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Células germinativas e células de Leydig em modelo de dano testicular no rato

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Mestre em Patologia.

Orientador: Dr. João Lauro Viana de Camargo
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Esta dissertação é composta pelos capítulos I e II, sendo o primeiro uma revisão da literatura redigida em português, e o segundo capítulo um manuscrito redigido em inglês, tanto para atender às normas do Programa de Pós-Graduação em Patologia como para agilizar a submissão do mesmo ao periódico de escolha.

Resumo

Evidências epidemiológicas sugerem a influência de xenobióticos no aumento da incidência de desordens do trato reprodutor masculino como criptorquidia, hipospadia, baixa qualidade do sêmen e tumores testiculares. Dentre elas, a criptorquidia é a anomalia congênita mais comum em meninos e é um fator de risco para o desenvolvimento de infertilidade e tumor testicular. O objetivo deste estudo foi avaliar morfologicamente os testículos de ratos criptorquídicos expostos *in utero* e no período pós-natal à acrilamida (AA) ou ao di-n-butil-ftalato (DBP). Durante a gestação e a lactação, as ratas prenhes foram expostas a AA 10 mg/kg/dia ou DBP 500 mg/kg/dia por gavagem e dieta, respectivamente. Após o desmame (dia pós-natal; DPN 21), a prole masculina foi submetida a cirurgia de criptorquidia (CPT), revertida por orquidopexia (R) após três semanas, continuamente expostos aos xenobióticos por dieta até a eutanásia quando com 16, 31 ou 58 semanas de idade. Os testículos foram avaliados por sistema de classificação de danos histológicos, imunohistoquímica para os antígenos tirosina quinase Kit (c-Kit) e fosfatase alcalina placentária (PLAP), e análise morfométrica das células de Leydig. Os resultados mostraram redução do peso corpóreo apenas no grupo AA/CPT-R na 16ª semana, mas ambos os grupos tratados apresentaram uma redução progressiva no peso testicular absoluto e no volume testicular em todos os momentos de estudo. As alterações histológicas apresentaram maior severidade nos grupos AA/CPT-R e DBP/CPT-R. Na 31ª e 58ª semanas, em animais do grupo AA/CPT-R foi encontrada predominância significativa de túbulos com células marcadas com c-Kit associada à redução da imunorreatividade para PLAP; o grupo DBP/CPT-R apresentou redução da imunorreatividade para PLAP na 58ª semana. Na 16ª semana, o volume total ocupado pelas células de Leydig apresentou-se diminuído em ambos os grupos expostos às substâncias químicas; o índice Leydigossomático (volume relacionado a massa corporal) apresentou-se diminuído no grupo DBP/CPT-R. O número de células de Leydig/ml apresentou-se aumentado no grupo DBP/CPT-R na 16ª semana, e também nos grupos AA/CPT-R e DBP/CPT-R nas 31ª e 58ª semanas. Os métodos de avaliação testicular adotados são individualmente capazes de detectar o dano testicular e, particularmente, o comprometimento da espermatogênese. Este modelo experimental mostrou-se útil para avaliar alterações testiculares induzidas por xenobióticos.

Abstract

Abstract

Epidemiological evidence suggests the influence of xenobiotics on the increased incidence of male reproductive tract disorders such as cryptorchidism, hypospadias, poor semen quality and testicular tumors. Among them, cryptorchidism is the most common congenital anomaly in boys and is a risk factor for the development of infertility and testicular tumor. The aim of this study was to evaluate morphologically the testes of cryptorchid rats exposed *in utero* and postnatally to the acrylamide (AA) or di-n-butyl-phthalate (DBP). During gestation and lactation, dams were exposed to AA 10 mg/kg/day or DBP 500 mg/kg/day by gavage and diet, respectively. After weaning (postnatal day; PND 21), male pups were surgically made cryptorchid (CPT), reverted by orchiopexy (R) three weeks later, continuously exposed to the xenobiotics by diet until euthanasia when 16-, 31- or 58-week old. The testes were evaluated by histological damage classification system, immunohistochemical for the antigens tyrosine kinase Kit (c-Kit) and placental alkaline phosphatase (PLAP), and morphometric analysis of Leydig cells. The results showed a decrease in the body weight only in the AA/CPT-R group at 16th week, but both treated groups presented a progressive decrease in the absolute testicular weight and testicular volume in all the moments of study. The histological alterations presented greater severity in the AA/CPT-R and DBP/CPT-R groups. A significant predominance of tubules with c-Kit strongly labeled cells was found in the AA/CPT-R group at the 31st and 58th weeks after birth, associated to reduction of the immunoreactivity for PLAP; DBP/CPT-R group presented decreased immunoreactivity for PLAP at the 58th week. The total volume occupied by Leydig cells decreased in both chemically-exposed groups at the 16th week; Leydig-somatic index (volume related to the body mass) was significantly decreased in the DBP/CPT-R group. The number of Leydig cells/ml was increased in the DBP/CPT-R group at the 16th week, and in the AA/CPT-R and DBP/CPT-R groups at the 31st and 58th weeks. The methods of testicular evaluation adopted are individually able to detect testicular damage and, particularly, the compromising of the spermatogenesis. This experimental model showed to be useful to evaluate testicular alterations induced by xenobiotics.

CAPÍTULO I

CAPÍTULO II

CAPÍTULO II

AA Acrilamida/Acrylamide
AP2γ proteína AP2γ
b.w. peso corpóreo/body weight
c-Kit proteína receptora tirosina quinase/tyrosine kinase receptor protein
CPT-R criptorquidia seguida de orquidopexia/cryptorchidism followed by orchiopexy
DBP di-n-butil-ftalato/di-n-butyl-phthalate
DPN Dia pós-natal/Postnatal Day
FSH Hormônio Folículo Estimulante
GD dia gestacional/gestational day
H&E hematoxilina e eosina/hematoxylin and eosin
i.e. latim, *id est*: isto é
Insl3 Hormônio Insulin-like 3
i.p. intraperitoneal
LH Hormônio Luteinizante
LSI índice Leydigossomático/Leydig-somatic index
NLC número de células de Leydig/number of Leydig cells
PLAP fosfatase alcalina placentária/placental-like alkaline phosphatase
POU5F1 proteína POU5F1
SALL4 proteína Sal-like protein 4
SCF fator de células-tronco/Stem Cell Factor
SCO somente células de Sertoli/Sertoli cells only
s.c. subcutâneo
SD desvio padrão/standard deviation
TGCT Tumor Testicular de Célula Germinativa/Testicular Germ Cell Tumor
TVOLC volume total ocupado pelas células de Leydig/Total volume occupied by Leydig cells

Sumário

CAPÍTULO I

CAPÍTULO II

Capítulo I

1. REVISÃO DA LITERATURA

1.1 Desordens do aparelho reprodutor masculino e fatores ambientais

A influência dos fatores ambientais no aparecimento de desordens do aparelho reprodutor masculino está cada vez mais evidente; países como Dinamarca e Suécia apresentam índices elevados de criptorquidia, baixa qualidade do sêmen, hipospadia e tumores testiculares quando comparados à Finlândia; entretanto, a segunda geração de migrantes da Finlândia para a Suécia apresenta risco aumentado para tumores testiculares (Montgomery et al., 2005; Myrup et al., 2008; Gilbert et al., 2011). De fato, a incidência do câncer testicular depende da região geográfica analisada; em 2002, as taxas foram de 7,8 na Nova Zelândia, 6,3 no Reino Unido, 6,1 na Austrália, 5,6 na Suécia, 5,2 nos Estados Unidos, 4,9 na Polônia e 3,8 na Espanha por 100.000 homens, enquanto Índia, China e Colômbia apresentaram menor incidência (0,5, 1,3 e 2,2, respectivamente) por 100.000 homens (Shanmugalingam et al., 2013). As maiores incidências são observadas nos países desenvolvidos, ou seja, com grande potencial industrial e, portanto, há maior risco de exposição a substâncias químicas exógenas (xenobióticos). Esses dados corroboram a hipótese de que fatores ambientais locais possam contribuir para a ocorrência de desordens testiculares.

Uma variedade de xenobióticos é capaz de induzir danos testiculares. Dentre os xenobióticos tóxicos para o testículo estão a acrilamida (AA) e o di-n-butil-ftalato (DBP) (Figura 1-I), aos quais a exposição ocorre constantemente devido à ampla aplicabilidade desses compostos em processos industriais.

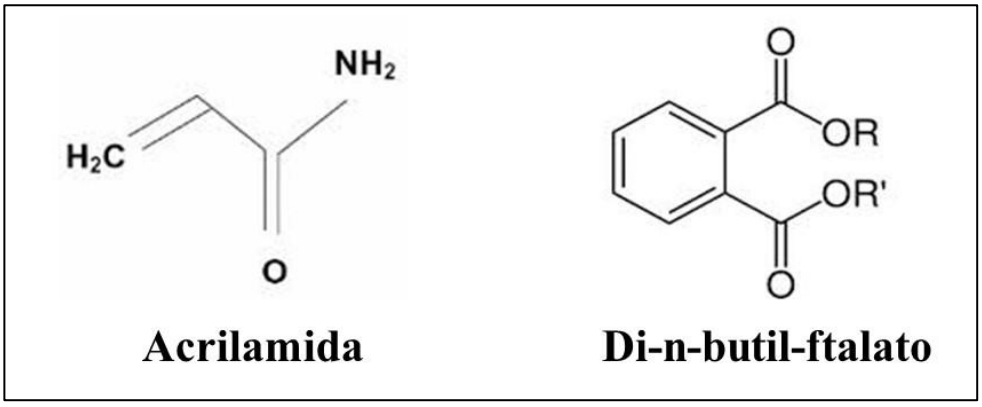

Figura 1-I: Estrutura química dos xenobióticos AA e DBP, tóxicos para células testiculares.

O DBP constitui um dos grupos químicos mais produzidos no mundo, os ftalatos, utilizados principalmente para conferir maleabilidade, transparência e durabilidade a produtos plásticos (Crinnion, 2010). Os ftalatos são líquidos viscosos, incolores e inodoros; possuem baixa solubilidade em água, alta solubilidade em óleo e baixa volatilidade (National Research Council, 2008). Já a AA, um polímero sintético com baixo peso molecular e alta solubilidade em água, é muito utilizada como floculante em tratamento de água ou selante em barragens e também pode ocorrer espontaneamente em processos de cozimento sob alta temperatura de alimentos ricos em carboidratos (USEPA, 2010).

