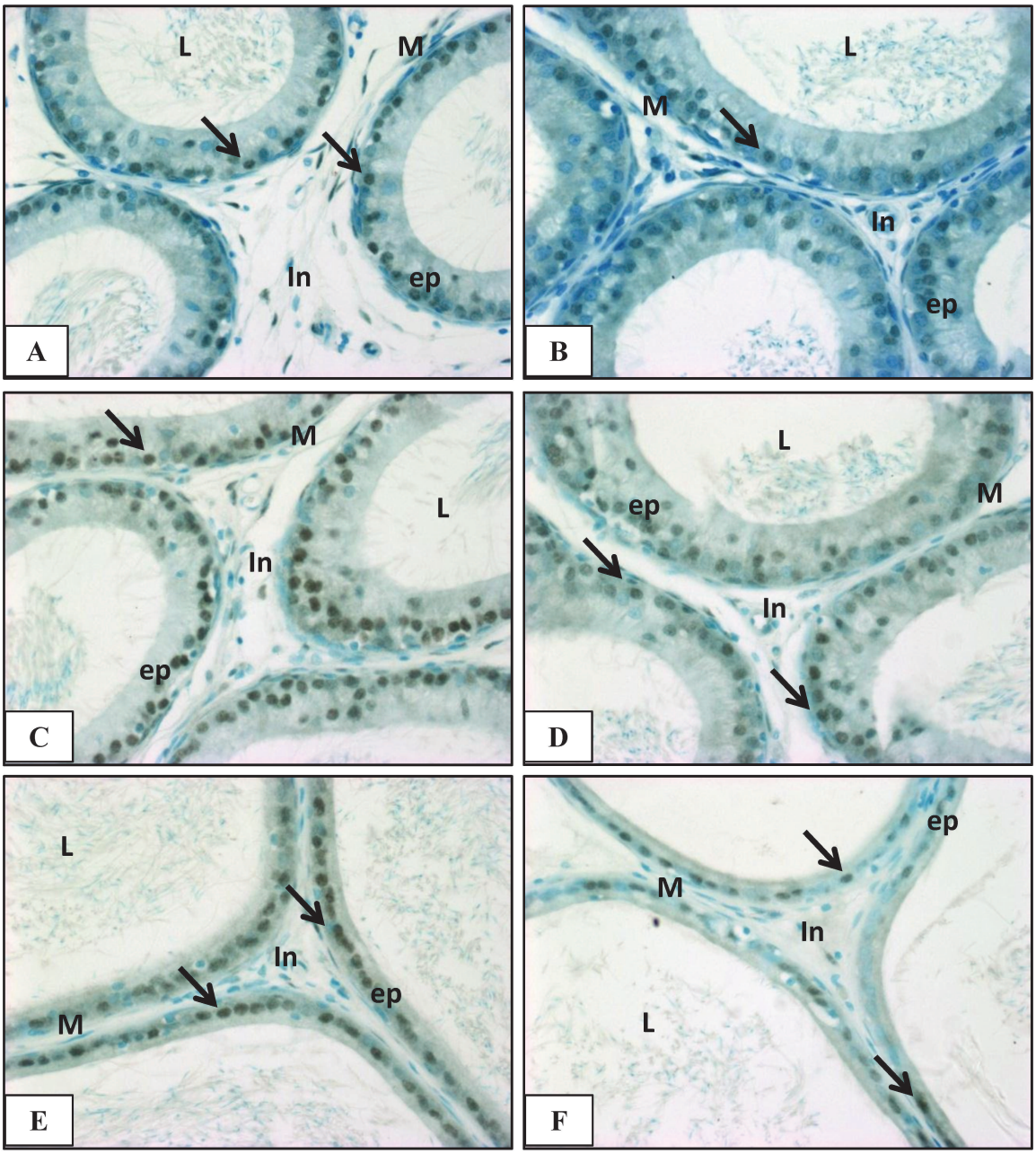

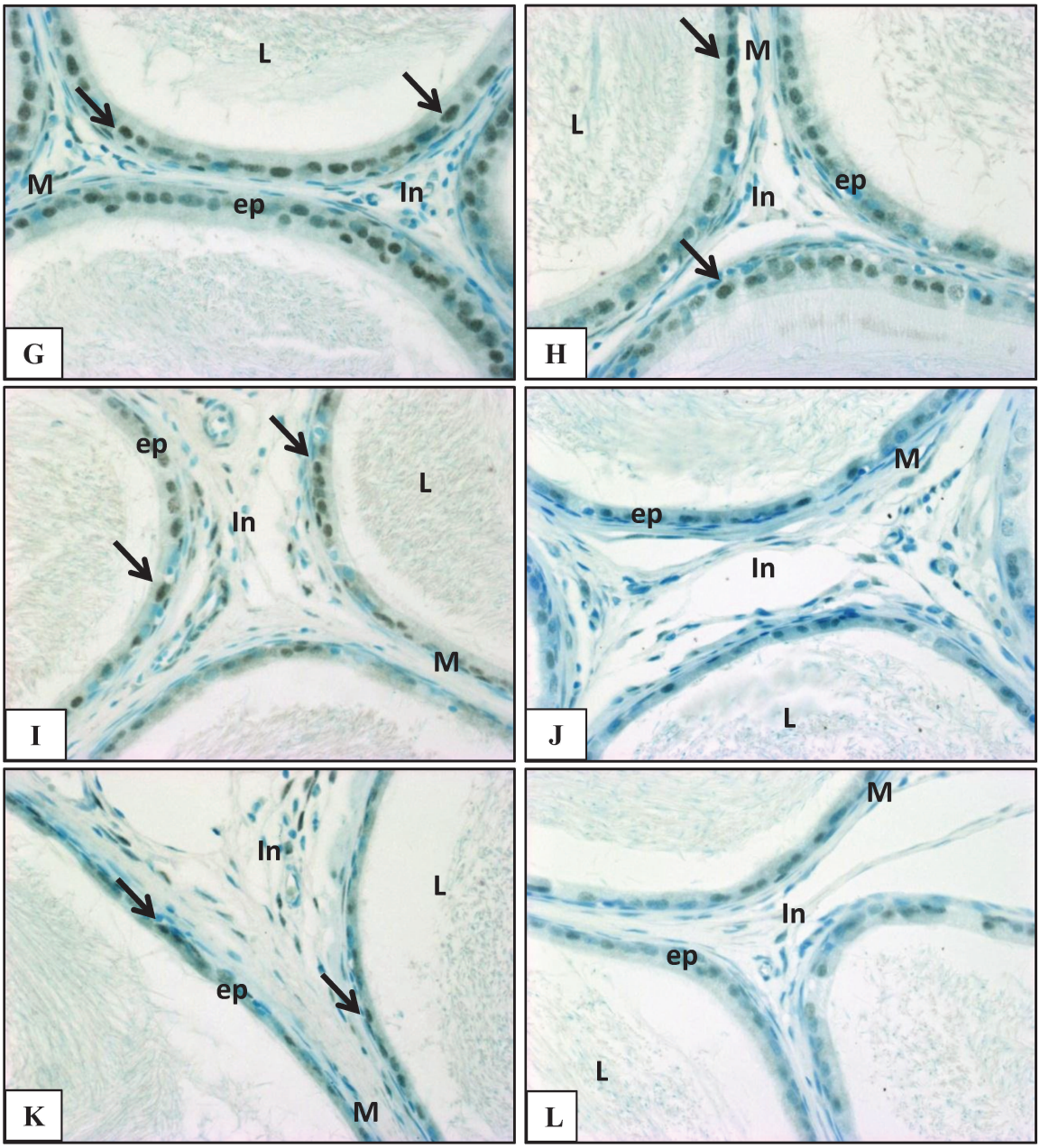
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Figure 4

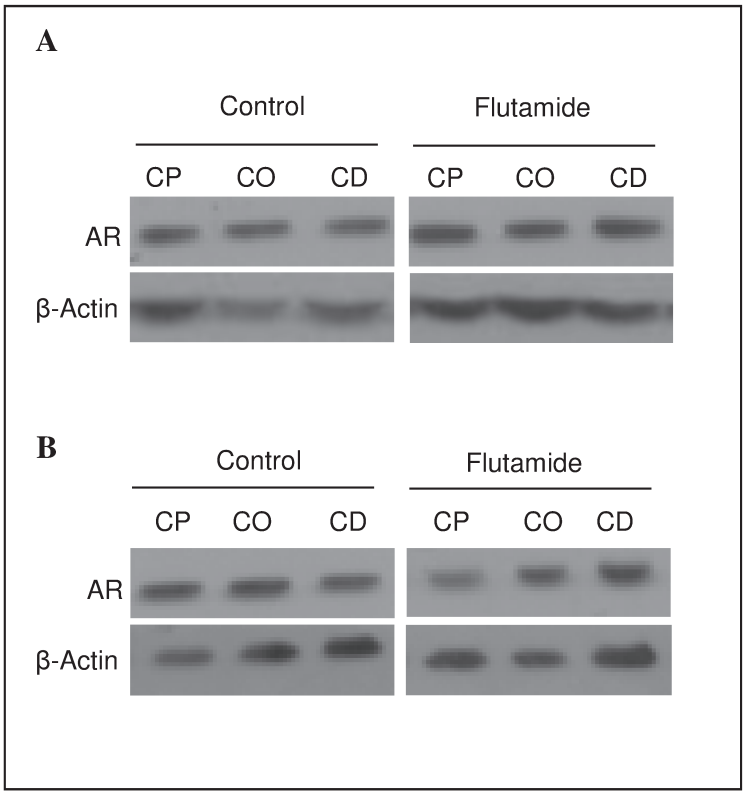
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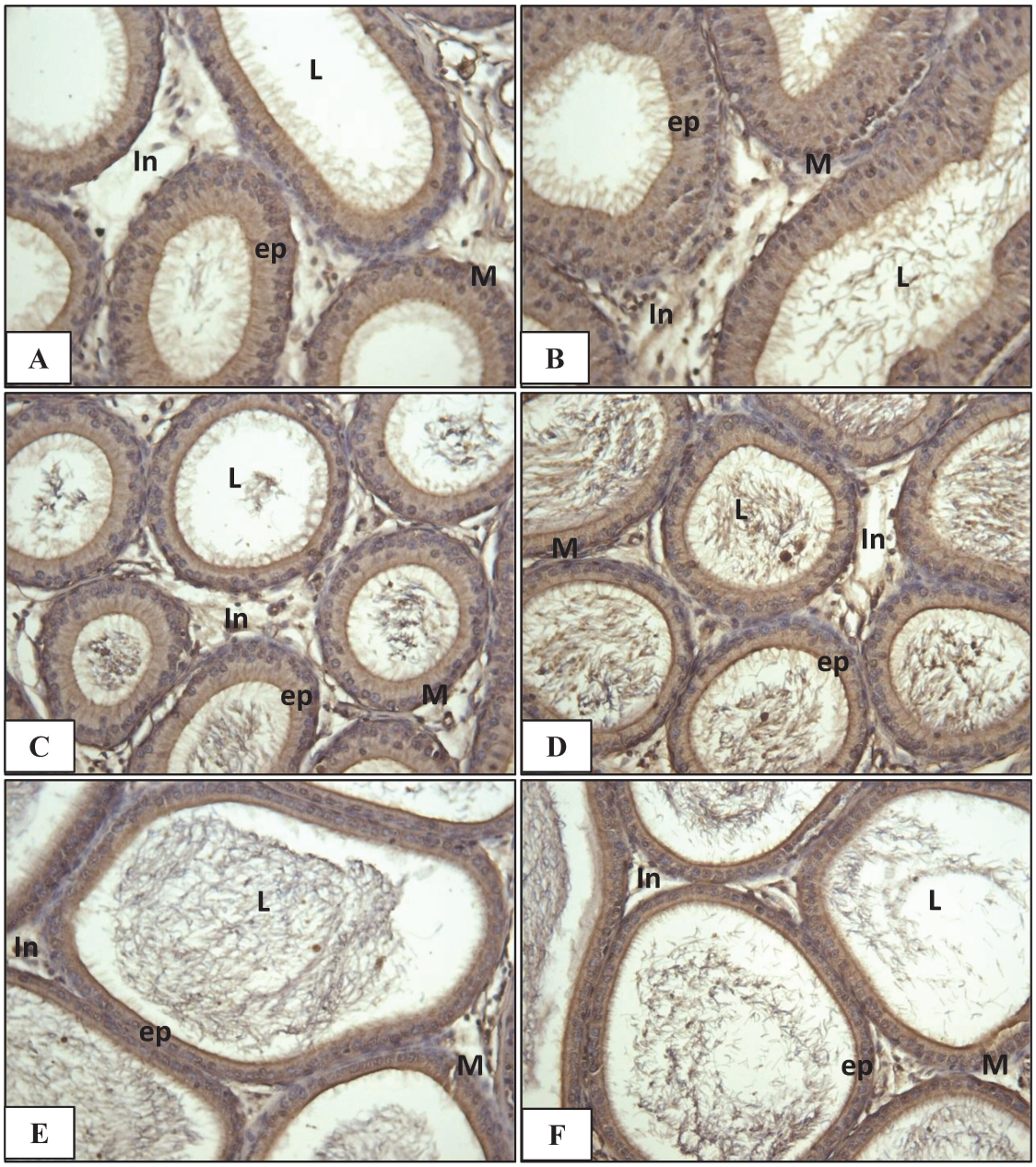
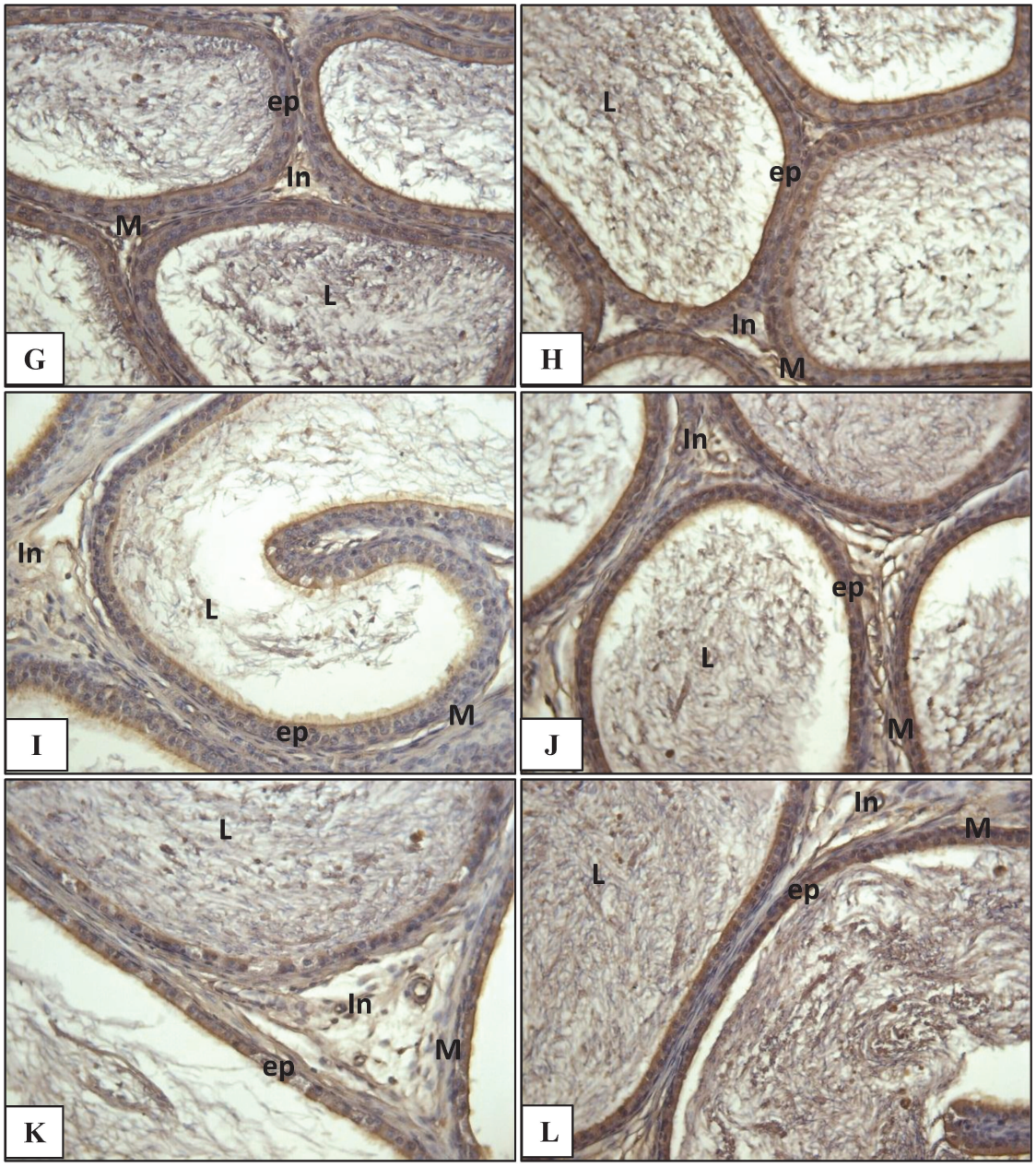
Figure 5

