UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

Nathália Pereira de Souza

Criptorquidia e exposição *in utero* ao di(n-butil)-ftalato e à acrilamida – avaliação morfológica do dano testicular

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

Orientador: Prof. Dr. João Lauro Viana de Camargo

Coorientadora: Dra. Merielen Garcia Nascimento e Pontes

Botucatu
2015

Nathália Pereira de Souza

Criptorquidia e exposição *in utero* ao di(n-butil)-ftalato e à acrilamida – avaliação morfológica do dano testicular

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

Orientador: Prof. Dr. João Lauro Viana de Camargo

Coorientadora: Dra. Merielen Garcia Nascimento e Pontes

Botucatu
2015

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP

BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Souza, Nathalia Pereira de.
Criptorquidia e exposição *in utero* ao di(n-butil)-ftalato e à acrilamida : avaliação morfológica do dano testicular / Nathalia Pereira de Souza. - Botucatu, 2015

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
Orientador: João Lauro Viana de Camargo
Coorientador: Merielen Garcia Nascimento e Pontes
Capes: 40105008

1. Aparelho genital masculino. 2. Testículos - Doenças. 3. Toxicologia. 4. Acrilamida. 5. Expressão gênica. 6. Espermatogênese.

Palavras-chave: acrilamida; criptorquidia; di(n-butil) ftalato; orquidopexia; rato.

Dedicatória

"Nascestes no lar de que precisavas.
Vestiste o corpo físico que merecias.
Moras no melhor lugar que Deus poderia te proporcionar, de acordo com o teu adiantamento.
Possuis os recursos financeiros coerentes com as tuas necessidades; nem mais nem menos, mas o justo para as tuas lutas terrenas.
Teu ambiente de trabalho é o que elegeste espontaneamente para a tua realização.
Teus parentes e amigos são almas que atraístes com tuas próprias afinidades.
Portanto, teu destino está constantemente sob teu controle.
Tu escolhes, recolhes, eleges, atrais, buscas, expulsas, modificas tudo aquilo que te rodeia a existência.
Teus pensamentos e vontades são a chave de teus atos e atitudes, são as fontes de atração e de repulsão na tua jornada vivencial.
Não reclames nem te faças de vítima. Antes de tudo, analisa e observa.
A mudança está em tuas mãos."
(Hammed)

Aos meus pais, Magda e Joel, pelo esforço e compreensão dispensados à minha formação.

A minha irmã Andréia, por fazer parte da minha vida e por aguentar minha frequente variação de humor.

Agradecimentos Especiais

Ao Prof. Dr. João Lauro Viana de Camargo, o meu mais sincero agradecimento pela oportunidade, disponibilidade e pelos conhecimentos transmitidos.

À Dra. Merielen Garcia Nascimento e Pontes, pelo empenho, pela paciência e credibilidade. Obrigada por estar sempre presente!

Agradecimentos

A Deus, pela oportunidade de realização deste trabalho.

Aos familiares que de alguma forma me incentivaram, em especial minhas tias Mara, Marta e Mariliza que torcem muito pelo meu sucesso. À minha vó Ercina que está sempre presente e aos meus avós, Santo e Lourdes, que mesmo no céu cuidam de mim.

À Universidade estadual Paulista, que me acolhe e me oferece estrutura desde à graduação.

Ao Conselho Nacional de Desenvolvimento Científico e tecnológico, CNPq, pela concessão da bolsa de mestrado.

À Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, pelo suporte financeiroo que permitiu a realização deste trabalho. Processo nº 2012/09873-4.

À Cristina, Vânia, Paulo e Paulo César (PC) que me auxiliaram durante todo esse período e se tornaram amigos.

Aos docentes e funcionários do Departamento de Patologia, pela convivência e apoio.

À Ligia, pelo convívio diário, incentivo e carinho, pelos momentos de desabafos, distração e gordices, pela amizade e confiança!

À Ana Paula, pelo agradável convívio, por fazer eu me sentir parte de sua adorada família, pelas sugestões e broncas essenciais para meu aprendizado e principalmente pela amizade.

À Viviane, pelos conselhos, pela atenção com que sempre me ouve, pelas risadas, por sempre se preocupar e por dividir comigo o título de quem fica mais doente no laboratório! Obrigada pela amizade.

À Rafaela que com seu jeito atrapalhado conquista o coração de todos ao seu redor. Obrigada por ter se tornado minha amiga!

À Gabi, por estar sempre disposta a me ajudar, pelo agradável convívio dentro e fora do laboratório e pela amizade.

À Thânia e ao Osvaldo pelo auxílio e pela companhia.