Dentre as desordens do aparelho reprodutor masculino que apresentam aumento considerável da incidência em várias regiões do mundo e podem resultar da exposição a xenobióticos, destaca-se a criptorquidia; ela ocorre em 2-9% dos meninos nascidos a termo e em mais de 30% dos pré-termos (Giwercman et al., 2011; Hauser et al., 2015; Fawzy et al., 2015; Skakkebaek et al., 2016). Trata-se da anomalia urogenital congênita mais comum em crianças do sexo masculino, consequência de falha na descida dos testículos para o escroto durante o desenvolvimento embrionário, provavelmente provocada por múltiplos fatores associados a distúrbios hormonais (Virtanen & Adamsson, 2012; Hutson, 2015). Também, a criptorquidia pode ser adquirida, consequência do alongamento anormal dos cordões espermáticos que leva a ascensão espontânea e criptorquídica, após o nascimento, do testículo já localizado no escroto (Hughes & Acerini, 2008; Hutson, 2015).

Tanto em humanos quanto em roedores, a temperatura do testículo no escroto é menor do que no canal inguinal e no abdômen. Portanto, a degeneração tubular no testículo criptorquídico possivelmente ocorra devido à temperatura mais elevada de seu sítio irregular; devido ao comprometimento testicular, a criptorquidia é um dos principais fatores de risco para infertilidade e desenvolvimento de tumores naquela gônada (Zakaria et al., 1998; Ferguson & Agoulnik, 2013; Hutson, 2015). Acredita-se que os principais fatores que contribuem para o aparecimento das desordens testiculares sejam a duração da condição criptorquídica e sua localização abdominal ou inguinal ao longo de seu trajeto em direção ao escroto, uma vez que essas características modulam a injúria gonadal, como inibição da espermatogênese, depleção de células germinativas, atrofia tubular e fibrose (Ferguson & Agoulnik, 2013).

É possível recuperar a função espermática e reduzir os riscos de alterações malignas pela correção cirúrgica da criptorquidia, ou seja, orquidopexia. A idade para realização desse procedimento é determinante para sua eficácia; tumores testiculares são quase 3,5 vezes mais

provável de ocorrer em garotos que se submeteram à orquidopexia após a puberdade do que naqueles que realizaram a cirurgia no período pré-púbere (Walsh et al., 2007).

1.2 Modelos experimentais

A elevada incidência de criptorquidia e evidências da influência da exposição a xenobióticos no desenvolvimento de desordens testiculares em humanos estimulam a realização de estudos experimentais sobre os efeitos dessas condições no testículo.

Como em humanos, a criptorquidia experimental em roedores resulta na redução da capacidade reprodutiva (Ivell & Hartung, 2003). Cirurgicamente, a criptorquidia experimental pode ser obtida por intervenção no gubernáculo, ligamento que orienta o deslocamento da gônada para o escroto, ou no canal inguinal, local de trajeto da gônada até o escroto (Bergh & Soder, 2007); ratos Wistar induzidos à criptorquidia unilateral no DPN 21 e eutanasiados 30, 45 ou 65 dias após a cirurgia apresentaram diâmetro e número de túbulos seminíferos com células germinativas maduras menores em relação ao testículo não criptorquídico (Dündar et al., 2001). No entanto, o procedimento mais comum de criptorquidia experimental compreende a retirada dos testículos que já se encontram no escroto e sua fixação na parede abdominal (Bergh & Soder, 2007); ratos Wistar adultos submetidos à criptorquidia cirúrgica bilateral apresentaram, após 1 dia, redução significativa nos níveis plasmáticos de FSH, LH, testosterona e inibina B. Além disso, após 5 dias, foi observada redução significativa dos parâmetros de qualidade espermática como motilidade, linearidade e velocidade (Ren et al., 2006).

Em mamíferos, a descida testicular é mediada pela substância anti-Mulleriana, secretada pelas células de Sertoli, e pelos hormônios Insl3 e andrógenos, especialmente a testosterona, secretados pelas células de Leydig (Hutson et al., 2013). Deste modo, além desses modelos mecânicos, é possível induzir criptorquidia pela exposição *in utero* a compostos estrogênicos ou antiandrogênicos tais como o dietilestilbestrol e a flutamida (Nef et al., 2000; Ma et al., 2011; Virtanen & Adamsson, 2012). Contudo, esse método possui baixa taxa de sucesso e pode provocar efeitos adicionais sobre os testículos, além de efeitos sistêmicos, decorrentes da desregulação endócrina pelas substâncias utilizadas (Bergh & Soder, 2007).

Apesar de algumas diferenças interespécies, a extrapolação de dados da criptorquidia experimental para humanos pode ser válida, pois o processo de descida testicular é semelhante entre mamíferos. Por exemplo, no período de migração do testículo até o escroto e na remodelação, migração e alongamento gubernacular através do púbis (Hutson et al., 2010).

Os danos testiculares resultantes da exposição *in utero* e ou pós-natal aos xenobióticos AA e DBP estão bem descritos na literatura. Ratos Sprague-Dawley expostos *in utero* a 50, 250 ou 500 mg/kg/dia de DBP durante os dias gestacionais (DG) 12.5 a 21.5 apresentaram diminuição da distância anogenital, atrofia tubular severa, vacuolização do epitélio seminífero e depleção de células germinativas, e alteração na expressão das proteínas Rasd1, MEK1/2, Bcl-2 e Bax no testículo, relacionadas com proliferação e apoptose (Ma et al., 2017). Esses efeitos parecem resultar de interferência na ação da testosterona e di-hidrotestosterona, o que pode causar redução da função das células de Sertoli e malformações do epidídimo e da genitália externa (Foster et al., 2001; Ferrara et al., 2006; Scott et al., 2008; Ma et al., 2017).

Por outro lado, a AA exerce clastogenicidade em diferentes orgãos de ratos e camundongos, incluindo o testículo, efeito adverso que aparentemente depende da dose e do protocolo de exposição (Adler et al., 2000; Gamboa da Costa et al., 2003; Manière et al., 2005). Injeções únicas i.p. de 50mg/kg em camundongos BALB/c e C3H/HENMTV aumentaram em até 380 vezes a formação de adutos de DNA no pulmão, fígado e rim (Gamboa da Costa et al., 2003), enquanto exposição oral de ratos a 18 ou 54 mg/kg de acrilamida induziu a formação de adutos de DNA no cérebro, fígado e nos testículos (Manière et al., 2005). Além disso, ratos e camundongos expostos cronicamente por 8 semanas a 5-8 e 7-14 mg/kg/dia dessa substância apresentaram supressão da espermatogênese e redução da reserva de espermatozóides na cauda do epidídimo, respectivamente (Wang et al., 2010).

1.3 Métodos de avaliação do dano testicular

Assim, tanto substâncias tóxicas para o testículo como a criptorquidia podem causar alterações morfológicas e quantitativas em células germinativas, comprometendo a função testicular (Lanning et al., 2002). Em mamíferos, as células germinativas em diferentes fases de diferenciação são organizadas no epitélio dos túbulos seminíferos em associações que definem os estágios do ciclo espermatogênico. Embora alterações estágio-específicas e a quantificação dos estágios sejam recomendadas para análise do processo espermatogênico e amplamente utilizadas em estudos experimentais (Bartlett et al., 1990; Jhonston et al., 2008; Nudmamud-Thanoi et al., 2012), essas análises podem auxiliar avaliações qualitativas, no reconhecimento de alterações comuns, como depleção de células germinativas, retenção de espermátides, células germinativas multinucleadas ou apoptóticas e exfoliação celular para o lúmen dos túbulos seminíferos (Lanning et al., 2002). A maioria dos sistemas de classificação de danos histológicos disponíveis na literatura consideram apenas a presença de células germinativas ou apenas alterações morfológicas ocorrendo no interior dos túbulos

seminíferos. Por exemplo, em Elshaari et al. (2012) foram avaliados 10 túbulos seminíferos para cada testículo de ratos Sprague-Dawley submetidos a modelo de torção-detorção utilizando-se uma pontuação que varia de 1 a 10, sendo que túbulos sem células evidentes pontuaram 1 e túbulos com espermatogênese completa e presença de espermatozóides maduros pontuaram 10. Todos os resultados abaixo de 10 foram considerados evidências de comprometimento da espermatogênese.

Além das alterações morfológicas, as expressões de algumas proteínas importantes para o processo espermatogênico podem estar desreguladas em testículos comprometidos. A imunohistoquímica permite determinar a distribuição tecidual de antígenos de interesse, visíveis ao microscópio óptico devido a aplicação de cromógenos (Duraiyan et al., 2012). Os resultados podem ser avaliados por métodos quali-quantitativos que se baseiam na localização, intensidade, distribuição e extensão da coloração imunohistoquímica (Fedchenko & Reifenrath, 2014). Vigueras-Villaseñor et al. (2015) avaliaram a imunorreatividade dos antígenos POU5F1, c-Kit, PLAP, AP2γ, SALL4 em testículos de pacientes com criptorquidia abdominal ou inguinal (uni ou bilateral) pela contagem de 30 túbulos seminíferos positivos/total de túbulos; a análise foi realizada utilizando uma escala semi-quantitativa que pontua de 0 a 3, onde 0 é ausência completa de imunorreatividade e 3 é imunorreatividade em mais de 20 túbulos seminíferos. Em particular, o receptor da tirosina quinase Kit (c-Kit) é essencial nos processos de migração, proliferação e sobrevivência celular, e a fosfatase alcalina placentária (PLAP) está relacionada a sobrevivência e pluripotência celular (Vigueras-Villaseñor et al., 2015; Zhang et al., 2014). Dessa forma, tais antígenos são comumente utilizados como marcadores no diagnóstico de tumores testiculares. Maior imunorreatividade para todas as proteínas avaliadas foi observada em pacientes com criptorquidia abdominal e, portanto, sugere-se que as células testiculares desses indivíduos são mais suscetíveis a malignidade.

Outra ferramenta comum na avaliação dos danos testiculares é a morfometria, método quantitativo utilizado em biologia para auxiliar interpretações funcionais de um órgão ou sistema (Campos-Junior et al., 2013). Particularmente nos testículos, alterações do número, do tamanho, e da estrutura dos túbulos seminíferos apresentam relação positiva com a atividade espermatogênica (Noorafshan et al., 2014). Por isso, parâmetros morfométricos como diâmetro, área e comprimento tubular, altura do epitélio germinativo, volume dos testículos, além de mensurações de tamanho e número de grupamentos celulares como células de Sertoli e de Leydig são utilizados na análise da função testicular em diferentes condições experimentais (Rajpurkar & Dhabuwala, 2000; Adamkovicova et al., 2014; Ma et al., 2016).