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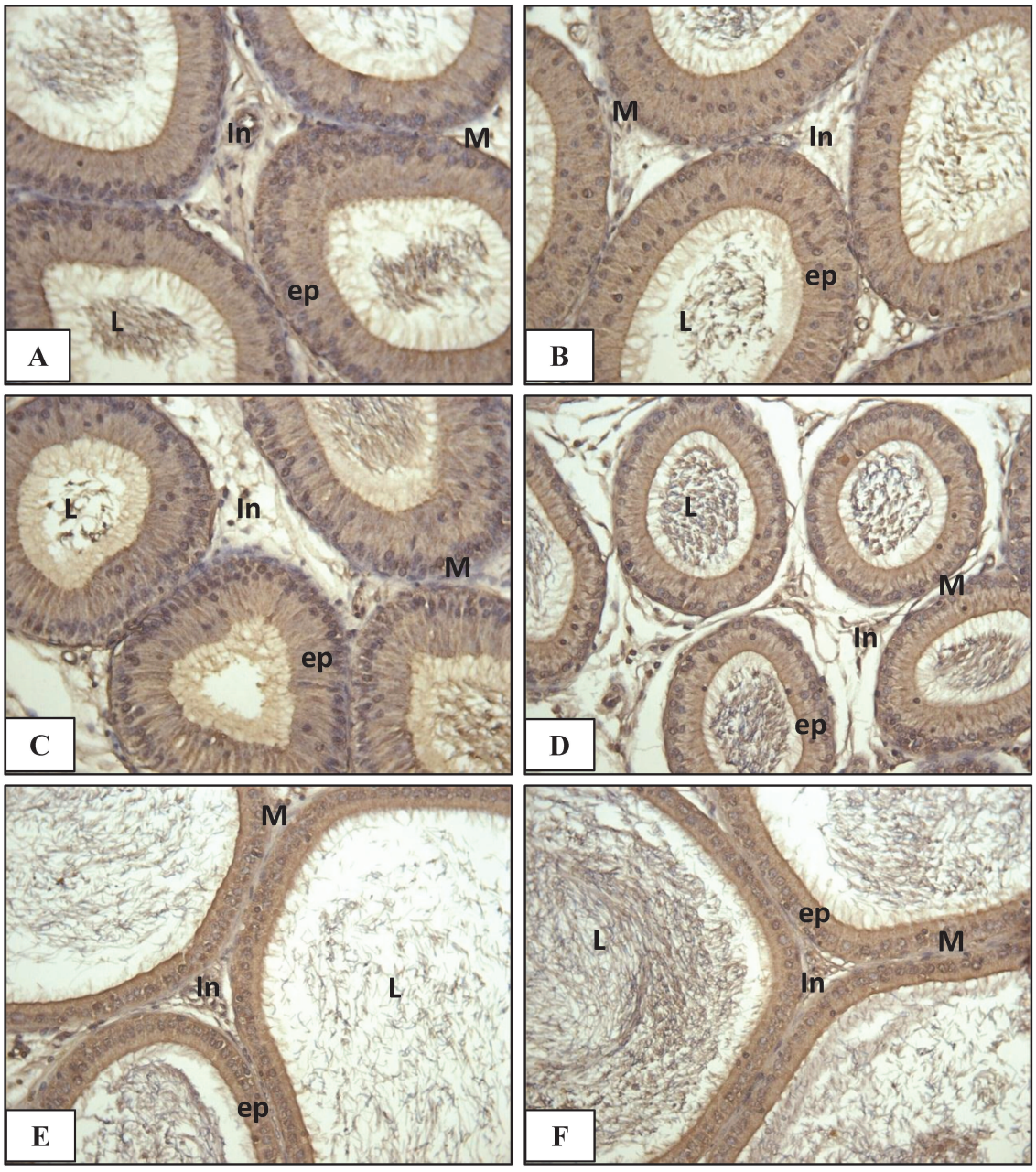

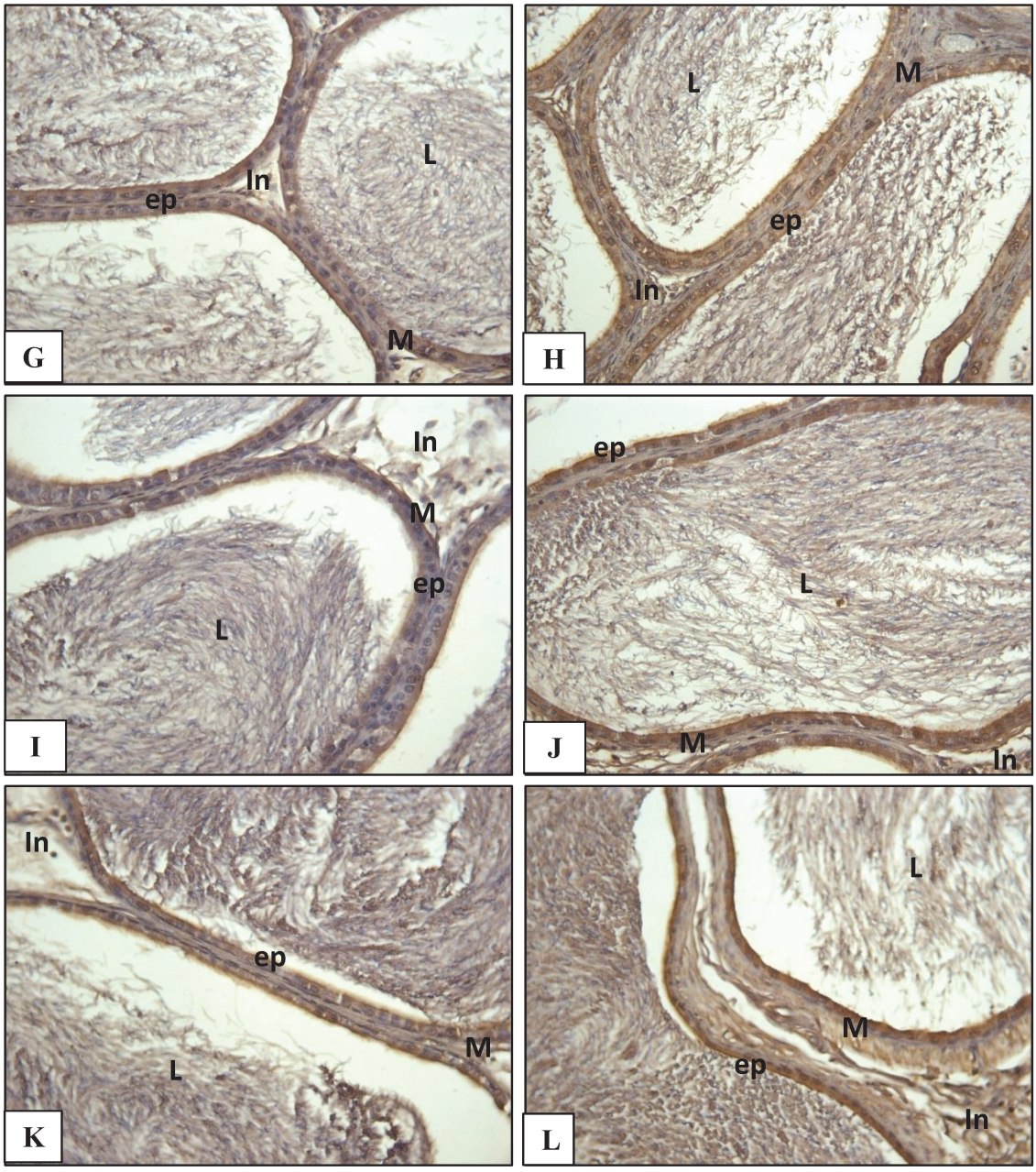
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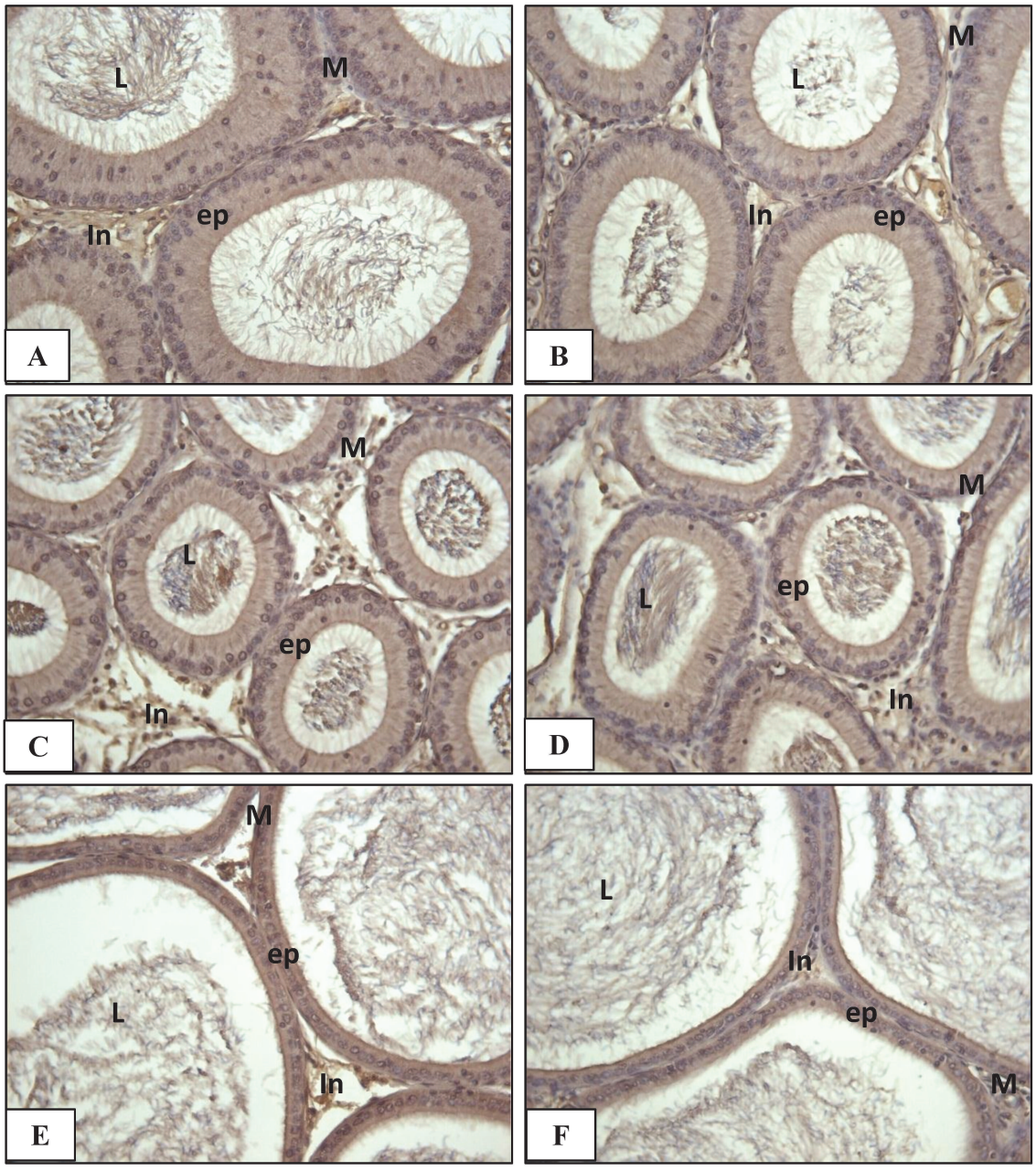