À Thálitta, por ouvir meus desabafos, pelos incentivos e conselhos. Tenho um carinho imenso por você.

À Marina (Perdiz), pela disponibilidade e torcida. Obrigada!

À Amanda, que sendo minha amiga desde sempre, foi fundamental para que eu chegasse até aqui. Nem a distância é capaz de diminuir nossa amizade. Obrigada por estar sempre comigo e ser minha irmã de coração!

Às amigas de Marília: Anelise, Raísa, Jéssica, Beatriz, Camila e Milena, pela torcida e pelos momentos de descontração. Obrigada, meninas!

À Jacutinga, por não me abandonar mesmo morando distante. Obrigada pelas conversas, pelo apoio e torcida!

Finalmente, a todos que de alguma forma contribuíram durante o desenvolvimento do meu mestrado.

Resumo

A Síndrome de Disgenesia Testicular (SDT) é caracterizada por baixa qualidade espermática, criptorquidia, hipospadia e tumores testiculares, sendo que essas condições podem aparecer isoladas ou variavelmente combinadas. Estudos recentes têm associado o aumento da incidência desta síndrome à exposição a agentes químicos exógenos e que sua origem esteja relacionada com distúrbios da esteroidogênese *in utero*. Modelos experimentais para o estudo da SDT devem utilizar substâncias tóxicas para células testiculares, como os ftalatos (DBP) e a acrilamida (ACR). Ainda, a criptorquidia, um componente da SDT, pode ser induzida experimentalmente e ser utilizada como ferramenta no estudo da patogênese desta síndrome. Exposição ao DBP, à ACR e a criptorquidia, quando estudados isoladamente, alteram o microambiente das células testiculares. Entretanto, os efeitos da combinação desta anomalia congênita com a exposição a tóxicos testiculares ainda não foram explorados. Este estudo teve como objetivo avaliar as alterações morfológicas e de imunoexpressão do fator de transcrição de ligação ao octâmero ¾ (OCT¾) e do receptor de hormônio luteinizante (LHR) nos testículos de ratos submetidos a um modelo de dano testicular que associa exposição química (DBP ou AA) e tratamentos cirúrgicos (criptorquidia/orquidopexia). Exposição *in utero* e pós-natal ao DBP e à AA associada à criptorquidia resultou em prejuízo da espermatogênese e aumentou a expressão de OCT¾ em células germinativas. A imunoexpressão de LHR foi qualitativamente reduzida no grupo expostos ao DBP e submetidos à criptorquidia. Exposição ao DBP ou à AA não alterou a distância anogenital. Todos os grupos experimentais apresentaram diminuição no peso relativo do epidídimo. O peso relativo da próstata ventral foi diminuído em animais criptorquídicos e o peso relativo do fígado diminuiu em animais expostos ao DBP e submetidos à criptorquidia. A orquidopexia realizada três semanas após criptorquidia foi eficaz para restabelecer a estrutura dos testículos e dos pesos dos órgãos reprodutivos. Nossos resultados indicam que o modelo utilizado, que associa exposição química a intervenções cirúrgicas, pode ser útil para entender a SDT. Alterações da diferenciação de células germinativas e de imunoexpressão sugerem semelhanças com células transformadas relacionadas ao câncer testicular humano.

Abstract

The testicular dysgenesis syndrome (TDS) encompasses conditions such reduced semen quality, hypospadia, cryptorchidism and testicular germ cell tumors (TGCT), which may occur isolated or in association. The influence of environmental factors on TDS occurrence has become increasingly evident, special attention being paid to exogenous chemicals such phthalates and acrylamide (AA), well known testicular toxicants. Experimentally induced cryptorchidism can be an useful experimental tool to understand TDS pathogenesis. It has been reported that the testicular microenvironment is critically modified when rodents are experimentally exposed to dibutyl-phthalate (DBP), to AA or to cryptorchidism. However, the combined influence of cryptorchidism and testicular toxicants has not been explored. This study aimed to evaluate the morphological changes and the immunoexpression of the transcription factor binding octamer ¾ (OCT¾) and the luteinizing hormone receptor (LHR) in the testes of rats submitted to a model of testicular damage that associated chemicals (DBP or AA) and surgical (cryptorchidism/orchidopexy) treatments. *In utero* and postnatal DBP or AA exposures associated with surgically-established cryptorchidism induced disruption of spermatogenesis and increased the expression of OCT¾ in germ cells. Immunoreactivity of LHR was qualitatively decreased in the cryptorchid group also exposed to DBP. Exposures to DBP or AA did not alter the anogenital distance. All experimental groups showed decreased in the epididymis weights. Relative ventral prostate weights was decreased in cryptorchid animals and relative liver weights was decreased in DBP-exposed animals. The orchidopexy performed 3 weeks after cryptorchidism was effective to reestablish fairly the testes structure and reproductive organs weights. Our results indicate that the model used, which associated chemical exposures to surgical interventions, may be useful to understand TDS pathogenesis. Alterations of germ cell differentiation and immunoexpression suggest similarities with transformed cells related to human testicular cancer.