Embora a histopatologia qualitativa seja um componente essencial na avaliação da toxicidade testicular, o sistema visual humano é pouco sensível para detectar alterações sutis quantitativas, especialmente no que se refere ao número de células (Boyce et al., 2010). Portanto, no caso das células de Leydig, a quantificação morfométrica é indicada e tem sido realizada em vários estudos. Souza et al. (2016) utilizaram o software Image-Pro Plus® na avaliação do número de células de Leydig por testículo de ratos Wistar adultos, estimado a partir do volume individual de células de Leydig e o volume total ocupado por esse tipo celular no parênquima testicular. O resultado foi dividido pelo peso gonadal para estimar o número de células de Leydig por grama de testículo, o que permite comparações entre diferentes espécies e tratamentos. Além desses parâmetros, o volume ocupado por essas células no testículo, a área individual média das células de Leydig, e o índice Leydigossomático também podem ser utilizados na avaliação da função testicular. De acordo com o exposto, todas as ferramentas metodológicas citadas permitem caracterizar o dano testicular sob diversas condições experimentais. A utilização de um sistema de análise adequado é fundamental para manter a precisão e acuracidade na obtenção dos dados.

1.4 Novo modelo de dano testicular no rato

Há na literatura científica diferentes estudos experimentais que objetivam investigar isoladamente os efeitos da criptorquidia ou da exposição à AA e DBP. Nosso grupo de pesquisa, TOXICAM, estabeleceu um modelo de dano testicular no rato que associou exposição *in utero* e pós-natal à AA ou DBP e criptorquidia experimental no momento do desmame (DPN 21). A criptorquidia foi induzida cirurgicamente, pela retirada dos testículos do escroto e sua fixação na parede abdominal, pela túnica albugínea. Para verificar o potencial de recuperação da gônada masculina, orquidopexia foi realizada pela retirada da sutura de fixação do testículo ao abdômen e sua realocação no escroto. Os procedimentos cirúrgicos estão apresentados na Figura 2-I.

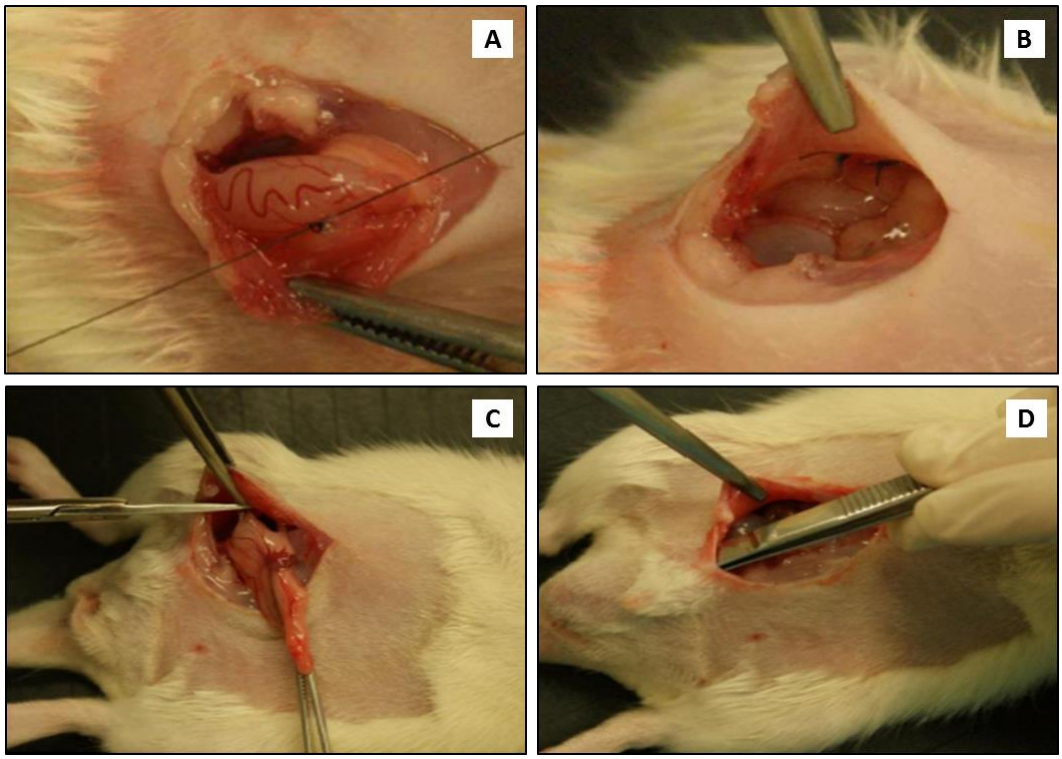

Figura 2-I: (A, B) Indução cirúrgica da criptorquidia e (C, D) orquidopexia.

A exposição à substâncias capazes de transformar células germinativas, associada à sensibilização testicular pela criptorquidia experimental, constitui ferramenta efetiva para indução de danos nestas células, favorecendo estudos sobre modos e mecanismos de agressão aos testículos por agentes variados. Deste modo, a avaliação morfológica dos testículos, o perfil imunohistoquímico das células germinativas e a análise quantitativa das células de Leydig podem fornecer informações importantes sobre o estado dos testículos de animais submetidos ao modelo experimental. No presente estudo, procuramos por alterações na expressão das proteínas c-Kit e PLAP e danos na morfologia testicular com foco nas células germinativas, além de alterações morfométricas nas células de Leydig que podem refletir possível desregulação da esteroidogênese, processo fundamental para o desenvolvimento e manutenção do sistema reprodutor masculino.

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2. OBJETIVOS

2.1 Geral

Avaliar o comprometimento de células testiculares germinativas e de Leydig em ratos submetidos à criptorquidia cirúgica pós-natal como forma de sensibilização testicular aos xenobióticos AA e DBP administrados *in utero* e no período pós-natal.

2.2 Específicos

- Verificar alterações histológicas no testículo dos animais submetidos ao modelo estabelecido utilizando sistema de avaliação semi-quantitativo.
- Verificar alterações no epitélio germinativo testicular desses animais pela análise imunohistoquímica dos marcadores de células germinativas indiferenciadas c-Kit e PLAP.
- Caracterizar quantitativamente as células de Leydig: quantidade, tamanho médio (área) individual, volume total ocupado no testículo e o índice Leydigossomático.

Capítulo II

Methods to evaluate rat testicular damage: *in utero*/postnatal exposures to xenobiotics and surgically-established cryptorchidism*

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Evaluation of testicular chemical and surgical-induced damages

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KEYWORDS

Rat, testis, cryptorchidism, acrylamide, di-n-butyl-phthalate.

ABSTRACT

Cryptorchid testes may be susceptible to environmental chemicals. The current study aimed to analyze morphologically testicular alterations induced in rats by combination of exposure to acrylamide (AA) or to di-n-butyl-phthalate (DBP) and surgical cryptorchidism/orchiopexy. For this purpose, the testes were evaluated for seminiferous tubules alterations by a semiquantitative morphological scoring system, by the immunohistochemical occurrence of the antigens c-Kit and PLAP and by morphometric analysis of the Leydig cells. Female Sprague-Dawley rats were exposed by gavage to AA 10 mg/kg/day or DBP 500 mg/kg/day during gestation and through diet during lactation. At the 21st postnatal day, male pups were submitted to surgical bilateral cryptorchidism and after 3 weeks to orchiopexy (CPT-R), but continued under exposure to xenobiotics by diet until they were 16, 31 or 58-week-old. Decrease in the body weight was seen only in the AA/CPT-R group at the 16th week, but all treated groups presented decreased absolute testicular weights and volumes. Control animals showed minimal score of histological alterations, while increased scores were seen in AA/CPT-R and DBP/CPT-R groups. A significant predominance of tubules with c-Kit strongly labeled cells was found in the AA/CPT-R group at the 31st and 58th weeks after birth, while at the same moments a reduction in the immunoreactivity for PLAP was observed. A significantly decreased immunoreactivity for PLAP was also found in the DBP/CPT-R group at 58th week. The total volume occupied by Leydig cells (TVOLC) decreased in both chemically-exposed groups at the 16th week, while the Leydig-somatic index (TVOLC/b.w.) significantly decreased only in the DBP/CPT-R group. The number of Leydig cells/ml was increased in animals of the DBP/CPT-R group exposed by 16th, 31st and 58th weeks, and in the AA/CPT-R group only in the 31st and 58th weeks. In conclusion, the applied methods were individually able to identify the compromising of the spermatogenesis being this experimental model useful to evaluate testicular alterations induced by xenobiotics.

1. INTRODUCTION

Modifications in testicular normal morphology may be associated with functional alterations, thus, histological evaluation is widely used for detect adverse effects in the testes; accordingly, several methods of structural analysis have been applied (Hayes, 2007; Noorafshan et al., 2014). In humans, the Johnsen method considers only cell types occurring within the germinative epithelium to evaluate infertility (Johnsen, 1970). In experimental studies, histomorphological investigation has been used to evaluate testicular damage (Hasanin et al., 2017; Hashish, 2015); Creasy (1997) proposed an approach focused on morphological changes of stage-specific spermatogenic cycle.

In combination to histological analysis, detection of immunohistochemical expressions of antigens by somatic and germ cells are commonly used to evaluate human and rodent damaged testes (Vigueras-Villaseñor et al., 2015). The tyrosine-protein kinase Kit (c-Kit) and placental-like alkaline phosphatase (PLAP), which are widely used as testicular germ cells tumor markers in humans, have poorly been explored in the evaluation of rat testicular toxicity by xenobiotics. The c-Kit protein is a cell membrane receptor in different tissues, where it phosphorylates tyrosine after interaction with its ligand *stem cell factor* (SCF) (Paul and Mukhopadhyay, 2004; Zhang et al., 2013). This is an important mechanism regulating migration, proliferation and cell survival processes in gametogenesis (Liang et al., 2013; Figueira et al., 2014; Zhang et al., 2014). On the other hand, the biological function of PLAP, a membrane-bound phosphotyrosine phosphatase, in spermatogenesis remains unclear. It has been proposed that PLAP participates in processes related to survival, pluripotency and other undifferentiated cell characteristics of gonocytes (Milose et al., 2012; Vigueras-Villaseñor et al., 2015).

Furthermore, histomorphometric methods allow quantitative comparisons of testicular changes intensities and their relationships with testicular function (Tenorio et al., 2010; Campos-Junior et al., 2013; Noorafshan, 2014). Morphometric evaluation of the testes was

applied in different experimental conditions. For example, testes of Wistar rats exposed to an electromagnetic field of 60Hz and 1mT from the 13th day of gestation to the 21st postnatal day, were evaluated by parameters such as testicular volume, seminiferous epithelial volume, diameter and area of the seminiferous tubule and height of the germinal epithelium. The results indicated a delay in testicular development in exposed animals (Tenorio et al., 2010). The same parameters were also appropriate to detect adverse effects after 5 weeks of exposure of Wistar rats to 100mg/kg i.p. of cyclophosphamide (Camargo et al., 2006).