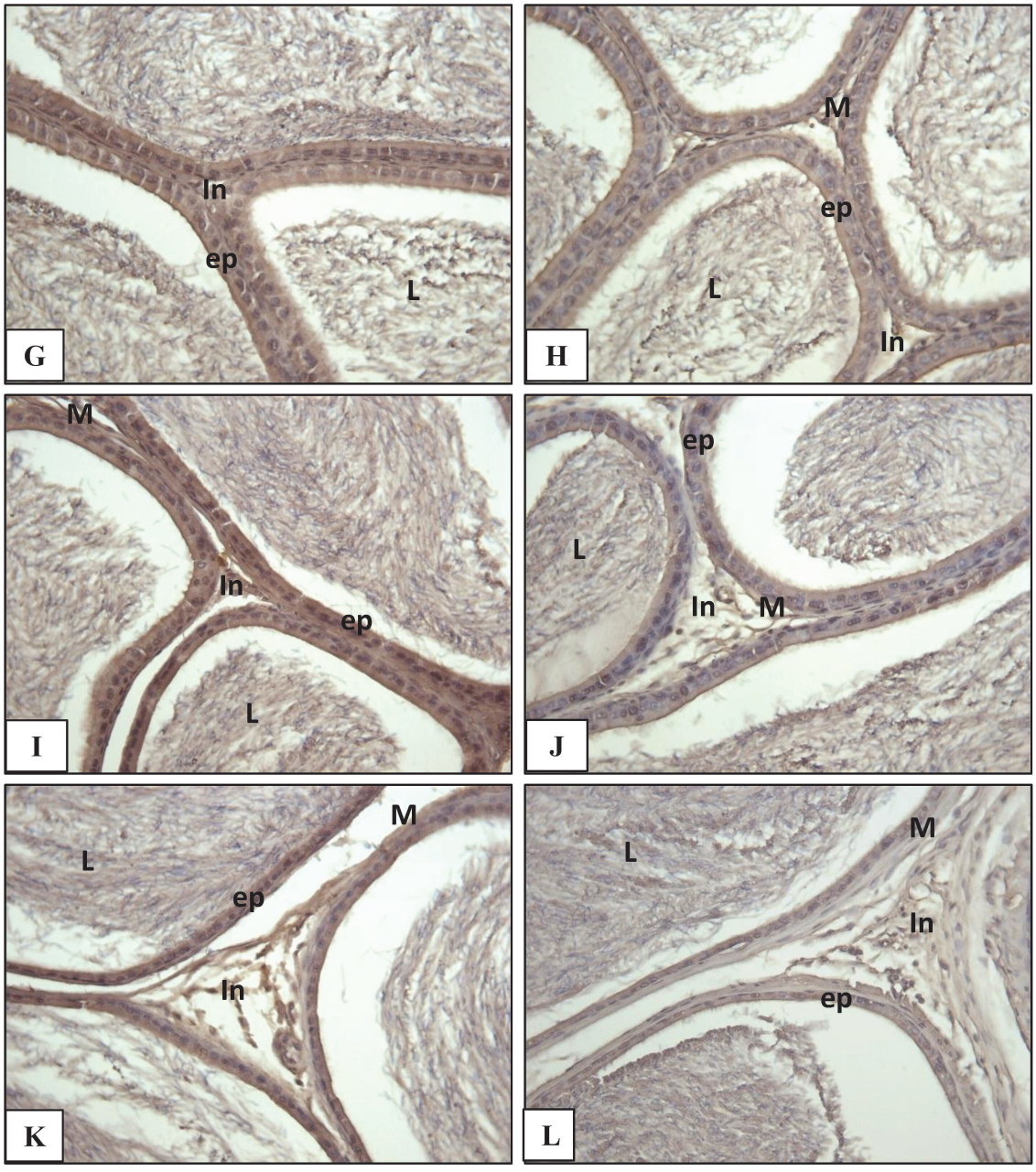
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Conclusões Finais

5. CONCLUSÕES FINAIS

A exposição a agente antiandrogênico durante o período pré-puberal prejudicou a homeostasia do organismo em desenvolvimento e acarretou efeitos adversos sobre parâmetros reprodutivos avaliados na puberdade e na idade adulta dos animais. A competência reprodutiva e a qualidade espermática dos ratos que sofreram privação androgênica durante a pré-puberdade foi alterada de maneira persistente. Tais resultados parecem estar associados a mudanças no perfil proteico da membrana dos espermatozoides e na expressão de determinadas proteínas no epitélio epididimário, o que poderia indicar um prejuízo aos processos de diferenciação e maturação dos espermatozoides e, consequentemente, à qualidade dos gametas.

Os resultados obtidos no presente estudo reafirmam a susceptibilidade de animais em desenvolvimento aos desreguladores endócrinos e ratificam a necessidade de mais estudos nesta subárea da toxicologia, uma vez que a maior parte das pesquisas desenvolvidas até o momento aborda exposição durante o período gestacional e lactacional ou na idade adulta.

Referências Bibliográficas

6. REFERÊNCIAS BIBLIOGRÁFICAS DA INTRODUÇÃO

Adamali H. I., Hermo L. 1996. Apical and narrow cells are distinct cell types differing in their structure, distribution, and functions in the adult rat epididymis. J. Androl., 17(3), 208-22.

Aitken R. J., Clarkson J. S. 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. Biol. Reprod., 40, 183-97.

Amann R. P., Johnson L., Thompson D. L., Pickett B. W. 1976. Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the rhesus monkey. Biol. Reprod., 15, 586-92.

Amann R.P. 1986. Detection of alterations in testicular and epididymal function in laboratory animals. Environ. Health. Perspect., 70, 149-58.

Auger J., Kunstmann J. M., Czyglik F., Jouannet P. 1995. Decline in semen quality among fertile men in Paris during the past 50 years. New Eng. J. Med., 332, 281-5.

Bardin C. W., Musto N., Gunsalus G., Kotite N., Cheng S. L., Larrea F., Becker R. 1981. Extracellular androgen binding proteins. Annu. Rev. Physiol., 43, 189-98.

Baumgarten H. G., Holstein A. F., Rosengren E. 1971. Arrangement, ultrastructure and adrenergic innervation of smooth musculature of the ductuli efferents, ductus epididymis and ductus deferens of man. Z zellforsch. Mikrosk. Anat., 120, 37-79.

Bedford J. M. Male reproductive System. In: Hamilton, D.W., Greep, R.O. (Eds). Handbook of physiology. American Physiological Society: Washington DC, 1975, 303-18p.

Blystone C.R., Lambright C.S., Furr J., Wilson V., Gray E. 2007. Iprodione delays male rat pubertal development, reduces serum testosterone levels, and decrease ex vivo testicular testosterone production. Toxicol. Lett., 174, 74-81.

Blystone C.R., Lambright C.S., Cardon M.C., Furr J., Rider C.V., Hartig P.C., Wilson V.S., Gray L.E. Jr. 2009. Cumulative and antagonistic effects of a mixture of the

antiandrogens vinclozolin and iprodione in the pubertal male rat. Toxicol. Sci., 111(1), 179-88.

Boekelheid K., Johnson K.J., Richburg J.H. Sertoli cell toxicants. In: Skinner, M.K., Griswold, M.D. (Eds). Sertoli cell Biology. San Diego: Elsevier Academic Press, 2005, 345-82p.

Breton S., Tyszkowski R., Sabolic I., Brown D. 1999. Postnatal development of H+ ATPase (proton-pump)-rich cells in rat epididymis. Histochem. Cell. Biol., 111(2), 97-105.

Brooks D. E. 1983. Epididymal functions and their hormonal regulation. Aust. J. Biol. Sci., 36, 205-21.

Carlsen E., Giwercman A., Keiding N., Skakkebaek N. 1992. Evidence for decreasing quality of semen during past 50 years. Brit. Med. J., 305, 609-13.

Carr D.W., Usselman M.C., Acott T.S. 1985. Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. Biol. Reprod., 33, 588-95.

Chapin R.E., Stevens J.T., Hughes C.L., Kelce W.R., Hess R.A., Daston G.P. 1996. Endocrine Modulation of Reproduction. Fundam. Appl. Toxicol.,. 29, 1-17.

Chapin R.E., Harris M.W., Davis B.J., Ward S.M., Wilson R.E., Mauney M.A., Lockhart A.C., Smialowics R.J., Moser V.C., Burka L.T., Collins B.J. 1997. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. Fundam. Appl. Toxicol., 40, 138-57.

Cheung K.H., Leung G.P., Leung M.C., Shum W.W., Zhou W.L., Wong P.Y. 2005. Cell-cell interaction underlies formation of fluid in the male reproductive tract of the rat. J. Gen. Physiol., 125(5), 443-54.

Chughtai B., Sawas A., O'Malley R.L., Naik R.R., Ali Khan S., Pentyala S. 2005. A neglected gland: a review of Cowper's gland. Int. J. Androl., 28 (2), 74-7.

Clegg E. 1960. The age at which male rats become fertile. J. Reprod. Fertil., 1, 119-20.

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

Colborn T., Vom Saal F.S., Soto A.M. 1993. Developmental effects of endocrine disrupting chemicals in wildlife and humans. Environ. Health. Perspect., 101, 378-84.

Comhaire F., Van Waeleghem K., De Clercq N., Schoonjans F. 1996. Declining sperm quality in European men. Andrologia., 28, 300-1.

Committee for the update of the guide for the care and use of laboratory animals. Key concepts. In: National Research Council of the National Academies (Eds).Guide laboratory animals for the care and use of laboratory animals, 8ed, Washington DC: The National Academies Press, 2011: 1-10p.

Cooper T G. 1995. Role of the epididymis in mediating changes in the male gamete during maturation. Adv. Exp. Med. Biol., 377, 87-101.

Cooper T.G. Epididymis. In: Knobil E., Neill J.D. (Eds). Encyclopedia of Reproduction, vol. 2, California: Academic Press, 1998: 1-17p.

Cornwall G.A., Lareyre J.J., Matusik R.J., Hinton B.T., Orgebin-Crist M.C. Gene expression and and epididymal function. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 169-99p.

Cosentino M. J., Cokett A.T. 1986. Structure and function of the epididymis. Urol. Res., 14(5), 229-40.

Cyr D.G., Finnson K., Dufresne J., Gregory M. Cellular interactions and the blood-epididymal barrier. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 103-18p.

Dacheux J.L., Castella S., Gatti J.L., Dacheux F. 2005. Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology., 63, 319-41.

Damgaard I.N., Main K.M., Toppari J., Skakkebaek N.E. 2002. Impact of exposure to endocrine disrupters in utero and in childhood on adult reproduction. Best. Pract. Res. Clin. Endocrinol. Metab., 16(2), 289-309.

Damgaard I.N., Skakkebæk N.E., Toppari J., Virtanen H.E., Shen H., Schramm K.W., Petersen J.H., Jensen T. K., Main K.M. The Nordic Cryptorchidism Study Group. 2006. Persistent pesticides in human breast milk and cryptorchidism. Environ. Health. Perspect., 114,1133–38.

Damgaard I., Jensen T.K., Petersen J.H., Skakkebæk N.E., Toppari, J., Main K.M. The Nordic Cryptorchidism Study Group. 2007. Cryptorchidism and maternal alcohol consumption during pregnancy. Environ. Health. Perspect., 115, 272–7.

David R.M. 2006. Proposed mode of action for in utero effects of some phthalate esters on the developing male reproductive tract. Toxicol. Pathol., 34 (3), 209-19.

De Larminant M., Monsalve A., Charreau E., Calandra R., Blaquier J. 1978. Hormonal regulation of 5α-reductase activity in rat epididymis. J. endocrinol., 79, 157-65.

Delongeas J., Gelly J., Leheup B., Grignon G. 1987. Influence of testicular secretions on the differentiation of the rat epididymis: ultrastructural studies after castration, efferent ducts ligation and cryptorchidism. Exp. Cell. Biol., 55, 74-82

Dyson A.L., Orgebin-Crist M.C. 1973. Effect of hypophysectomy, castration and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. Endocrinology., 93(2), 391-402.

Ezer N., Robaire B. Androgenic Regulation of the structure and function of the epididymis. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 297-316p.

Fan X., Robaire, B. 1998. Orchidectomy Induces a Wave of Apoptotic Cell Death in the Epididymis. Endocrinology., 139(4), 2128-36.

Fawcett D.W., Hoffer A.P. 1979. Failure of exogenous androgen to prevent regression of the initial segments of the rat epididymis after efferent duct ligation or orchidectomy. Biol. Reprod., 20(2), 162-81.

Fernandes G.S., Arena A.C., Fernandez C.D.B., Mercadante A., Barbisan L.F., Kempinas W.G. 2007. Reproductive effects in male rats exposed to diuron. Reprod. Toxicol., 23(1), 106-12.

Fisher J.S., Macpherson S., Marchetti N., Sharpe R.M. 2003. Human 'testicular dysgenesis syndrome': a possible model using in utero exposure of the rat to dibutyl phthalate. Hum. Reprod., 18, 1383-94.

Fisher J.S. 2004. Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. Reproduction., 27(3): 305-15.

Foley G.L. 2001. Overview of Male Reproductive Pathology. Toxicol. Pathol. 29(1), 49-63.

Fossato da Silva D.A., Teixeira C.T., Scarano W.R., Favareto A.P., Fernandez C.D.B., Grotto D., Barbosa F. Jr, Kempinas W.G. 2011. Effects of methylmercury on male reproductive functions in Wistar rats. Reprod. Toxicol., 31(4), 431-9.

Foster P.M.D. 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Intern. J. Androl., 29, 140-7.

França, L.R., Avelar, G.F., Almeida, F.F.L. 2005. Spermatogenesis and transit through the epididymis in mammals with empashis on pigs. Theriogenology., 63, 300-18.

Gatti J.L., Castella S., Dacheux F., Ecruyd H., Métayer S., Thimon V., Dacheux J.L. 2004. Post-testicular sperm environment and fertility. Anim. Reprod. Sci., 82-83, 321-39.

Golub M.S., Collman G.Q.W., Foster P.M., Kimmel C.A., Rajpert-De Meyts E., Reiter E.O. 2008. Public health implications of altered puberty timing. Pediatrics., 121(3), S218-30.

Gray L.E. Jr, Ostby J., Furr J., Wolf C.J., Lambrigth C., Parks L. 2001. Effects of environmental antiandrogens on reproductive development in experimental animals. Hum. Reprod. Update., 7, 248-64.

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

Gyllenborg J., Skakkebaek N.E., Nielsen N.C., Keiding N., Giwercman A. 1999. Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977-1995. Int. J. Androl., 22, 28-36.