Experimental exposures to xenobiotics, particularly acrylamide (AA) or to di-n-butyl-phthalate (DBP), have induced testicular disorders (Foster et al., 2016; Bonde et al., 2017). When exposed to 10 mg/kg b.w i.p. of AA for 4 or 6 weeks, adult male albino rats presented histological and ultrastructural testicular damage such as germ cell degeneration and spermatogenesis arrest (Hasanin et al., 2017). *In utero* exposure of Wistar rats to 750 mg/kg/day DBP, mainly during the masculinization programming window (MPW; embryonic days 15.5-18.5), induces extensive and severe dysgenetic areas in the seminiferous tubules, i.e., congenital abnormal development of the gonads characterized by ectopic Sertoli and germ cells (Lara et al., 2017).

On the other hand, cryptorchidism is a condition where the testicular microenvironment is modified and the susceptibility of the testes to exogenous chemicals may be increased. In an experimental model established by our research group, TOXICAM, which associated cryptorchidism and exposure to AA or DBP, impairment of spermatogenesis and of Sertoli cells were registered.

Taking these observations into consideration, and the availability of testes of rats exposed to two different chemicals and surgically-made cryptorchid the present study was developed to characterize the testicular lesions according different methodological approaches. The current study describes morphologically and morphometrically testicular

alterations induced in Sprague-Dawley rats by the association of testicular chemical toxicants and cryptorchidism.

2. MATERIAL AND METHODS

2.1 General

All analyses of current study (approved by the local Committee for Ethics in Animal Experimentation, Protocol No. 1209/2017) were performed in the testes of male offspring generated from matrices obtained from the Multidisciplinary Center of Biological Investigations (CEMIB UNICAMP, Campinas, SP, Brazil). Female Sprague-Dawley rats were exposed daily by gavage during gestational days (GD) 12 to 21 to 10 mg/kg b.w/day of acrylamide 99% pure (AA; CAS 79-06-1; Sigma-Aldrich cat. A9099) or to 500 mg/kg b.w/day of di-n-butyl-phthalate 99% pure (DBP; CAS 84-74-2; Sigma-Aldrich, cat. 524980). After birth, dietary concentrations corresponding to those doses were provided to the dams (AA, 120 ppm; DBP, 6000 ppm), the pups being potentially exposed by maternal milk.

After weaning, at the 21st postnatal day (PND), male pups were allocated to their respective groups and submitted to the surgical procedures for cryptorchidism and, after 3 weeks, to orchiopexy (CPT-R), but remained under exposure through diet to the chemicals until they were 16, 31 or 58-week-old. These groups were designated AA/CPT-R or DBP/CPT-R (Figure 1-II). The surgical procedures were described in details elsewhere (Cardoso et al., 2017). Briefly, the rats were anesthetized, and through an abdominal midline incision both testes were translocated from the scrotum into the abdominal cavity through the inguinal rings. Care was taken to avoid twisting of the spermatic cord, which could lead to testicular damage. The testes were fixed to the inner dorsolateral abdominal wall; after closure of the abdomen, the animals were kept at 30°C for 30 minutes to minimize the deleterious effects of hypothermia induced by anesthesia. For orchiopexy, the anesthetic, analgesic and asepsis procedures were the same as for the cryptorchidism surgery. At 6th week of age, the

sutures which held both testes fixed in the dorsolateral abdominal wall were carefully removed. The testes were gently guided into the scrotum and sutured through the tunica albuginea to the inner wall of the scrotum. All animals received antibiotic (enrofloxacin 5 mg/kg/day s.c.) during the three days following orchiopexy (Cardoso et al., 2016). Control animals received 1 ml of corn oil by gavage during the gestational period and xenobiotic-free basal diet during postnatal life in all periods of study. They were sham-operated; only the skin and muscle layers of the abdomen were opened and then sutured under the same anesthesia, analgesia and aseptic conditions as described above. At the end of the experiment, the animals were anesthetized between 8:00 and 10:00 a.m. with ketamine (30 mg/kg i.p.) and xylazine (4 mg/kg i.p.) and euthanized by exsanguination via heart puncture. The testes were immediately removed, weighed, fixed in modified Davidson's fixative for 24h (Latendresse et al., 2002) and then embedded in paraffin blocks.

2.2 Histologic examination

Histological sections of 5 µm thicknesses obtained from paraffin blocks were processed for hematoxylin and eosin (H&E) staining. Histological analyses were performed according to a system score established by our laboratory which was modified from the Johnsen score (Cardoso et al., 2017). The whole section of each testis was analyzed and all round seminiferous tubules were classified in one of the four grades as follows: Class 1, tubules showing normal spermatogenesis according to Creasy (1997); Class2, tubules with spermatids and sperm mature cells, but also showing alterations such as epithelial vacuoles, apoptotic bodies and/or giant germ cells; Class3, tubules with the same elements of epithelial damage as Class 2 but presenting only spermatocytes and spermatogonias; and Class 4, Sertoli cells-only tubules (SCO). The incidence (%) of tubules in each class per animal was calculated by multiplying the number of tubules in that class times 100 and dividing by the total number of tubules counted in the testis section. Then, the score for each

group was generated by adding the number of tubules in class 1 (1x), the number of tubules in class 2 (2x), the number of tubules in class 3 (3x), and the number of tubules in class 4 (4x), and dividing by the total number of tubules counted in the respective section.

2.3 c-Kit and PLAP immunohistochemistry

Histological sections of 4 μm thickness were mounted on silanized slides (Sigma Chemical Corporation, Saint Louis, MO, USA) and subjected to immunohistochemical staining for detection of the antigens c-Kit (Bioss Inc., Woburn, MA, EUA, rabbit pAb, bs-0672R) or PLAP (LSBioSciences Inc., Seattle, WA, EUA, rabbit mAb, LS-C189575), according to specific protocol for each marker.

After deparaffinization, rehydration and antigen retrieval in Trilogy solution® (Cell Marque Corp., Rocklin, CA, EUA), the endogenous peroxidase specifically and proteins in general were blocked in the tissue sections with Bloxall® (Vector Corp., Burlingame, CA, EUA) and by Sniper (Biocare Medical, LLC, CA, EUA) in the case of c-Kit and by 3% H_2O_2(Sigma, St. Louis, MO) and horse serum (Vector Corp., Burlingame, CA, USA) in the case of PLAP, respectively. The sections were then incubated overnight at 4°C with each of the primary antibodies diluted at 1:100. For c-Kit signal amplification was performed by biotin-free polymer detection system using the MACH 4® universal HRP-Polymer kit (Biocare Medical, Pike Lane Concord, CA, USA) according to the manufacturer's instructions. Lastly, 3-3' diaminobenzidine (DAB Substrate kit, Vector Laboratories, Burlingame, CA, USA) was used as the chromogenic substrate and the sections were counterstained with Harris hematoxylin.

Histological samples of human classical seminoma were used as positive controls for immunohistochemistry. For negative controls, the same material was processed without application of the respective primary antibodies. c-Kit and PLAP immunoexpression were qualitative and quantitatively assessed by the location of positive germ cells within the

seminiferous epithelium and the number of tubules with positive cells/total seminiferous tubules.

2.4 Morphometric analysis of Leydig cells

For correct morphometric evaluation it is necessary to standardize all steps that comprise this process: image capture, storage, lighting and magnification. c-Kit immunostained slides were used to facilitate the visualization of Leydig cells delimitations and, thus, to assist in obtaining the morphometric data (Noorafshan, 2014). The slides were digitalized using Pannoramic MIDI (3DHISTECH Ltd.) hardware; the images were captured by Pannoramic Viewer software (1.15.4 version) at 400x magnification and saved in BMP format. All morphometric analyses were performed in ImageJ software (1.48v version). To determine the volume occupied by Leydig cells within the testes a reticulated grid with 504 points of intersection was allocated randomly over fifteen histological fields of the testis and the number of intersections on the Leydig cells were counted using the ImageJ "point or multi-point" tool; the number of points was divided by the total number of points generated by the reticulated grid (7560) and multiplied by the total testicular volume (Tenorio et al., 2010; Noorafshan, 2014), which was assumed to be equal to its net weight since the density of the testis was assumed to be 1.0. In order to obtain the total net testicular volume, 6.5% (albuginea relative weight) was subtracted from the gross testicular weight (Silva et al., 2006; Tenorio et al., 2010).

The somatic index of Leydig cells (LSI) was calculated according to the formula $LSI = \frac{TVOLC}{BW}$x100, where TVOLC corresponds to the total volume occupied by Leydig cells in the testis and BW is the body weight of the animal (Souza et al., 2016). From the number of Leydig cells (NLC) in 15 histological fields (μm²), the average number of these cells per ml (NLC/ml) was estimated according to the formula $NLC/ml = 10^{10} xCells/$

μm^2[1,5]. The result was multiplied by the testicular volume of each animal to determine the number of Leydig cells/testis.

Further, the average area occupied by an individual Leydig cell was obtained by evaluating 30 cells/animal. After calibration between the software and the image of the histological section, each Leydig cell was delimited with the aid of the "polygon" tool; the average area of these 30 cells (Tenorio et al., 2010) was considered the average area of the individual Leydig cells in each animal.

All results were stored in XLS (Microsoft Excel) format until the statistical analysis.

2.5 Statistical analysis

Since the experimental groups were composed by more than one pup from the same litter, both the pups and dams were included in the statistical analyses in order to eliminate the "litter effect"; accordingly, the study experimental units were the rats, adjusted by their dams. Variables were analyzed by a mixed effect generalized linear model followed by a post-hoc Sidak correction (Norman and Streiner, 2008) and presented as mean ± standard deviation or as median (p25-p75) depending on the distribution pattern of the data. All analyses were performed with the IBM SPSS 22.0 Statistics software (Statistical Package for Social Science; SPSS Incorporation). The level of significance was considered as $p<0.05$.

3. RESULTS

3.1 Body and testicular weights

At the end of the study, a decreased body weight was seen only in the AA/CPT-R group at 16th week. However, the absolute testicular weight and testicular volume (net weight of the testes), were decreased in all treatment groups when compared to the control ($p<0.05$); AA/CPT-R animals showed a significant decrease in all periods of study, despite the slight recovery in the 31stweek, whereas a progressive reduction was seen in the DBP/CPT-R

animals. In control animals, these parameters remained similar throughout the experiment (Table 1-II).