Hamzeh M., Robaire B. 1982. Effect of Testosterone on Epithelial Cell Proliferation in the Regressed Rat Jaakkola um, talo a. relation of electrical activity to luminal transport in the cauda epididymis of the raa epididymis. J. Reprod. Fertil., 64, 121-6.

Haschek W., Rousseaux C.G. Male reproductive system. In: Haschek W, Rousseaux CG (Eds). Fundamentals of toxicologic pathology. San Diego: Academic Press, 1998: 443-84.

Hermo L. 1995. Structural features and functions of principal cells of the intermediate zone of the epididymis of adult rat. Anat. Rec., 242, 515-30.

Hermo L., Smith C.E. 1998. The structure of the Golgi apparatus: a sperm's eye view in principal epithelial cells of the rat epididymis. Histochem. Cell. Biol., 109(5-6), 431-47.

Hermo L., Robaire B. Epididymal cell types and their functions. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 81-102.

Hess RA. The efferent ductules: structure and functions. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 49-80

Hinton B.T., Palladino M.A., Rudolph D., Labus J.C. 1995. The epididymis as protector of maturing spermatozoa. Reprod. Fertil. Dev., 7(4), 731-45.

Hinton B.T., Lan Z.J., Rudolph D.B., Labus J.C., Lye R.J. 1998. Testicular regulation of epididymal gene expression. J. Reprod. Fertil., 53, 47-57.

Hoei-Hansen C.E., Holm M., Rajpert-De Meyts E. and Skakkebaek N.E. 2003. Histologic evidence of testicular dysgenesis in contralateral biopsies from 218 patients with testicular germ cell cancer. J. Pathol., 200, 370-4.

Hoffer A.P., Karnovsky M.L. 1981. Studies on zonation in the epididymis of the ginea pig :ultrastructural and biochemical analysis of the zone rich in large lipid droplets (zone II) Anat. Rec., 201, 623-33.

Hotchkiss A.K., Ostby J.S., Vandenbergh J.G., Gray Jr. 2002. Androgens and environmental antiandrogens affect reproductive development and play behavior in the Sprague-Dawley rat. Environ. Health. Perspect., 110 (3), 435-9.

Imperato-McGinley J., Sanchez R.S., Spencer J.R., Yee B., Vaughan E.D. 1992. Endocrinology., 131, 1149-56.

Jaakkola U.M. 1983. Regional variations in the transport of the luminal contents of the rat epididymis in vitro. J. Reprod. Fertil., 68, 465-70.

Jensen T.K., Jørgensen N., Punab M., Haugen T.B., Suominen J., Zilaitiene B. 2004. Association of in utero exposure to maternal smoking with reduced semen quality and testis size in adulthood: a cross-sectional study of 1,770 young men from the general population in five European countries. Am. J. Psychol., 159, 49–58.

Jensen T.K., Jørgensen N., Asklund C., Carlsen E., HolmM., Skakkebæk N. E. 2007. Fertility treatment and reproductive health of male offspring: a study of 1,925 young men from the general population. Am. J. Epidemiol., 165, 583–90.

Johnson L., Welsh T.J., Wilker C. Anatomy and physiology of the male reproductive system and potential target of toxicants. In: Boekleheide K., Chapin R.E., Hoyer P.B., Harris C. (Eds). Comprehensive Toxicology. New York: Pergamon, 1997:5-61p.

Jones R., Mann T., Sherins R.J. 1979. Peroxidative breakdown of phospholipids by human spermatozoa, spermicidal properties of fatty acid peroxides and protective action of seminal plasma. Fertil. Steril., 31, 531-7.

Jones R.C. 1999. To store or mature spermatozoa? The primary role of the epididymis. Int. J. Androl., 22, 57-67.

Kaleczyc J., Majewski M., Calka J., Lakomy M. 1968. Adrenergic innervation of the epididymis, vas deferens, accessory genital glands and urethra in the boar. Folia. Histochem. Cytobiol., 31, 117-23.

Kelce W.R., Gray E. Jr. Environmental antiandrogens as endocrine disruptors. In. Naz R.K. (Ed.). Endocrine Disruptors. Boca Raton: CRC Press, 1999: 247-77p.

Kempinas W.D.G., Klinefelter G.R. The epididymis as a target for toxicants. In Charlene A. McQueen (Ed.). Comprehensive Toxicology, Oxford: Academic press, 2010: 149-66p.

Kierszenbaum A.L. Transporte e maturação de espermatozóides. In: Kierszenbaum A.L. (Ed.). Histologia e Biologia Celular, 2ed, Rio de Janeiro: Elsevier, 2008: 601-39p.

Klinefelter G.R., Suarez, J.D. 1997. Toxicant-induced acceleration of epididymal sperm transit: androgen-dependent proteins may be involved. Reprod. Toxicol., 11(4), 511-19.

Klinefelter G.R. Actions of toxicants on the structure and function of the epididymis. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 353-69.

KomárekV., Gembardt C., Krinke A., Mahrous T., Schaetti P. Synopsis of the organ anatomy. In: Krinke G.J. (Ed.). The laboratory rat – The Handbook of Experimental Rats. London: Academic Press, 2000: 283-322.

Limanowski A., Miskowiak B., Otulakowski B., Partyka M., Konwerska A. 2001. Morphometric studies on rat epididymis in the course of postnatal development (computerised image analysis). Folia. Histochem. Cytobiol., 39(2), 201-2.

Maeda K., Ohkura S., Tsukamura H. Physiology of Reproduction. In: Krinke G.J. (Ed.). The laboratory rat – The Handbook of Experimental Rats. London: Academic Press, 2000: 145-76p.

Main K. M., Rajpert-De Meyts E., Toppari J., Skakkebæk N.E. Endocrine disrupters and development of the reproductive system: a paediatric perspective. In: Pescovitz O. H., Eugster E.A. (Eds). Pediatric Endocrinology: Mechanisms, Philadelphia: Lippincott Williams & Wilkins, 2004:376–90p.

Main K.M., Mortensen G.K., Kaleva M., Boisen K., Damgaard I., Chellakooty M. 2006. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in three months old infants Environ. Health. Perspec., 114, 270–6.

Main K.M., Kiviranta H., Virtanen H.E., Sundqvist E., Tuomisto J.T., Tuomisto J., Vartiainen T., Skakkebæk N.E., Toppari J. 2007 Flame retardants in placenta and

breast milk and cryptorchidism in newborn boys. Environ. Health. Perspec., 115, 1519–26.

Markkula-Viitanen M, Nikkanun V., Talo A. 1979. Electrical activity and intraluminal pressure of the cauda epididymis of the rat. J. Reprod. Fertil., 57, 431-5.

Marty M.S., Chapin R.E., Parks L.G., Thorsrud B.A. 2003. Development and maturation of the male reproductive system. Birth Defects Res. B Dev. Reprod. Toxicol., 68, 125-36.

McCarthy CR. 1999. Bioethics of laboratory animal research. ILAR J., 40, 1-37.

McNeal J.E. 1981. The zonal anatomy of the prostate. Prostate., 2, 35-49.

Menchini-Fabris F., Rossi P., Palego P., Simi S., Turchi P. 1996. Declining sperm counts in Italy during the past 20 years. Andrologia., 28, 304.

Moore H.D.M., Bedford J.M. 1979. Short-term effects of androgen withdrawal on the structure of different epithelial cell in the rat epididymis. Anat. Res., 193, 293-312.

Mylchreest E., Cattley R.C., Foster P.M. 1998. Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? Toxicol Sci., 43(1), 47-60.

Mylchreest E., Wallace D.G., Cattley R.C., Foster P.M.D. 2000. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. Toxicol. Sci., 55, 143-51.

Mylchreest E., Sar M., Wallace D.G., Foster P.M.D. 2002. Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di (n-butyl) phthalate during late gestation. Toxicol. Sci., 55, 143-51.

Nassr A.C., Arena A.C., Toledo F.C., Bissacot D.Z., Fernandez C.D., Spinardi-Barbisan A.L., Pires P.W., Kempinas W.G. 2010. Effects of gestational and lactational fenvalerate exposure on immune and reproductive systems of male rats. J. Toxicol. Environ. Health A., 73(13-14), 952-64.

Nicander L., Osman D.I., Ploen L., Bugge H.P., Kvisgaard K.N. 1983. Early effects of efferent ductule ligation on the proximal segment of the rat epididymis. Int. J. Androl., 6(1), 91-102.

Nicander L. 1956. Studies on the regional histology and citochemistry of the ductus epididymis in stallions, rams, and bulls. Acta. Morphol. Neerl. Scand., 1, 337-62.

Orgebin-Crist M.C. 1969. Studies on the function of the epididymis. Biol. Reprod., 1, 155-75.

Orgebin-Christ M.C., Danzo B.J., Davies J. Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. In: Greep R.O., Astwood E.B. (Eds.). Handbook of Physiology, Washington DC: American Physiology Society, vol. 5, 1975: 319-38p.

Ojeda S.R., Andrews W.W., Advis J.P., White S.S. 1980. Recent advances in the endocrinology of puberty. Endocr. Rev., 1(3), 228-57.

Ojeda SR, Skinner MK. Puberty in the rat. In: Knobil E., Neil JD. (Eds.). The physiology of reproduction. New York: Elsevier, 2006: 2061-126p.

Pajarinen J., Laippala P., Penttila A., Karhunen P.J. 1997. Incidence of disorders of spermatogenesis in middle aged Finnish men, 1981- 91: two necropsy series. Br. Med. J., 314, 13-8.

Patrão M.T.C.C., Silva E.J.R., Avellar M.C.W. 2009. Androgens and the male reproductive tract: an overview of classical roles and current perspectives. Arq. Bras. Endocrinol. Metab., 53, 934-45.

Perobelli J.E., Martinez M.F., Franchi C.A.S., Fernandez C.D.B., Camargo J.L.V., Kempinas W.D.G. 2010. Decreased sperm motility in rats orally exposed to single or mixed pesticides. J. Toxicol. Environ. Health A., 73(13), 991-1002.

Perry P. 2007. The ethics of animal research: A UK perspective. ILAR J., 48, 42-6.

Pflieger-Bruss S., Schuppe H.C., Schill W.B. 2004. The male reproductive system and its susceptibility to endocrine disrupting chemicals. Andrologia. 36(6),337-45. Review.

Pietrement C., Sun-Wada G.H., Silva N.D., Mckee M., Marshansky V., Brown D., Futai M., Breton S. 2006. Distinct expression patterns of different subunit isoforms of the V-ATPase in the rat epididymis. Biol. Reprod., 74(1), 185-94.

Poulos A., Darin-Bennett A., White L.G. 1973. The phospholipids-bound fatty acids and aldehydes of mammalian spermatozoa. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 46, 541-9.

Pujol A., Bayard F. 1979. Androgen receptors in the rat epididymis and their hormonal control. J. Reprod. Fertil., 56, 217-22.

Rajalakshmi M. 1985. Appearance of specific proteins in rat epididymis during postnatal development. Arch. Androl., 15(1), 49-52.

Reid B.L., Cleaned K.W. 1957. The structure and function of the epididymis. The histology of the rat epididymis. Aus. J. Zool., 5(3), 223-51.

Ricker D.D., Crone J.K., Chamness S.L., Klinefelter G.R., Chang T.S.K. 1997. Partial sympathetic denervation of the rat epididymis permits fertilization but inhibits emryo development. J. Androl., 18, 131-8.

Ricker D.D. 1998. The Autonomic Innervation of the epididymis: its effects on epididymal function and fertility. J. Androl., 19, 1-4.

Robaire B., Ewing L.L., Zirkin B.R., Irby D.C. 1977. Steroid delta4-5alpha-reductase and 3alpha-hydroxysteroid dehydrogenase in the rat epididymis. Endocrinology., 101(5), 1379-90.

Robaire B., Hermo L. Efferent ducts, epididymis and vas deferens: structure, functions and their regulation. In: Knobil E., Neil JD. (Eds.). The physiology of Reproduction. New York: New York: Raven Press Ltd, 1ed, 1988: 999-1080p.

Robaire B., Hinton B., Orgebin-Crist M.C. The Epididymis. In: Knobil E., Neil JD. (Eds.). The physiology of reproduction. New York: Elsevier, 2006: 1071-148p.

Robaire B., Seenundun S., Hamzeh M., Lamour S.A. 2007. Androgenic regulation of novel genes in the epididymis. Asian J. Androl., 9(4), 545-53.

Robb G.W., Amman R.P., Killian G.J. 1978. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J. Reprod. Fertil., 54(1), 103-7.

Roberts K.P. What are the components of male reproductive system? In: The American society of andrology (Eds.). Handbook of Andrology, 2ed., Lawrence: Allen Press, 2010: 1-5p.

Rodríguez C.M., Kirby J.L., Hinton B.T. The Development of the Epididymis. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 251 – 68p.

Rodriguez, J. A., Favaretto, A. L. V. Sistema reprodutor. In: Aires M.M. (Ed.). Fisiologia, Rio de Janeiro: Guanabara Koogan,1999: 877-917p.

Roy-Burman P., Wu H., Powell W.C., Hagenkpord J., Cohen M.B. 2004. Genetically defined mouse models that mimic natural aspects of human prostate cancer developmental. Endocr. Relat. Cancer., 11, 225-324.

Ruiz-Bravo N. 1988. Tissue and cell specificity of immobilin biosynthesis. Biol. Reprod., 39, 901-11.

Russell W.M.S., Burch R.L. The Principles of Humane Experimental Technique. London: Methuen and Co. [Reissued: 1992, Universities Federation for Animal Welfare, Herts, UK]. 1959.

Russell L.D., Ettlin R.A., Sinhahikin A.T., Clegg E.D. Mammaliam spermatogenesis. In: Russel L.D., Ettlin R., Hikim A.P.S., Clegg E.D. (Eds.). Histologycal and histopathologycal evaluation of the testis. Clearwater: Cache River Press, 1990: 1-40p.

Scarano W.R., Toledo F.C., Guerra M.T., Pinheiro P.F., Domeniconi R.F., Felisbino S.L., Campos S.G., Taboga S.R., Kempinas W.G. 2010. Functional and morphological reproductive aspects in male rats exposed to di-n-butyl phthalate (DBP) in utero and during lactation. J. Toxicol. Environ. Health A., 73(13-14):972-84

Serre V., Robaire B. 1999. Distribution of immune cells in the epididymis of the aging Brown Norway rat is segment-specific and related to the luminal content. Biol. Reprod., 61(3), 705-14.

Serre V., Robaire B. Interactions of the Immune System and the Epididymis. In: Robaire B., Hinton B.T. (Eds). The epididymis – from molecules to clinical practice. New York: Kluwer Academic/ Plenum Publisher, 2002: 219-32p.

Setchell B.P., Breed W.G. Anatomy, vasculature, and innervations of the male reproductive tract. In: Knobil E., Neil JD. (Eds.). The physiology of reproduction. New York: Elsevier, 2006:771-825p.

Sharpe R.M. 1993. Declining sperm counts in men – is there an endocrine cause? J. Endocrinol., 136, 357-60.

Sharpe R.M. 2006. Pathways of endocrine disruption during male sexual differentiation and masculinization. Best Pract. Res. Clin. Endocrinol. Metab. 20(1), 91-110. Review

Shum W.W., Da Silva N., Mckee M., Smith P.J., Brown D., Breton S. 2008.Transepithelial projections from basal cells are luminal sensors in pseudostratified epithelia. Cell., 135 (6), 1108-17.

Stoker T.E., Parks L.G., Gary E., Cooper R.L. 2000. Endocrine-disrupting chemicals: Prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. A focus on the EDSTAC recommendations. Crit. Rev. Toxicol., 30 (2), 197-252.

Sullivan R., Saez F., Girouard J., Frenette G. 2005. Role of exossomes in sperm maturation during the transit along the male reproductive tract. Blood Cells Mol. Dis., 35, 1-10.

Sun E.L., Flickinger C.J. 1979. Development of cell types and of regional differences in the postnatal rat epididymis. Am. J. Anat., 154(1), 27-55.

Sun E.L., Flickinger C.J. 1982. Proliferative activity in the rat epididymis during postnatal development. Anat. Rec., 203(2), .273-84.

Swan S.H., Elkin E.P., Fenster L. 1997. Have sperm densities declined? A reanalysis of global trend data. Environ. Health Perspec., 105, 1228-32.

Swan S.H., Main K.M., Liu F., Stewart S.L., Kruse R.L., Calafat A.M. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ. Health Perspec., 113, 1056–61.

Talo A., Jaakkola UM, Markkula-Viitasmen M. 1979. Spontaneous eletrical activity of the rat epididymis in vitro. J. reprod. Fertil., 57, 423-9.

Trasler J., Hermo L., Robaire B. 1988. Morphological changes in the testis and epididymis of rats treated with cyclophosphamide: a quantitative approach. Biol. Reprod., 38(2), 463-79.

Turner T.T. 1995. On the epididymis and its role in the development of the fertile ejaculate. J. Androl., 16(4), 292-8.

Turner T.T., Riley T.A. 1999. p53 independent, region-specific epithelial apoptosis is induced in the rat epididymis by deprivation of luminal factors. Mol. Reprod. Dev., 53(2), 188-197.

Turner T.T., Bomgardner D., Jacobs J.P., Nguyen Q.A. 2003. Association of segmentation of the epididymal interstitium with segmented tubule function in rats and mice. Reproduction., 125(6), 871-8.

Turner T.T. 2008. De Graaf's thread: the human epididymis. J. Androl., 29(3), 237-50.

Usselman M.C., Cone R.A. 1983. Rat sperm are mechanically immobilized in the caudal epididymidis by immobilin, a high molecular weight glycoprotein. Biol. Reprod., 29, 1241-53.

Vernet P., Aitken R.J., Drevet J.R. 2004. Antioxidant strategies in the epididymis. Moll. Cell. Endocrinol., 216, 31-9.

Vierula M.E., Rankin T.L., Orgebin-Crist M.C. 1995. Electron microscopic immunolocalization of the 18 and 29 kilodalton secretory proteins in the mouse epididymis: evidence for differential uptake by clear cells. Microsc. Res. Tech., 30(1), 24-36.

Viger R.S., Robaire B. 1994. Immunocytochemical localization of 4-ene steroid 5 alpha-reductase type 1 along the rat epididymis during postnatal development. Endocrinology., 134(5), 2298-2306.

Wilson V.S., Blystone C.R., Hotchkiss A.K., Rider C.V., Gray E. 2008. Diverse mechanisms of anti-androgen action: impact on male rat reproductive tract development. Int. J. Androl., 31, 178-87.

Zhu L.J., Hardy M.P., Inigo I.V., Huhtaniemi L., Bardin C.W., Moo-Young A.J. 2000. Effects of Androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. Biol. Reprod., 63, 368-76.

Zorn B., Virant-Klun I., Verdenik I., Meden-Vrtovec H. 1999. Semen quality changes among 2343 healthy Slovenian menincluded in an IVF-ET programme fom 1983-1996. Int. J. Androl., 22, 178-83.

Apêndices

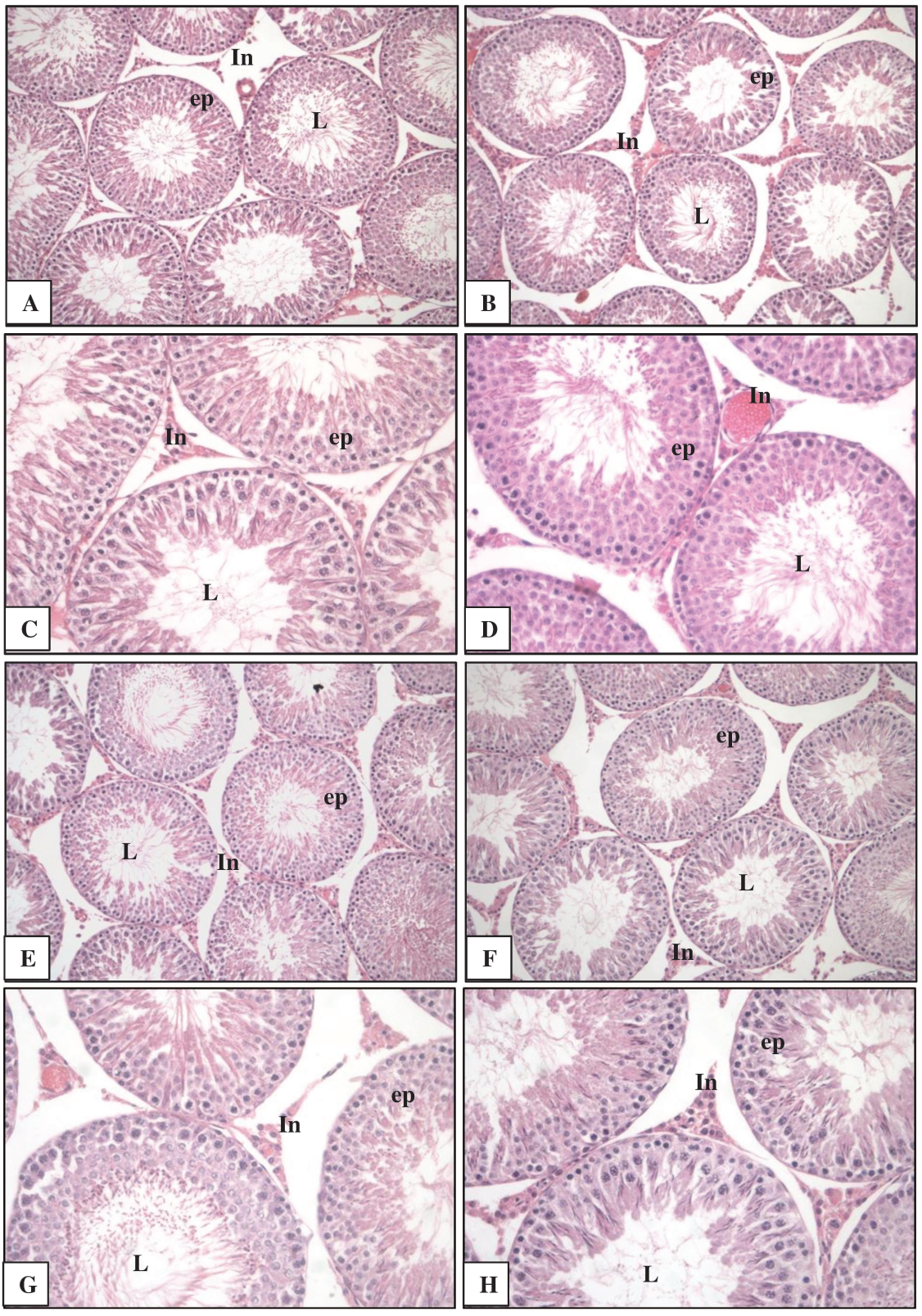
7. Apêndices

Neste item serão incluídos os resultados citados porém não mostrados no corpo dos manuscritos gerados pelo presente estudo.

7.1.Apêndice I

Histopatologia testicular. Fotomicrografias de cortes transversais do testículo de rato corados com hematoxilina e eosina; (A-D) animais aos 50 dias de idade, (E-H) animais aos 75 dias de idade (fixação Bouin). Coluna à esquerda: grupo controle; coluna à direita: grupo flutamida. (A, B, E, F): aumento final de 200X; (C, D, G, H) aumento final de 400X. Ep: Epitélio; In: Interstício; L: Lúmen.