3.2 Histology of the seminiferous tubules

Histologically, control groups presented testes with normal tubules and complete spermatogenesis (Figure 2-II). Treated rat testes presented tubules with severe histologic alterations which include seminiferous tubules with multinucleated giant cells, vacuolated Sertoli cells (Figure 2-II – AA/CPT-R, 16 weeks) and germ cell exfoliation (Figure 2-II – AA/CPT-R, 31 weeks). Moreover, some testes also showed "apparently" Leydig cells hyperplasia (Figure 2-II – AA/CPT-R, 58 weeks), interstitial edema (Figure 2-II – DBP/CPT-R, 16 weeks), tubular atrophy with or without SCO pattern (Figure 2-II – DBP/CPT-R, 31 weeks and Figure 3-II) and intraluminal calcification (Figure 2-II – DBP/CPT-R, 58 weeks). In the AA/CPT-R group, some isolated and enlarged putative germ cells showed hyperchromatic nuclei and pleomorphic cytoplasm (Figure 2-II – AA/CPT-R, 16 weeks). Frequently, the altered tubules occurred among apparently normal tubules.

The histologic examinations allowed classifying the testicular alterations into four classes according to the scoring system described in Materials and Methods. Non-chemically treated sham-operated controls presented minimal histological score (Table 2-II). In the AA/CPT-R groups the histological scores increased equally in the 16^{th} and 31^{st} weeks, progressing in the 58^{th} week; DBP/CPT-R groups showed a progressive increase in the histological scores during the study ($p<0.05$) (Table 2-II).

3.3 c-Kit and PLAP immunoexpression

In the control groups germ cells, since basal spermatogonias until primary spermatocytes, c-Kit and PLAP were weakly stained. AA/CPT-R and DBP/CPT-R animals also presented stained germ cells occurring diffusely in the germinative epithelium (c-Kit, Figure 3-II; PLAP, Figure 4-II).

Since spermatogenesis was severely compromised in this study, determination only of the number of positive cells for the evaluated markers is not representative of the ongoing process. By estimating the ratio of positive tubules/total seminiferous tubules we determine the immunoreactivity (%) of the germ cells for these markers. In c-Kit evaluation, although all experimental groups had increased immunoreactivity relatively to control, significant predominance of strongly labeled cells was found only in the AA/CPT-R group at the 31st and 58th weeks (12.5% and 32.1%, respectively) (Table 3-II). On the other hand, a decreased immunoreactivity was observed for PLAP in most experimental groups when compared with respective controls; statistically differences were observed in the AA/CPT-R group at the 31st and 58th weeks (66.9% and 23.8%, respectively) and in the DBP/CPT-R group at the 58th week (6.1%) (Table 4-II).

3.4 Morphometry of Leydig cells

Both AA/CPT-R and DBP/CPT-R groups presented a decrease in the total volume occupied by Leydig cells at the 16th week. In the same moment, only animals of the DBP/CPT-R group had decreased Leydig-somatic index and increased number of Leydig cells/ml. The number of Leydig cells/ml also increased in AA/CPT-R and DBP/CPT-R groups at the 31st and 58th weeks ($p<0.05$). There was no statistical difference in the number of Leydig cells/testis and individual area of these cells (Table 5-II).

4. DISCUSSION

This study aimed to compare morphologically rat testicular alterations induced by *in utero*/postnatally exposures to xenobiotics, DBP or AA, associated with surgically-established cryptorchidism and orchiopexy.

According to the final body weights, the association of surgical procedures and chemical exposures did not negatively influence the growth of the animals. In contrast to the present study, depressed body weights have been reported in rats following oral exposure to

acrylamide. For example, Sprague-Dawley male rats with 21th day-old exposed to 5 or 10 mg/kg/day of acrylamide by drink water for 8 consecutive weeks, presented decreased body weight compared to their respective control (ATSDR, 2012; Wang et al., 2010). In this study, only animals of 16 week in the AA/CPT-R group presented a decreased body weight, being this reduction probably related to systemic toxicity of the acrylamide. In the presence of systemic toxicity in mammals, is it common to see reduction in body weight and/or the presence of clinical signs of toxicity, effects on survival, as well as decreased weight and histopathological changes in gonads (ECETOC, 2016).

All experimental groups presented significant decrease of testes weights and testicular volumes, besides morphological testicular changes in different degrees of intensity, such as germ cells exfoliation, multinucleated germ cells, vacuolization of the germinative epithelium and tubules with SCO pattern. Previous study with an experimental cryptorchid rat model showed recover of the testicular weight and structure after performing orchiopexy 4 weeks after birth (Mizuno et al., 2008). This indicates that, in the present study, exposures to DBP or to AA negatively interfered with the male gonads development since the beneficial effects of orchiopexy were not fully observed. Since Sertoli cells support the normal spermatogenesis process, providing nutrients and blood products to facilitate the progression of imature germ cells to spermatozoa, the occurrence of morphological alterations of the germinative epithelium may be due to Sertoli cell malfunction (Hutchison et al., 2008), and could be the basis for the testicular alterations seen in our study.

The histological classification developed by our laboratory (Cardoso et al. 2017) allowed refining the observed testicular changes. The most evident histologic alterations occurred in DBP/CPT-R animals, which also presented early damages compared with AA/CPT-R animals. At the 16th week, most tubules of the DBP/CPT-R group were concentrated in Class 2, with a histological score of 2.54. At the 31st week, most tubules were Class 4 followed by Class 2, with an average score of 3.54 that remained similar at the 58th

week (3.63), but with the most tubules class 4 followed by class 3. Similarly, increased score was observed in AA/CPT-R animals only in the last moment of study (58th week; 3.48).

Morphological alterations as a result of the individual exposure to AA or DBP have been documented (Lebda et al., 2014; Van den Driesche et al., 2012). However, there are no studies about the association between these chemicals exposure and experimental cryptorchidism/orchiopexy. In the present study, although considerable testicular changes were seen in both experimental groups, higher damages were observed with shorter exposure time in the DBP/CPT-R group. This difference may be related to the main mode of action of these substances in the male reproductive tract: while DBP exerts an anti-androgenic effect in rats, AA is clastogenic for rodent germ cells (Scott et al., 2007; Pourentezari et al., 2014).

Histopathological examination is a sensitive method to identify the effects of toxicants on the testis (Hess and Moore, 1993). However, the accuracy of the assessment method applied is pivotal to maintain this sensitivity. Our four-class system, based on the predominant germ cell type within the tubules according to their differentiation stage, and their eventual alterations (Cardoso et al., 2017), was useful for detecting histological alterations induced by our experimental model since it allowed to detect variance between groups and experimental moments.

Immunohistochemistry allows determining tissue distribution of an antigen of interest and may provide clues about cells differentiation and functional status. It is widely used as a complementary method for the conventional histopathological analysis with hematoxylin-eosin staining (Duraiyan et al., 2012; Kabiraj et al., 2015). Our evaluations showed increased immunoreactivity for c-Kit in all experimental animals compared to the control, but significant difference was found only in the AA/CPT-R group at the 31st and 58th weeks. For PLAP, significant decrease in the immunoreactivity was observed in the AA/CPT-R group at 31st and 58th weeks and in the DBP/CPT-R group at 58th week. In humans, cryptorchidism is a defined risk factor associated with testicular germ cell tumors (TGCT) and the influence of

environmental agents also have been investigated. It is expected that TGCT originate from an embryonic germ cell blocked in its maturation process, initially generating carcinoma *in situ*. In these lesions, testes express some proteins characteristic of embryonic cells, including c-Kit and PLAP (Elzinga-Tinke et al., 2015); overexpression of c-Kit protein is related with a malignant phenotype in several tissues (Barbaei et al., 2016), whereas PLAP immunostaining is variable and may be absent depending on the type of testicular neoplasm (Manivel et al., 1987; Bahrami et al., 2007). Literature data about expression of these proteins in healthy or damaged postnatal rat testes are controversial. In this study, although no neoplastic changes were found, there was a deregulation in the immunoexpression of both evaluated proteins, especially for PLAP in the AA/CPT-R group at the 31st week. Accordingly, our immunohistochemical findings suggest functional damage occurring in the germ cells, since those proteins are required for normal spermatogenesis. The immunoreactivity (%) for c-Kit and PLAP was quantified by counting of tubules with positive cells/total seminiferous tubules. Although most studies determine the number of positively labeled cells, this information would not represent the actual testicular state, as depletion of germ cells occurred in many seminiferous tubules of the exposed animals. There are no studies that relate exposure to testicular toxicants with changes in the number of seminiferous tubules per section. Therefore, the immunohistochemical method of analysis applied was adequate to detect functional alterations induced by our model of testicular damage in the rat. The progressively increased expression of c-Kit and decreased expression of PLAP as the experiment grew longer might be indicating cellular indifferentiation and, we speculated that, in the persistence of testicular aggression, may be possible progression to pre-neoplastic or neoplastic alteration. This occurred earlier and was more expressive in AA-exposed animals.

In addition to damaging germ cells, *in utero* and postnatal exposure to testicular toxicants may also compromises Leydig cells (Guo et al., 2013; Ivell et al., 2003). Thus, morphometry was performed to better describe the Leydig cells alterations. Body weight and

testicular volume data were used to support morphometric parameters of the Leydig cells. Reduction in the volume occupied by Leydig cells in the testis was observed only in animals exposed to AA/CPT-R and DBP/CPT-R until 16 weeks of age. When the volume occupied by Leydig cells in the testis was related to body mass, i.e., Leydig-somatic index, a significative decrease was seen only in DBP/CPT-R animals with 16weeks-old. The number of Leydig cells/ml increased in DBP/CPT-R animals at 16 weeks and in DBP/CPT-R and AA/CPT-R animals at 31 and 58 weeks. In the same way, exposure of Sprague-Dawley rats to 500 mg/kg and 750 mg/kg of di- (2-ethylhexyl) phthalate (DEHP) for 30 days led to an increase in the number of Leydig cells and reduction in the testosterone levels, that was associated with abnormal biosynthesis or hormonal clearance (Ha et al., 2016). However, increase in the individual sectional area and reduction in the volume occupied by Leydig cells in the testis was observed after *in utero* and postnatal exposure of Wistar rats at low frequency of electromagnetic field (60Hz and 1mT), maintaining plasma levels of testosterone (Tenorio et al., 2010).

Morphometric analysis has often been used to aid functional interpretations of different organs (Campos-Junior et al., 2013). Particularly in the testes, the cell number or size is positively related to spermatogenic activity (Noorafshan et al., 2014). In the case of Leydig cells, changes in cell structure may be related to their steroidogenic function (França et al., 2005; Tenorio et al., 2010). This is important because the development and maintenance of the male reproductive system depend on the synthesis of androgen hormones, mainly testosterone by Leydig cells. Therefore, those morphometric changes observed in this study suggests an attempt to maintain homeostasis in response to disruption on steroidogenesis affecting the spermatogenesis process. To confirm this possible impairment in the steroidogenesis, hormonal dosage is required.

In summary, the histologic and morphometric analyses indicate that AA/CPT-R and DBP/CPT-R have similar testicular toxicity, but altered immunoexpression of c-Kit and

PLAP showed slightly greater functional damage in germ cells of the AA/CPT-R groups. This difference was observed as early as in the 31st week of age and, therefore, it is not necessary to extend the experimental model until the 58th week of age to establish severe alterations of testes. Finally, all methods of testicular evaluation were individually able to identify impairment of spermatogenesis. However, performing analyzes with different methodological tools were crucial to properly characterize the testicular damage.

5. CONFLICT OF INTEREST STATEMENT

The employment affiliation of each author is shown on the cover page. All the authors were associated with academic institutions when the paper was prepared. The authors have no conflicts of interest to declare.

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9. FIGURES

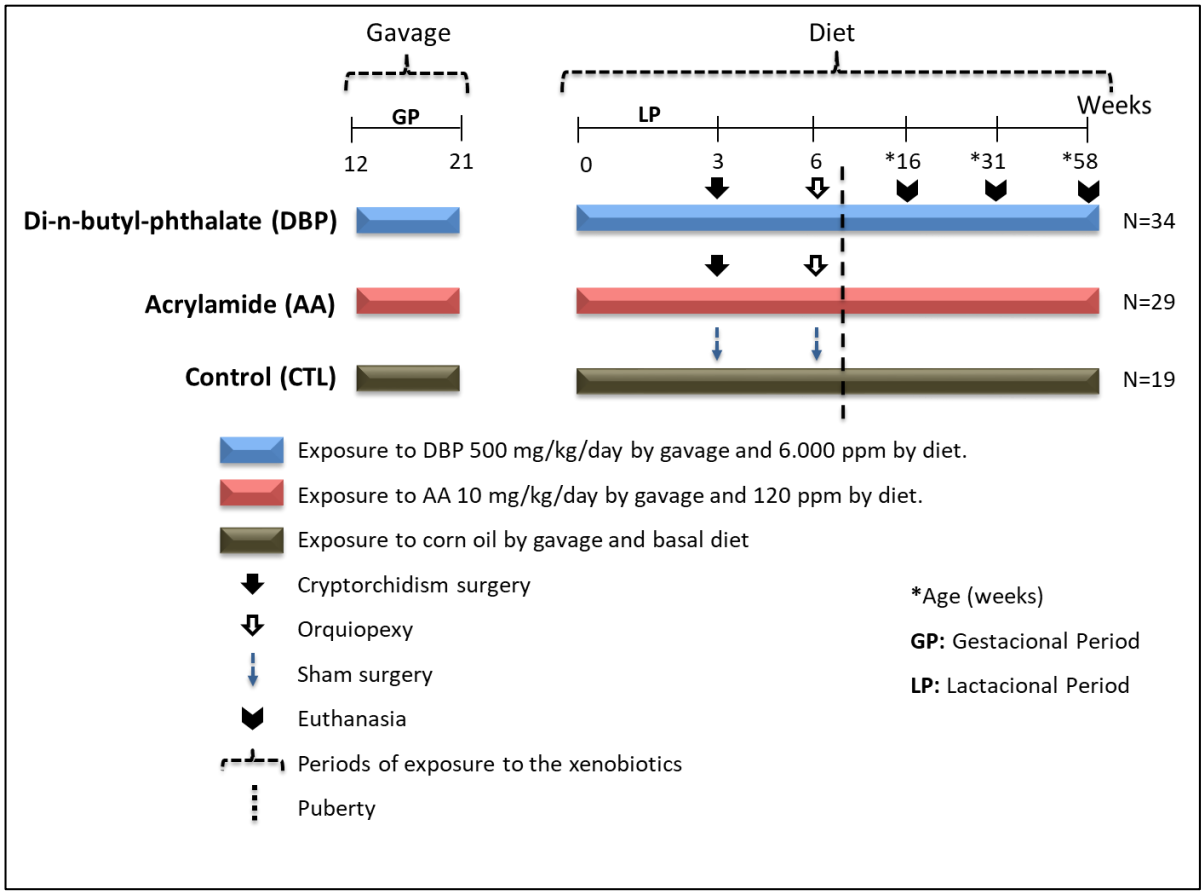

Figure 1-II: Experimental design of the present study.

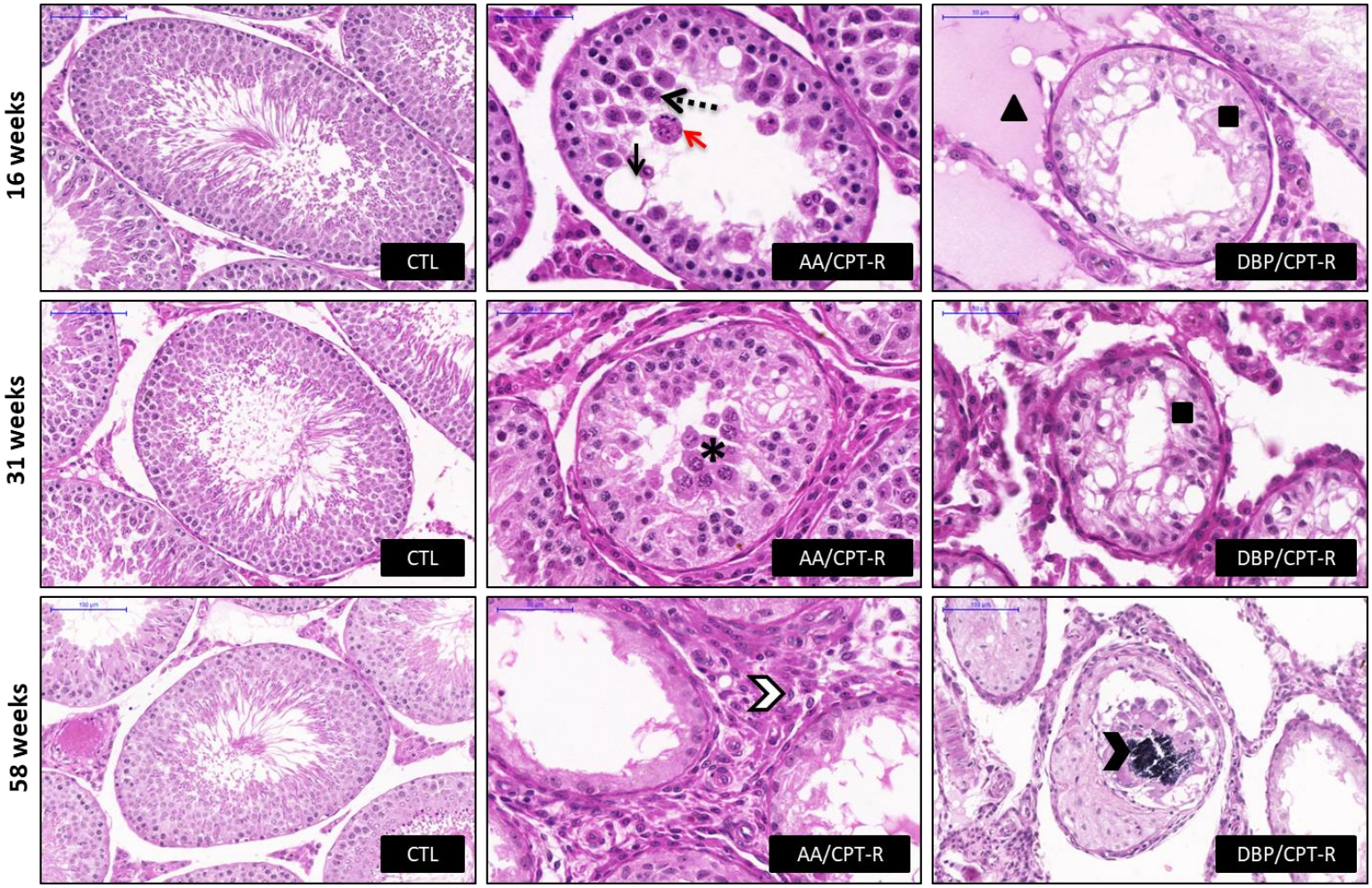

Figure 2-II: Testes of Sprague-Dawley rats (H&E). **(CTL)** 100 µm **-** Control groups submitted to sham surgeries showing preserved spermatogenesis. Treated groups with different histopathological changes: **(AA/CPT-R)** 50 µm – Vacuolization (black arrow), multinucleated germ cells (red arrow), hyperchromatic nuclei and pleomorphic cytoplasm (dotted arrow), germ cells exfoliation (*) and "apparent" Leydig cell hyperplasia (white arrowhead). **(DBP/CPT-R)** 50 µm – Interstitial edema (triangle), tubules with "Sertoli cells only" (square) and, 100 µm - calcification (black arrowhead).

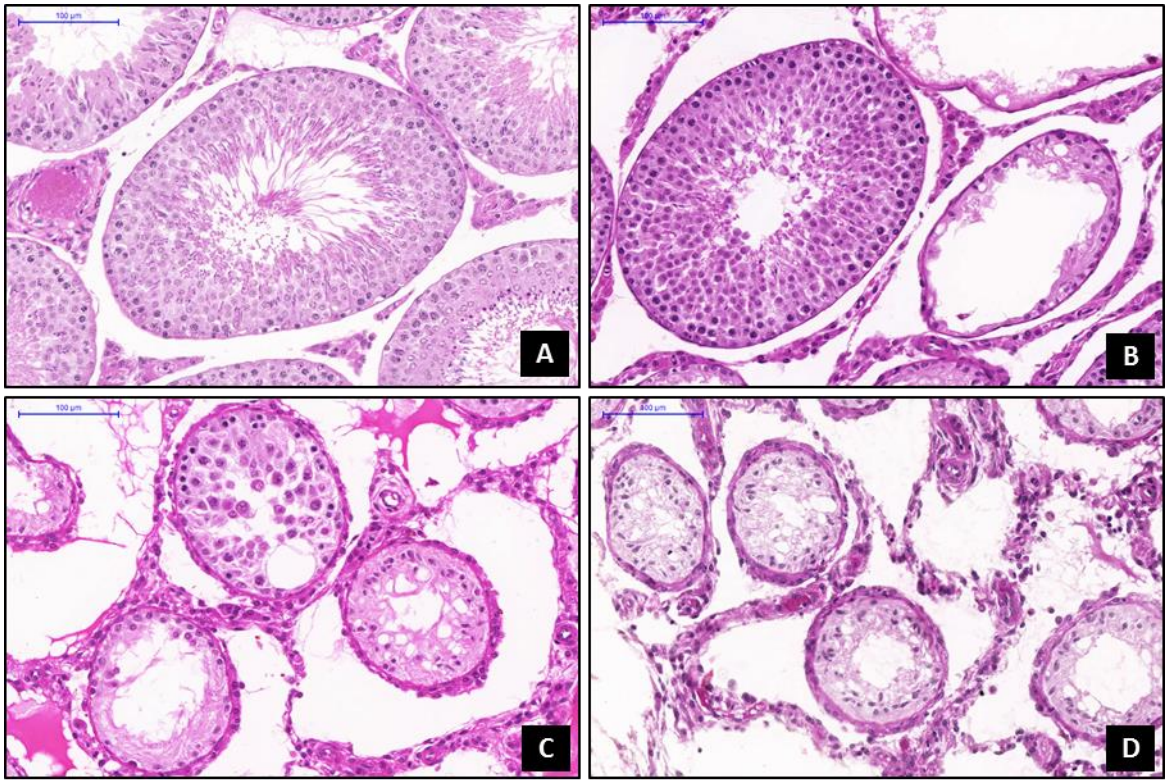

Figure 3-II: Testis of Sprague-Dawley rats (H&E, 100µm). **(A)** Control - normal seminiferous tubules with complete spermatogenesis. Exposure to acrylamide until 16 **(B)**, 31 **(C)** and 58 **(D)** weeks-old - tubular atrophy with spermatogenesis arrest.