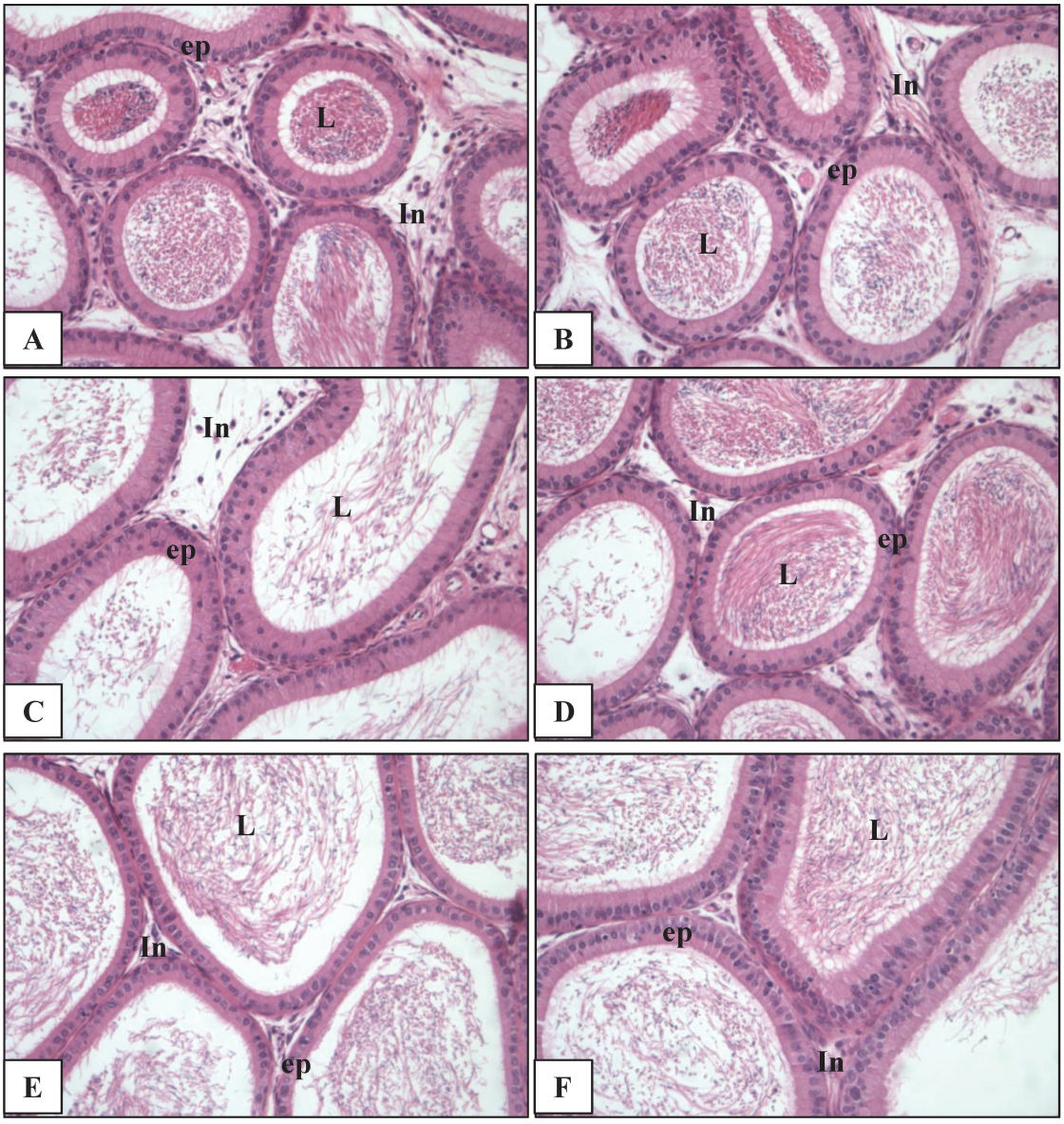
Apêndice I

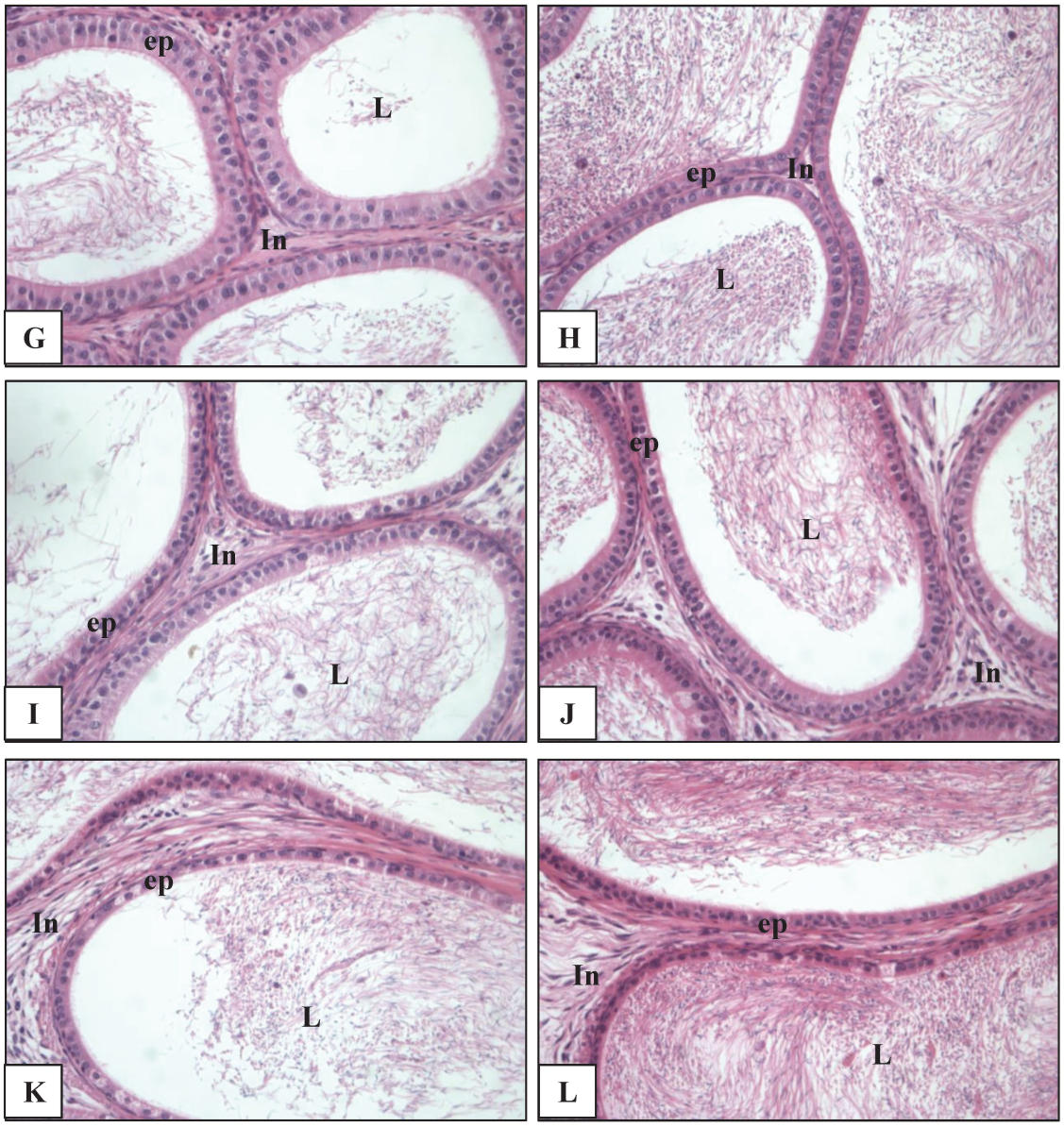


7.2. Apêndice II

7.2.1. Fotomicrografias de cortes longitudinais de epidídimo de rato aos 50 dias de idade corados com hematoxilina e eosina (Fixação Bouin). (A e B) SI proximal; (C e D) SI distal; (E e F) cabeça; (G e H) corpo; (I e J) cauda proximal; (K e L) cauda distal. Ep: epitélio epididimário; In: interstício epididimário; L: luz do ducto. Coluna à esquerda: grupo controle. Coluna à direita: grupo flutamida. Aumento final 400X.

Apêndice II
7.2.1.

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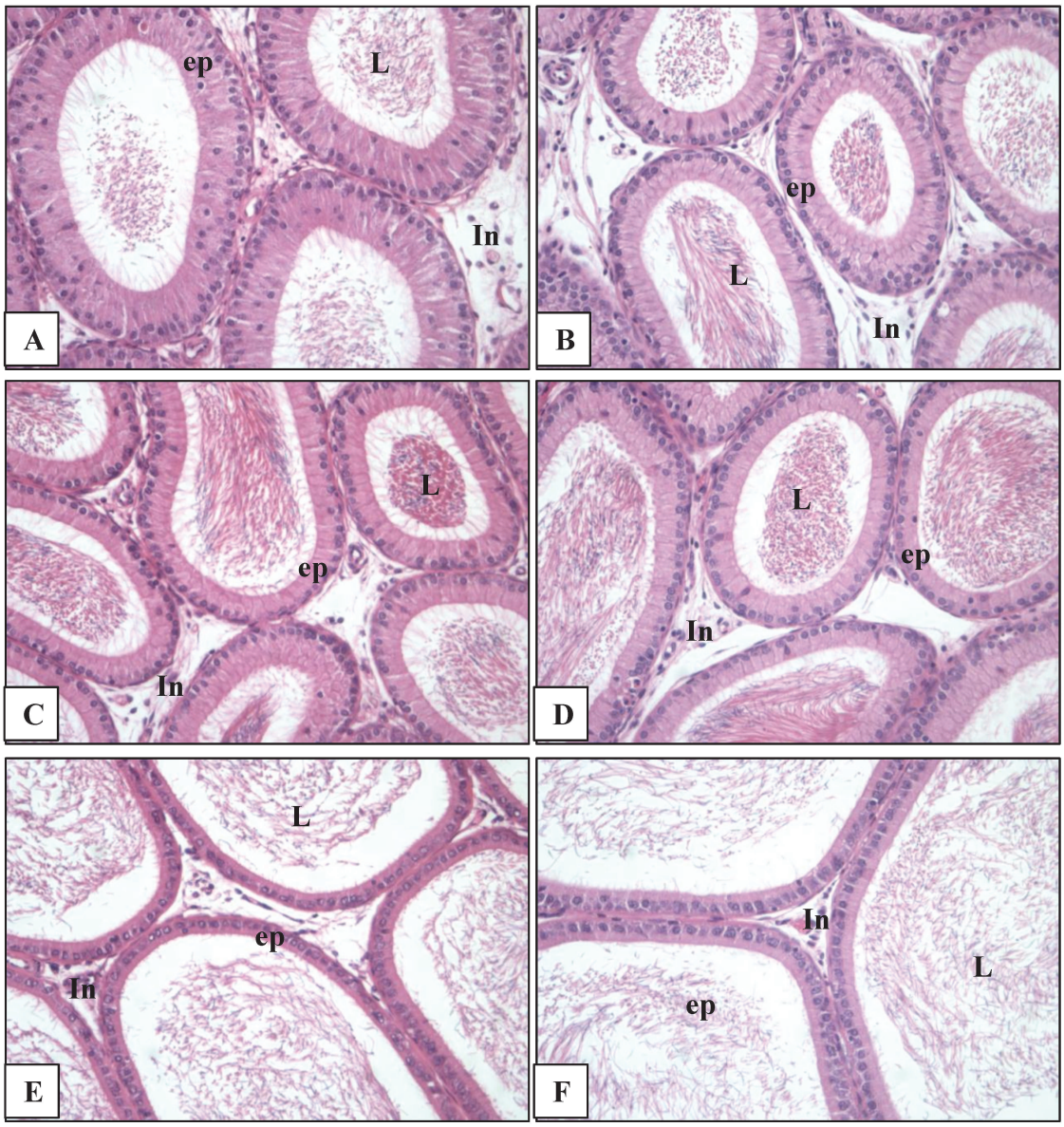
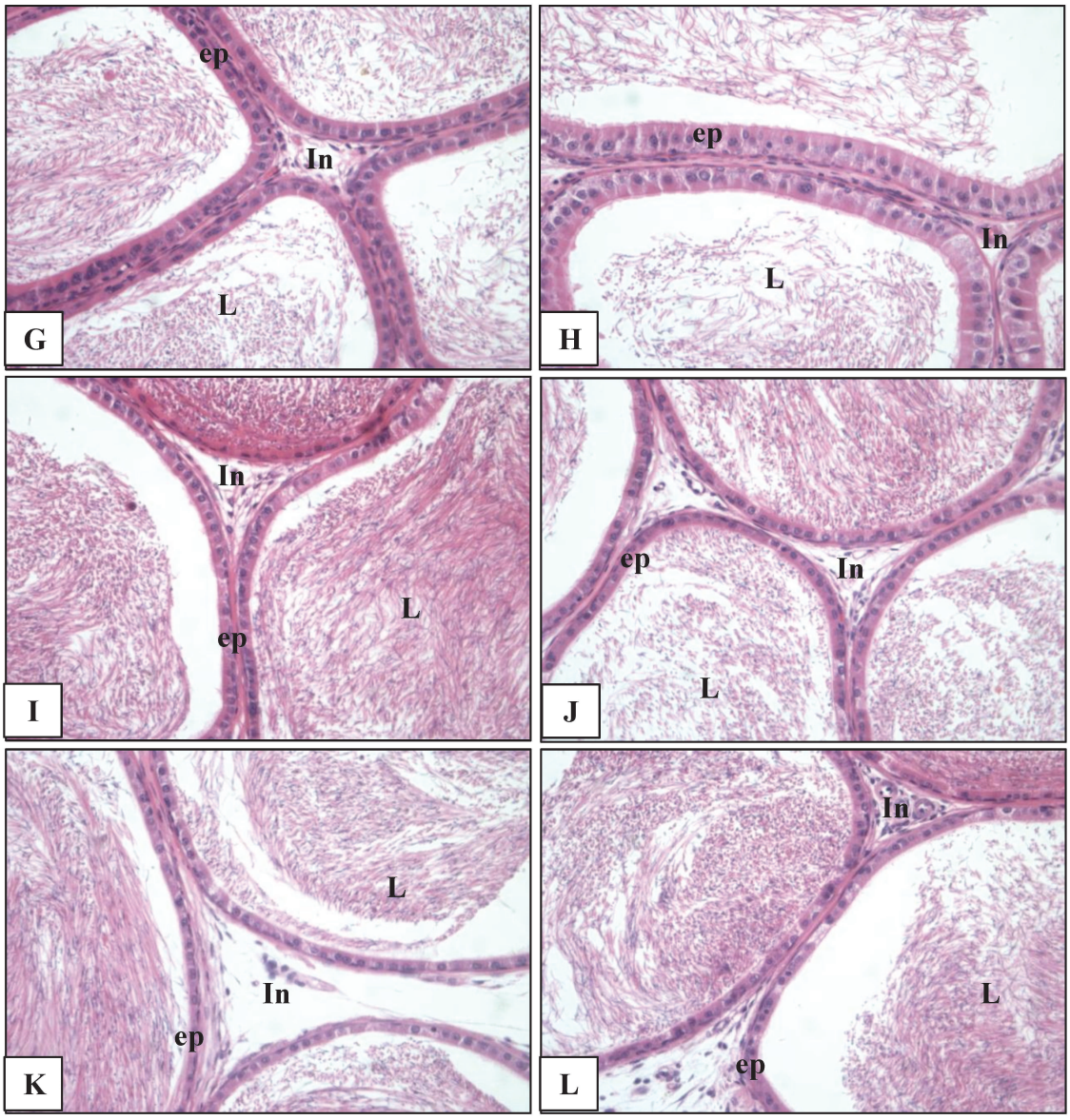
7.2.2. Fotomicrografias de cortes longitudinais de epidídimo de rato aos 75 dias de idade corados com hematoxilina e eosina (Fixação Bouin). (A e B) SI proximal; (C e D) SI distal; (E e F) cabeça; (G e H) corpo; (I e J) cauda proximal; (K e L) cauda distal. Ep: epitélio epididimário; In: interstício epididimário; L: luz do ducto. Coluna à esquerda: grupo controle. Coluna à direita: grupo flutamida. Aumento final 400X.

Apêndice II
7.2.2.

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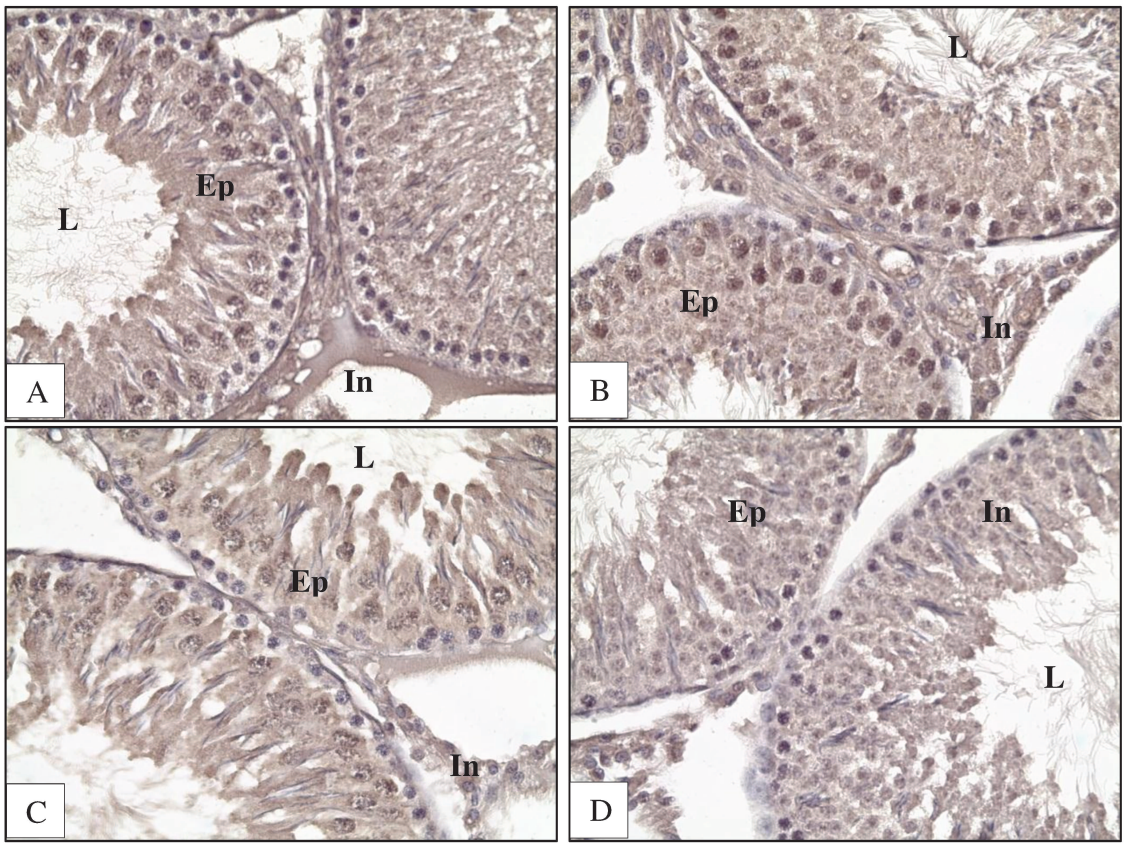
7.3. Apêndice III

Imuno-marcação da Calmodulina (CALM) em testículo de rato. (A-B) animais aos 50 dias de idade, (C-D) animais aos 75 dias de idade. Coluna à esquerda: grupo controle; coluna à direita: grupo flutamida. aumento final de 400X. Ep: Epitélio; In: Interstício; L: Lúmen.