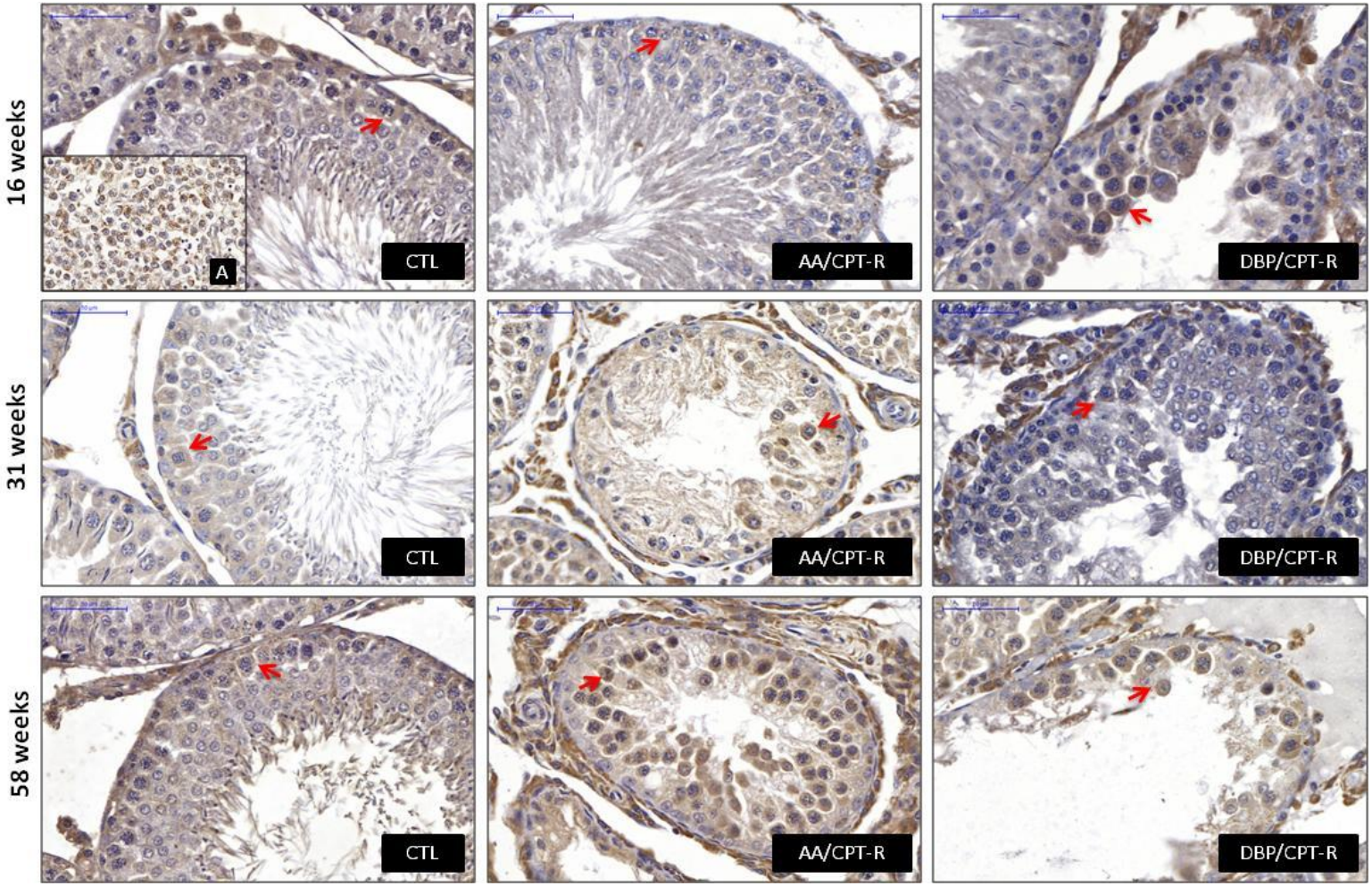

Figure 4-II: Immunohistochemistry staining for c-Kit (red arrows). **(A, insert)** -Human classical seminoma, positive control of the reaction; **(CTL)** 50 µm - Control Sprague-Dawley rat, spermatogonias with weak positive cytoplasmic staining; **(AA/CPT-R and DBP/CPT-R)** 50 µm -Sprague-Dawley rats submitted to cryptorchidism/orchiopexy and to AA or DBP exposures: Positive cytoplasmic staining in germ cells, with a predominance of strongly labeled cells in the AA/CPT-R group at 31th and 58th weeks.

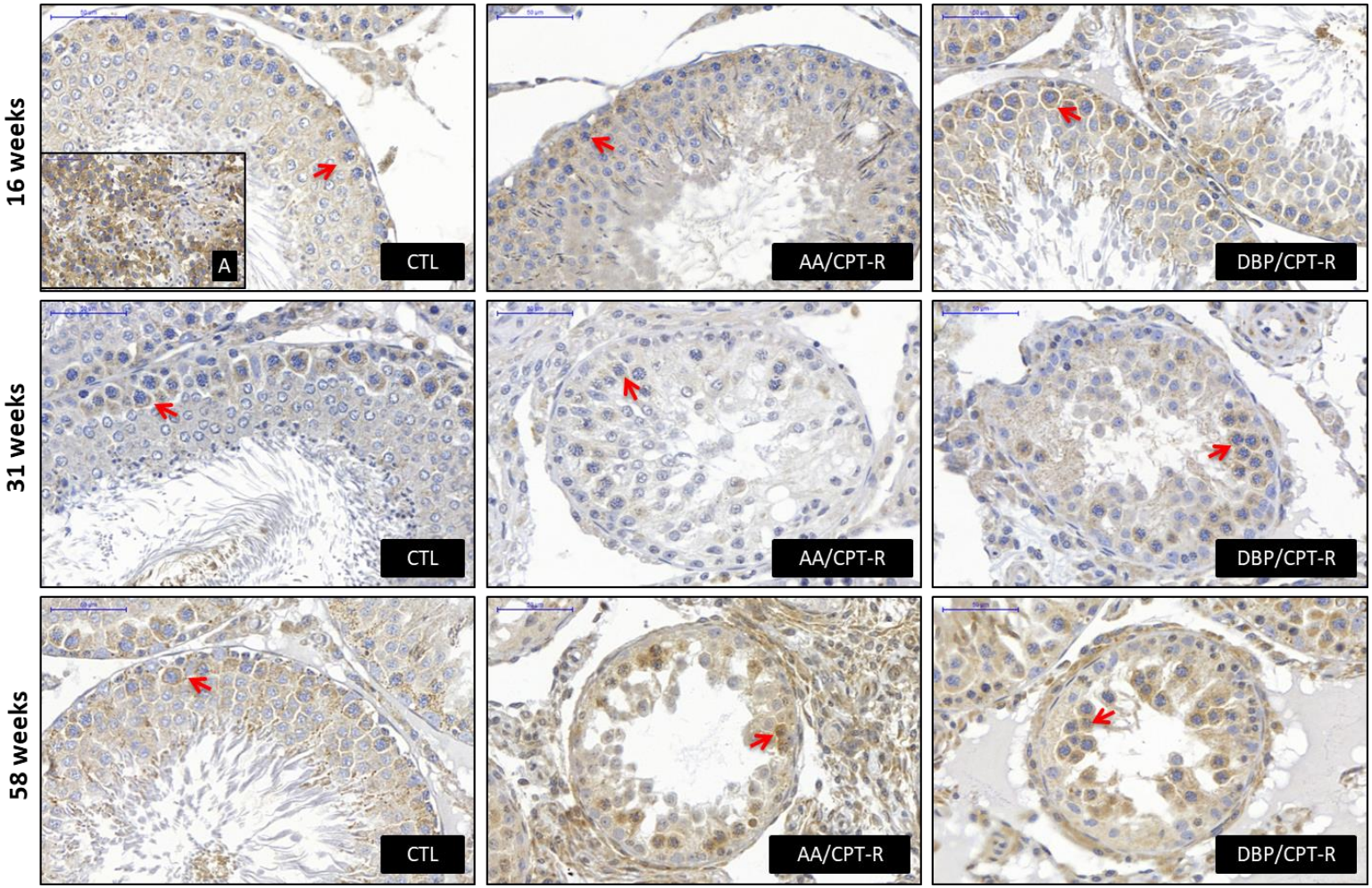

Figure 5-II: Immunohistochemistry staining for PLAP (red arrows). **(A, insert)** -Human classical seminoma, positive control of the reaction; **(CTL)** 50 µm - Control Sprague-Dawley rat with spermatogonias with slight cytoplasmic staining; **(AA/CPT-R** and **DBP/CPT-R)** 50 µm -Sprague-Dawley rats submitted to cryptorchidism/orchiopexy and to AA or DBP exposures: Positive cytoplasmic stainings in germ cells, with a gross decrease in the number of labeled tubules in the AA/CPT-R group at 31th week.

10. TABLES

Table 1. Body and absolute testicular weights of rats exposed in utero and postnatal to xenobiotics **(AA or DBP)** and submitted to cryptorchidism and orchiopexy **(CPT-R)**.