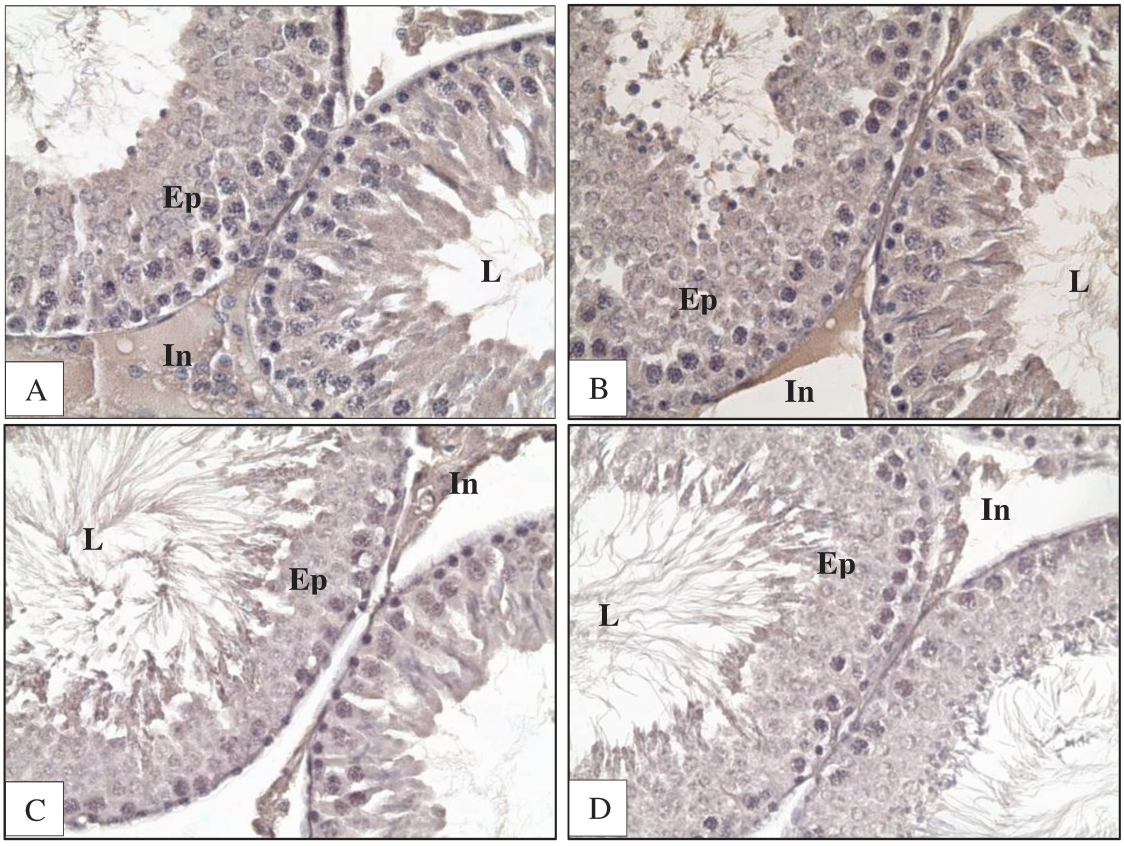
Apêndice III



7. 4. Apêndice IV

Imuno-marcação da Rab11A em testículo de rato. (A-B) animais aos 50 dias de idade, (C-D) animais aos 75 dias de idade. Coluna à esquerda: grupo controle; coluna à direita: grupo flutamida. aumento final de 400X. Ep: Epitélio; In: Interstício; L: Lúmen

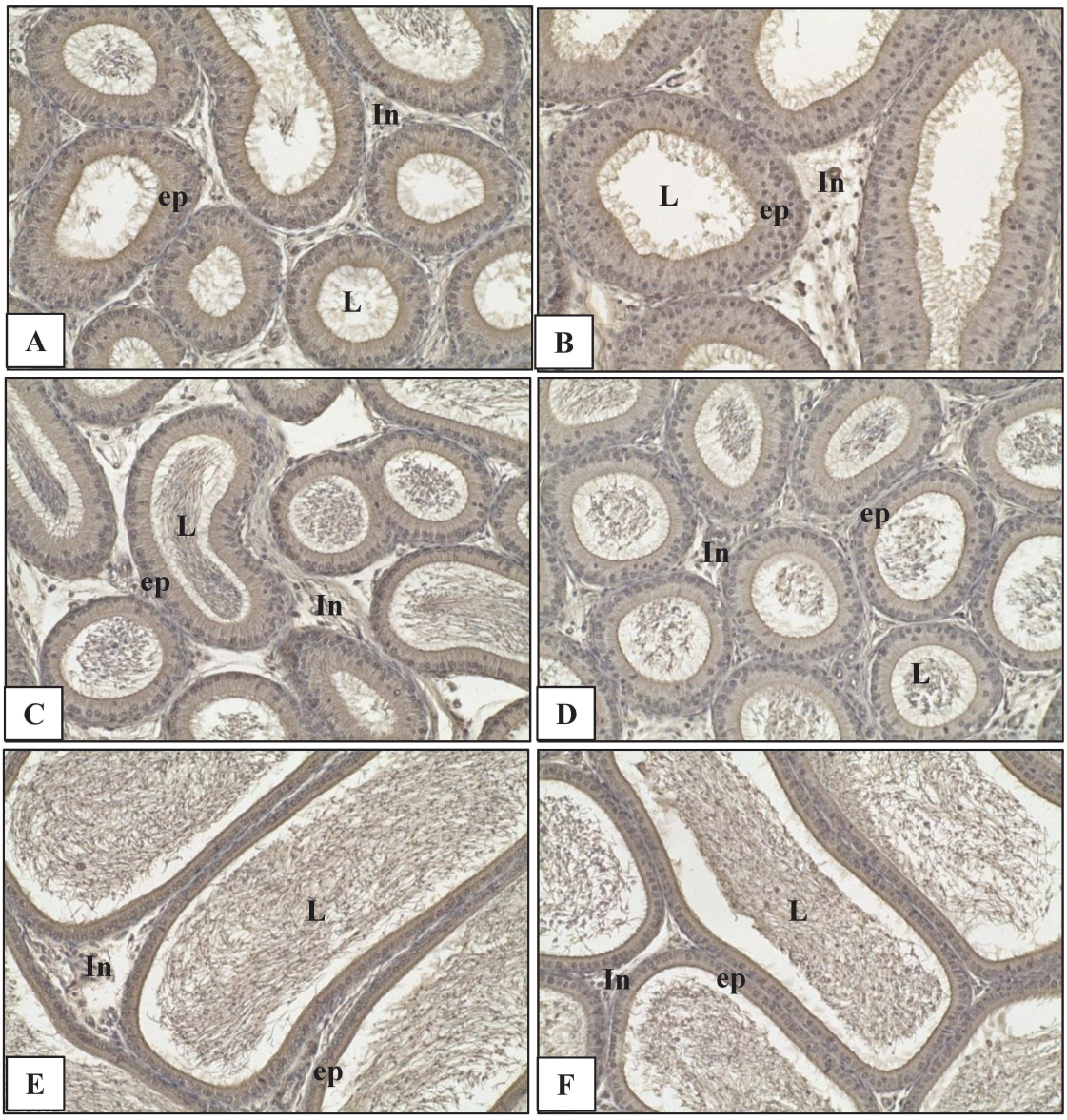
Apêndice IV

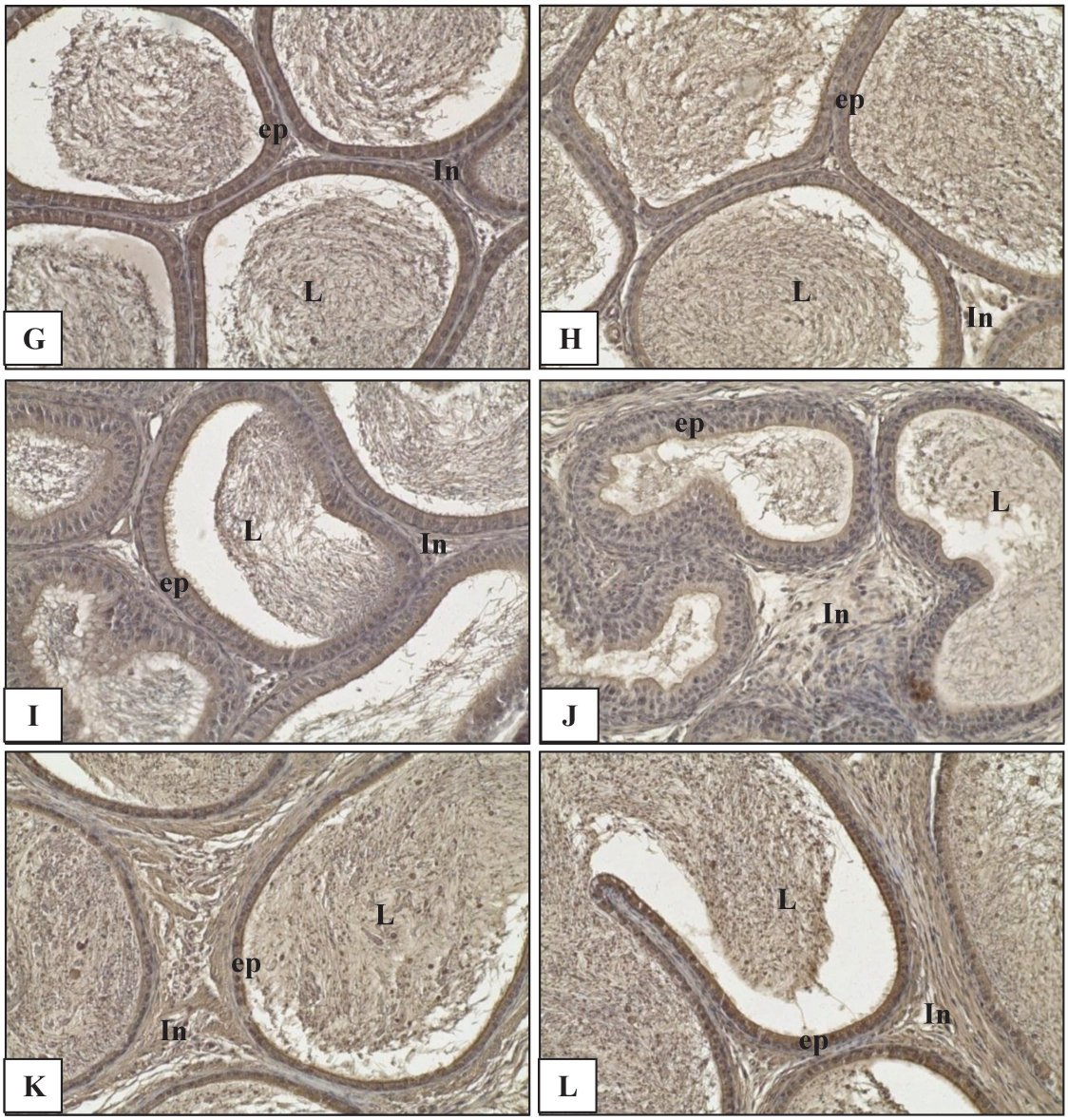


7.5. Apêndice V

7.5.1. Imuno-marcação da proteína espermática 22 (SP22) em epidídimo de rato aos 50 dias de idade (Fixação Bouin). (A e B) SI proximal; (C e D) SI distal; (E e F) cabeça; (G e H) corpo; (I e J) cauda proximal; (K e L) cauda distal. Aumento final 400X. Ep: epitélio epididimário; In: interstício epididimário; L: luz do ducto.

Apêndice V
7.5.1.