Groups		Body weight (g)[a]	Absolute testis weight (g)[a,b]	Testicular volume (ml)[b]
16 weeks	**CTL (n)**	455.8 ± 30.4 (6)	1.7 ± 0.1 (6)	1.6 (1.5-1.7) (6)
	AA/CPT-R (n)	**375.7 ± 25.9*** (11)	**0.7 ± 0.3*** (9)	**0.7 (0.6-0.8)*** (6)
	DBP/CPT-R (n)	449.2 ± 21.5 (10)	**0.8 ± 0.3*** (9)	**0.8 (0.7-0.8)*** (6)
31 weeks	**CTL (n)**	536.4 ±37.5 (7)	1.8 ± 0.1 (7)	1.7 (1.6-1.7) (7)
	AA/CPT-R (n)	526.3 ± 33.2 (9)	**1.0 ± 0.4*** (7)	**0.9 (0.5-0.9)*** (5)
	DBP/CPT-R (n)	519.3 ± 41.7 (10)	**0.7 ± 0.2*** (8)	**0.7 (0.6-0.9)*** (7)
58 weeks	**CTL (n)**	553.8 ± 48.6 (6)	1.7 (1.6-1.7) (6)	1.6 (1.5-1.6) (6)
	AA/CPT-R (n)	496.7 ± 64.7 (8)	**0.7 (0.7-0.8)*** (5)	**0.6 (0.6-0.7)*** (5)
	DBP/CPT-R (n)	579.5 ± 91.2 (14)	**0.6 (0.5-1.3)*** (7)	**0.8 (0.5-1.2)*** (5)

[a]Values are expressed as mean ± SD or [b]median (p25-p75); generalized linear mixed-effects model with Gama distribution followed by post-hoc Sidak correction. n: number of animals/group. Testicular volume was calculated only for c-Kit immunostained testes that were submitted to morphometric analyses. *Different from control (p<0.05).

Table 2. Histologic classification[a] of the testes of rats exposed in utero and postnatal to xenobiotics **(AA or DBP)** and submitted to cryptorchidism and orchiopexy **(CPT-R)**.

	Score[b]		
Groups	**16 weeks**	**31 weeks**	**58 weeks**
CTL **(n)**	1.02 (1.01-1.02) (5)	1.03 (1.02-1.03) (6)	1.03 (1.03-1.06) (6)
AA/CPT-R **(n)**	**2.55 (2.26-3,28)*** (10)	**2.55 (1.81-3.33)*** (10)	**3.48 (3.11-3.60)*** (9)
DBP/CPT-R **(n)**	**2.54 (2.29-2.94)*** (10)	**3.54 (2.77-3.97)*** (10)	**3.63 (2.31-3.90)*** (14)

[a]Class 1: Tubules containing spermatid and sperm; Class 2: Tubules with spermatid and sperm with vacuoles, apoptotic bodies and giant cells; Class 3: Tubules showing spermatocytes and spermatogonia with vacuoles, apoptotic bodies and giant cells; Class 4: Tubules with Sertoli cells only. [b]Values are expressed as median (p25-p75); generalized linear mixed-effects model with Gama distribution followed by post-hoc Sidak correction. n: number of animals/group. *Different from control (p<0.05).

Table 3. c-Kit immunoexpression in the testes of rats exposed in utero and postnatally to acrylamide **(AA)**or to di-n-butyl-phthalate **(DBP)**, also submitted to cryptorchidism and orchiopexy **(CPT-R)**.

	Immunoreactivity (%)[a]		
Groups	**16 weeks**	**31 weeks**	**58 weeks**
CTL	29.4 ± 16.0	74.7 ± 6.1	58.5 ± 22.9
(n)	(6)	(7)	(6)
AA/CPT-R	49.2 ± 21.5	**87.2 ± 10.6***	**90.6 ± 6.7***
(n)	(6)	**(7)**	**(6)**
DBP/CPT-R	40.6 ± 21.4	80.5 ± 11.2	75.0 ± 9.4
(n)	(6)	(5)	(5)

[a]Values are expressed as mean of tubules with positive cells/total seminiferous tubules ± SD; generalized linear mixed-effects model with Gama distribution followed by post-hoc Sidak correction. n: number of animals/group.*Different from control (p<0.05).

Table 4. PLAP immunoexpression in the testes of rats exposed in utero and postnatally to acrylamide **(AA)** or to di-n-butyl-phthalate **(DBP)**, also submitted to cryptorchidism and orchiopexy **(CPT-R)**.

	Immunoreactivity (%)[a]		
Groups	**16 weeks**	**31 weeks**	**58 weeks**
CTL	92.3 ± 3.6	94.9 ± 4.6	99.3 ± 1.1
(n)	(4)	(4)	(4)
AA/CPT-R	79.2 ± 29.4	**28.0 ± 33.2***	**75.5 ± 22.4***
(n)	(8)	**(8)**	**(6)**
DBP/CPT-R	94.2 ± 2.2	93.7 ± 4.9	**93.2 ± 4.8***
(n)	(7)	(6)	**(8)**

[a]Values are expressed as mean of tubules with positive cells/total seminiferous tubules ± SD; generalized linear mixed-effects model with Gama distribution followed by post-hoc Sidak correction. n: number of animals/group.*Different from control (p<0.05).

Table 5. Morphometric parameters of Leydig cells of rats exposed in utero and postnatally to acrylamide **(AA)** or to di-n-butyl-phthalate **(DBP)**, also submitted to cryptorchidism and orchiopexy **(CPT-R)**.

Groups		Volume occupied by Leydig cells (µl)[b]	Leydig cell somatic index (LSI) (%)[b]	Number of Leydig cells/ml ($x10^5$)[b]	Number of Leydig cells/testis ($x10^5$)[b]	Individual Leydig cell area (µm²)[a]
16 weeks	**CTL (n)**	143.4 (135.1-149.3) (6)	30.5 (28.4-35.5) (6)	2.8 (2.7-2.8) (6)	4.2 (3.9-4.5) (6)	94.7 ± 8.0 (6)
	AA/CPT-R (n)	**88.9 (76.8-119.1)*** (6)	24.2 (21.1-31.6) (6)	4.0 (2.6-8.0) (6)	2.6 (2.5-4.9) (6)	77.1 ± 2.0 (6)
	DBP/CPT-R (n)	**95.4 (82.2-111.9)*** (6)	**20.9 (17.9-24.8)*** (6)	**4.7 (3.5-6.7)*** (6)	3.3 (3.2-4.3) (6)	86.8 ± 3.9 (6)
31 weeks	**CTL (n)**	122.9 (96.6-136.4) (7)	23.7 (18.7-24.6) (7)	1.9 (1.2-2.3) (7)	3.2 (2.1-3.8) (7)	83.2 ± 7.5 (7)
	AA/CPT-R (n)	109.4 (93.4-133.8) (5)	21.3 (17.9-24.2) (5)	**5.9 (3.9-7.9)*** (7)	4.8 (3.8-4.9) (5)	67.4 ± 9.7 (7)
	DBP/CPT-R (n)	115.4 (92.9-129.5) (7)	19.3 (17.9-28.3) (7)	**6.5 (3.0-1.1)*** (7)	4.6 (2.8-5.9) (7)	66.2 ± 10.4 (7)
58 weeks	**CTL (n)**	120.4 (111.6-130.8) (6)	21.9 (20.4-23.5) (6)	2.1 (1.8-2.5) (6)	3.3 (2.8-3.5) (6)	72.7 ± 7.7 (5)
	AA/CPT-R (n)	130.0 (73.9-157.2) (5)	22.4 (15.2-31.2) (5)	**9.4 (5.3-1.3)*** (6)	5.7 (2.2-6.8) (5)	65.2 ± 9.9 (6)
	DBP/CPT-R (n)	116.0 (102.6-149.5) (5)	19.5 (18.8-21.6) (5)	**5.5 (3.1-1.4)*** (6)	3.6 (3.2-7.4) (5)	61.3 ± 5.6 (6)

[a]Values are expressed as mean ± SD or [b]median (p25-p75); generalized linear mixed-effects model with Gama distribution followed by post-hoc Sidak correction. n: number of animal/group; LSI: volume occupied by Leydig cells in the testis related to body mass. *Different from control (p <0.05).

Conclusão

Nossos resultados sugerem que a associação exposição *in utero* e pós-natal à AA ou DBP e criptorquidia/orquidopexia com eutanásia na 31ª semana de idade constitui um bom modelo para estabelecer danos testiculares. Embora, como todo modelo, não traduza as condições naturais que ocorrem em homens, esse protocolo experimental pode ser útil para o estudo e compreensão dos fenômenos envolvidos no aparecimento de alterações testiculares neoplásicas ou pré-neoplásicas. No presente estudo, os métodos de avaliação histológica, imunohistoquímica e morfométrica utilizados foram capazes de detectar o comprometimento da morfologia testicular e da espermatogênese.

Anexos

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

unesp

FMB

Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO Nº 1209/2017-CEUA

Certificamos que a proposta intitulada **"Células germinativas e células de LEYDIG em modelo de dano testicular no rato"**, registrada com o n. 1209/2017, sob a responsabilidade da Thania Rios Rossi Lima, orientada pelo Prof. Titular João Lauro Vianna de Camargo, coorientada pela Dra. Merielen Garcia Nascimento, Nathalia Pereira de Souza e Samuel Cohen – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n. 11.794, de 8 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião de 23/02/17.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	24/03/2018
Espécie/Linhagem/Raça	Ratos Sprague Dawley
Nº de animais	84
Peso/Idade	até 700 gramas/até 58 semanas
Sexo	Macho/Fêmea
Origem	Biotério do CEMIB - UNICAMP

Prof. Dr. Guilherme Antonio Moreira de Barros
Presidente da CEUA

Kleber M. Camargo
Kleber Messias de Camargo
Secretário da CEUA

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Prof. João Lauro Viana de Camargo

CPF 513.355.736-87

Distrito Rubião Jr, s/n - RUBIÃO JUNIOR

BOTUCATU - SP

Atestado de Saúde Animal

Atestamos que os Ratos (30 machos e 50 fêmeas) da linhagem **NTacUnib:SD,** provenientes da Divisão de Produção de Animais S.P.F. (Specific Pathogen Free) deste Centro, pertencem à categoria sanitária S.P.F. e apresentam-se isentos dos agentes patogênicos pesquisados pelo laboratório de controle de qualidade sanitária. Informamos que os mesmos encontram-se livres de outros agentes infecciosos capazes de causarem riscos à saúde humana. A validade deste Atestado consta da data dos últimos testes do programa de monitorização sanitária, rotineiramente realizados pelo Laboratório de Controle de Qualidade Animal - C.Q.S.(*).

Observação - O estado sanitário dos animais retirados do CEMIB nesta data será mantido se os mesmos forem acondicionados em equipamento adequado e o mesmo não for violado durante o transporte. A Instituição receptora deverá oferecer infra-estrutura e condições adequadas para a manutenção de animais da Categoria Sanitária livres de agentes patogênicos especificados (S.P.F.), alojando os animais em equipamentos e/ou salas dotadas de sistema de barreiras de proteção sanitária. Torna-se necessário manejo correto e a esterilização de todo material utilizado na rotina como: rações, maravalha/cama, bebedouros, água, gaiolas, tampas, e outros.

(*) Data dos últimos testes de monitorização sanitária realizados em Abril de 2012.

Campinas, 04 de junho de 2012.

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