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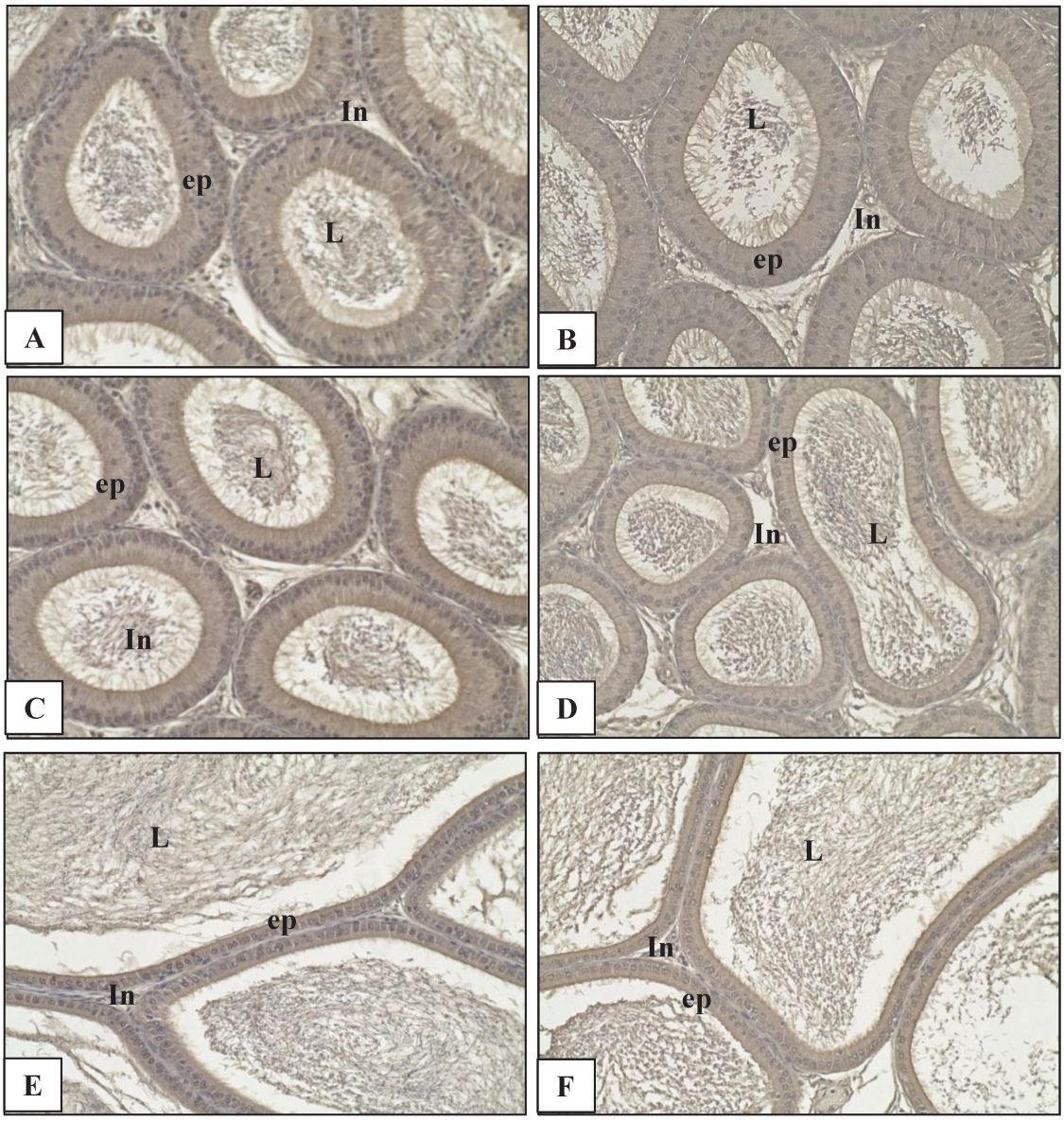
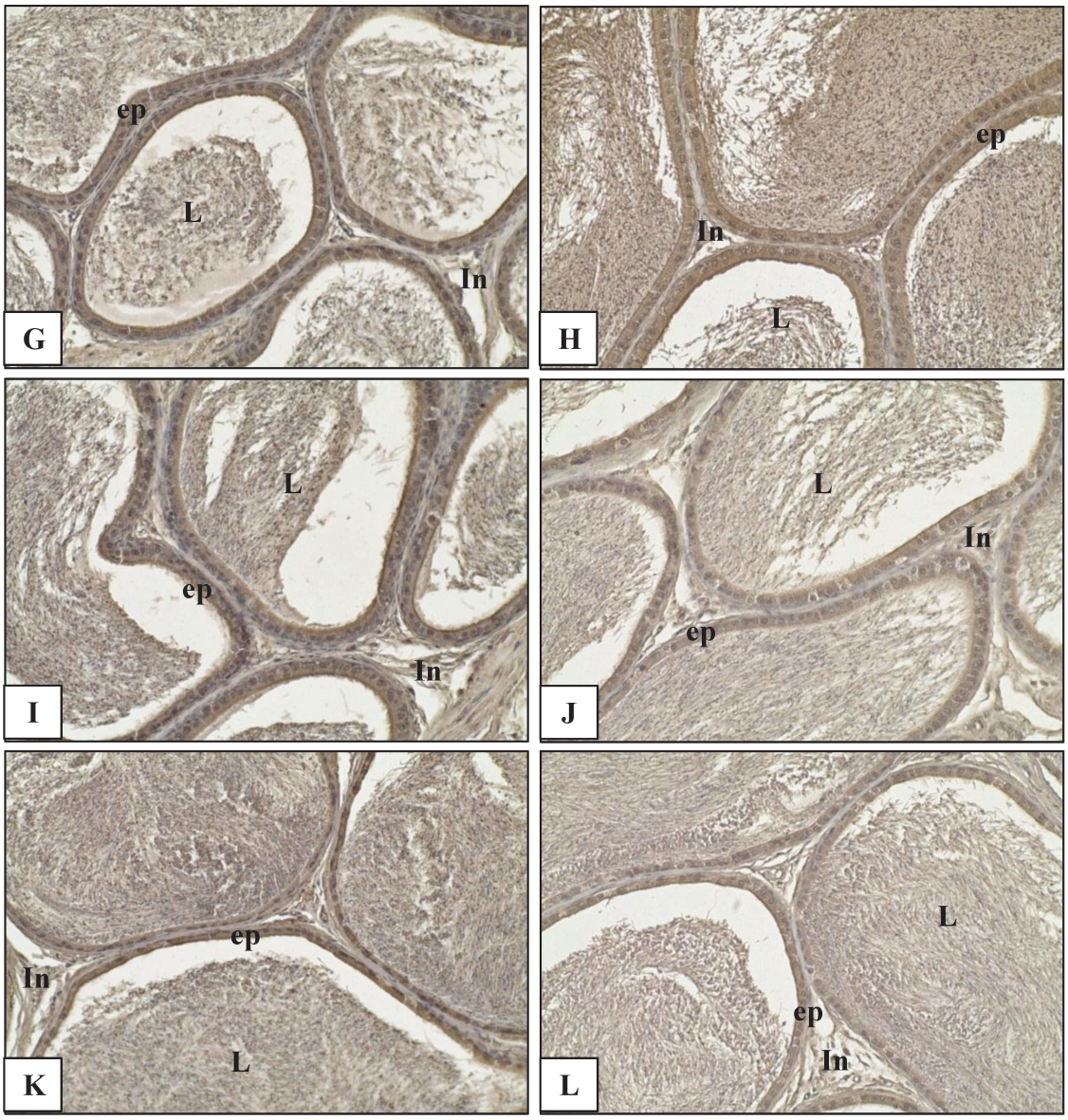
7.5.2. Imuno-marcação da proteína espermática 22 (SP22) em epidídimo de rato aos 75 dias de idade (Fixação Bouin). (A e B) SI proximal; (C e D) SI distal; (E e F) cabeça; (G e H) corpo; (I e J) cauda proximal; (K e L) cauda distal. Aumento final 400X. Ep: epitélio epididimário; In: interstício epididimário; L: luz do ducto.

Apêndice V
7.5.2.

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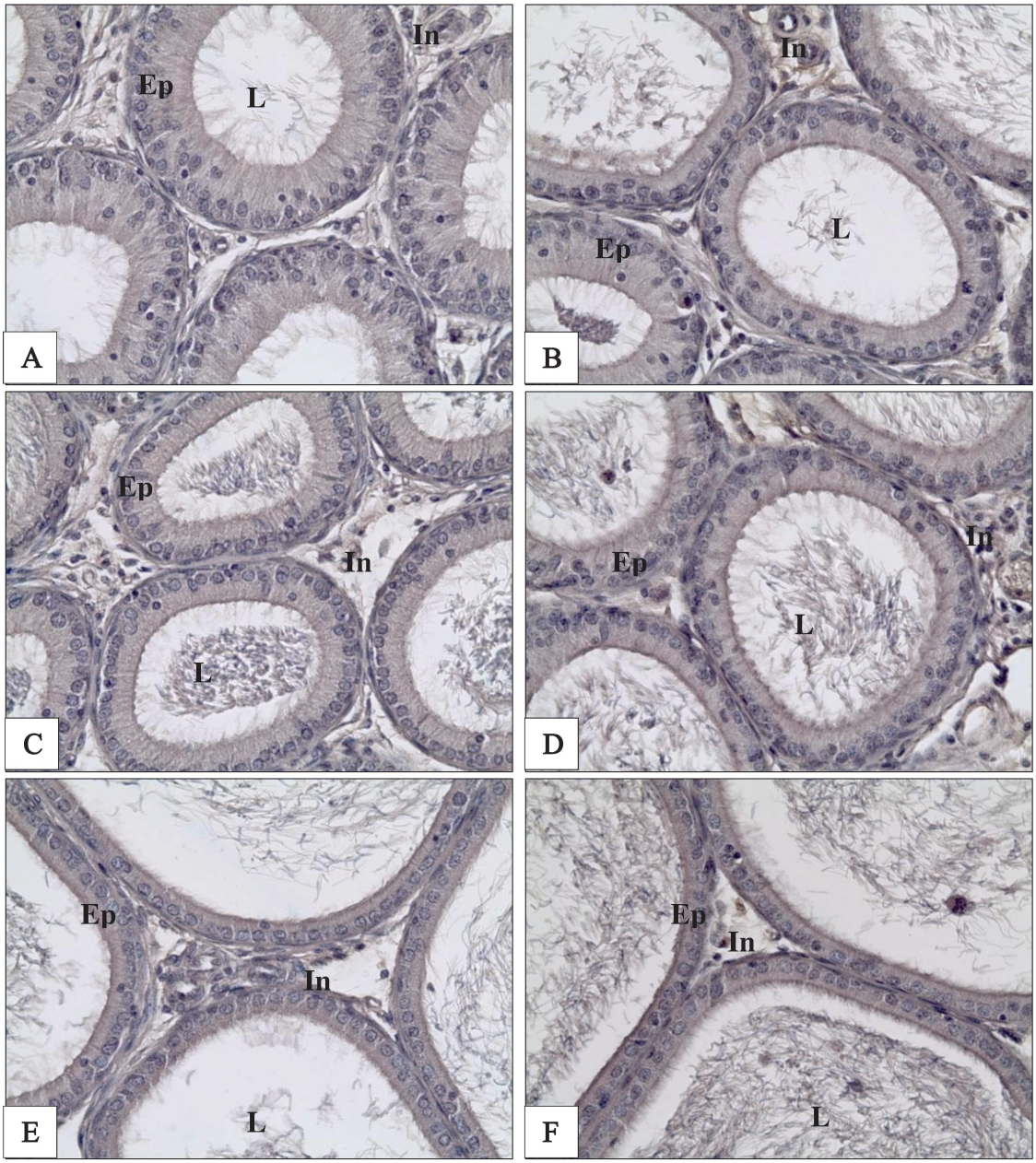
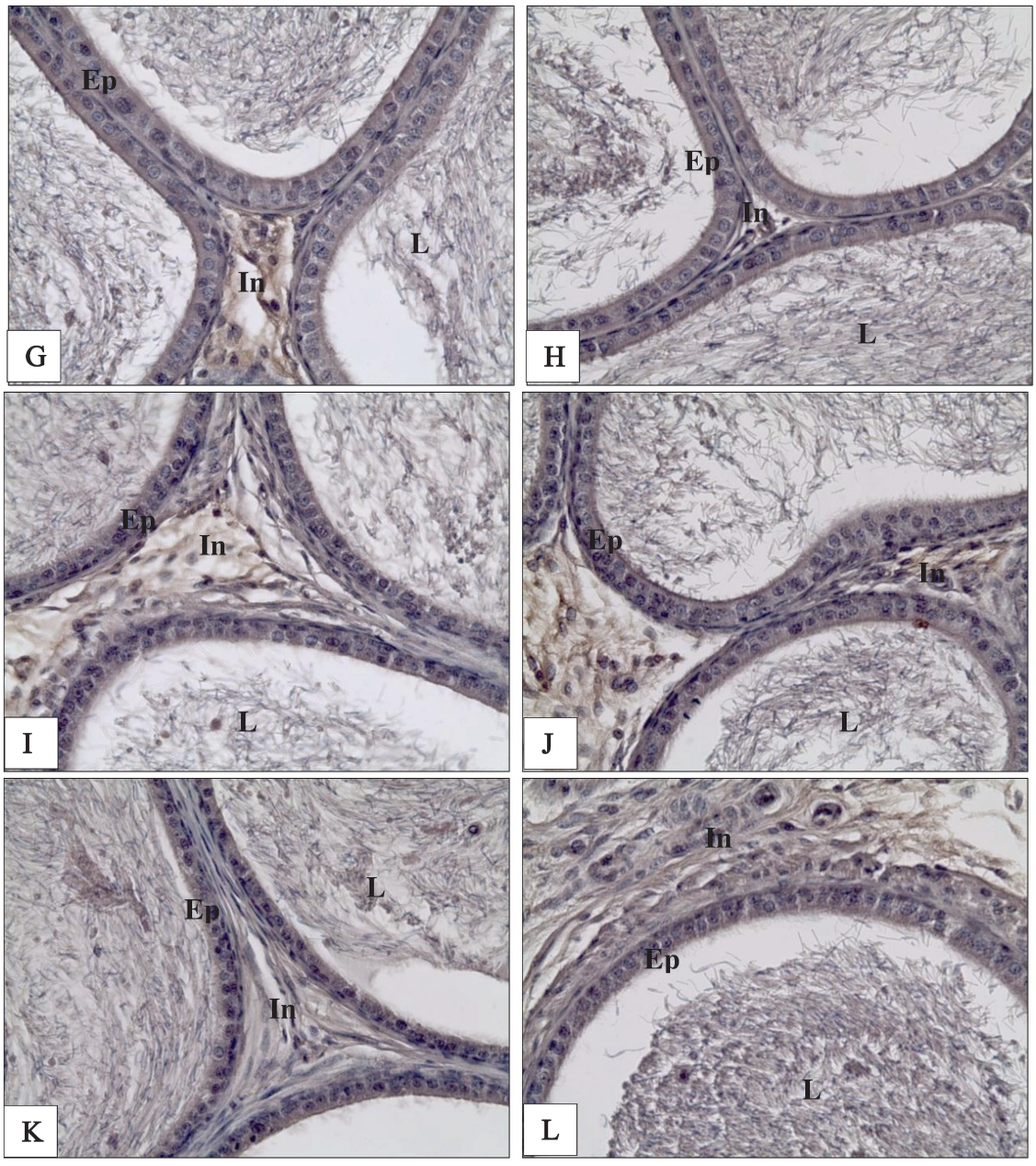
7.6. Apêndice VI

Imuno-marcação da Rab11A em epidídimo de rato aos 50 dias de idade (Fixação Bouin). (A e B) SI proximal; (C e D) SI distal; (E e F) cabeça; (G e H) corpo; (I e J) cauda proximal; (K e L) cauda distal. Aumento final 400X. Ep: epitélio epididimário; In: interstício epididimário; L: luz do ducto.

Apêndice VI

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Anexos

Anexos

8.1. Certificado da Comissão de Ética em experimentação animal.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **""Morfofisiologia do sistema genital masculino de ratos púberes e adultos após privação androgênica durante a pré-puberdade"**

( ) 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 21/08, Instituição: Comissão de Ética em Experimentação Animal do Instituto de Biociências de Botucatu/UNESP.

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

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

Juliana Elaine Perobelli
Aluno(a): Juliana Elaine Perobelli

Wilma De Grava Kempinas
Orientador(a): Wilma De Grava Kempinas

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura Ana Maria Aparecida Guaraldo

Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

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"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

CERTIFICADO

Certificamos que o Protocolo nº **21/08-CEEA**, sobre "Morfo-fisiologia epididimária de ratos púberes e adultos sujeitos à privação de andrógeno durante o período pré-puberal", 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 **07/05/2008**.

Botucatu, 7 de maio de 2008.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

NADIA JOVÊNCIO COTRIM
Secretária – CEEA

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