Sumário

Sumário

Capítulo I

Revisão da Literatura

Epidemiologia e fatores de risco para Síndrome de Disgenesia Testicular

A Síndrome de Disgenesia Testicular (SDT) humana é caracterizada por baixa qualidade espermática, criptorquidia, hipospadia e tumores testiculares, sendo que essas condições podem aparecer isoladas ou variavelmente combinadas (FIGURA 1) (Skakkebaek et al., 2001; Ulbright and Emerson, 2008). Estudos epidemiológicos sugerem que essas quatro condições possuem origem comum na vida fetal, durante o desenvolvimento gonadal, e que são indicadoras do risco uma das outras (Skakkebaek et al., 2001; Jorgensen et al., 2010). Essa síndrome resulta de disfunções de células de Leydig e/ou Sertoli durante a vida fetal, aumentando o risco de distúrbios reprodutivos (Sharpe and Skakkebaek, 2008). A SDT pode estar associada tanto a alterações cromossômicas (45,X/46,XY) quanto à exposição a agentes químicos exógenos; entretanto, o rápido aumento das incidências dessas desordens reprodutivas sugere que fatores ambientais e/ou estilo de vida são causas mais prováveis do que as alterações genéticas (FIGURA 1) (Skakkebaek et al., 2001).

Dados epidemiológicos evidenciam aumento considerável das incidências de doenças no trato reprodutor masculino, que se manifestam tanto ao nascimento quanto na idade adulta jovem. Dentre elas destacam-se o declínio da qualidade espermática em várias regiões do mundo, com aumento consequente na demanda de serviços de reprodução assistida (Andersen et al., 2000, Giwercman et al., 2011), e o aumento das incidências de criptorquidia, hipospadia e de tumores testiculares (Adami et al., 1994; Paulozzi et al., 1997; Skakkebaek et al., 2001). Nos últimos 30 anos, a incidência de tumores testiculares, dos quais 90% são de células germinativas (TTCG), passou de 4,1 para 6,6 em 100.000 habitantes no Reino Unido e nos Estados Unidos (Richiardi et al., 2007; Thayer and Foster, 2007; Gilbert et al., 2011). No Brasil, esses tumores correspondem a 5% do total de neoplasias em homens; estima-se que ocorreram 11.593 novos casos em 2009 (INCA, 2011). No mundo, a incidência e a mortalidade pelo câncer testicular dependem da região geográfica analisada, corroborando a hipótese da interferência de fatores ambientais. As maiores incidências ocorrem no Noroeste da Europa, como na

Dinamarca e na Suécia, em que a incidência desta neoplasia é de 10 casos/100 mil habitantes (Myrup et al., 2008).

Embora os fatores de risco para TTCG não sejam totalmente conhecidos, as malformações do trato genital que ocorrem na SDT estão fortemente associadas a eles. Dentre elas, destaca-se a criptorquidia, que ocorre em cerca de 10% dos casos de TTCG, sendo que pacientes criptorquídicos têm risco de desenvolvimento de TTCG de cinco a dez vezes maior do que homens sem essa condição (McKiernan et al., 1999; Ferguson and Agoulnik, 2013). Acredita-se que os TTCG em humanos originem-se de gonócitos iniciados para carcinogênese durante a vida intrauterina, gerando inicialmente a neoplasia intratubular germinativa (NITG), ou carcinoma *in situ,* e depois o TTCG (Skakkebaek et al., 2001; Thayer and Foster, 2007). Nestas lesões, as células germinativas expressam uma variedade de proteínas específicas, como o fator de transcrição de ligação ao octâmero ¾ (OCT ¾), um reconhecido marcador de seminomas humanos (Ferrara et al., 2006). O OCT ¾ é uma proteína expressa em células germinativas primordiais durante a vida fetal, nas quais exerce regulação de sua pluripotência. Sua expressão diminui gradativamente a partir da 20ª semana de gestação em humanos (Rajpert-De Meyts et al., 2004) e do 17º dia embrionário em ratos (Ferrara et al., 2006), até a ausência de sua imunoexpressão em células germinativas adultas de ambas as espécies.

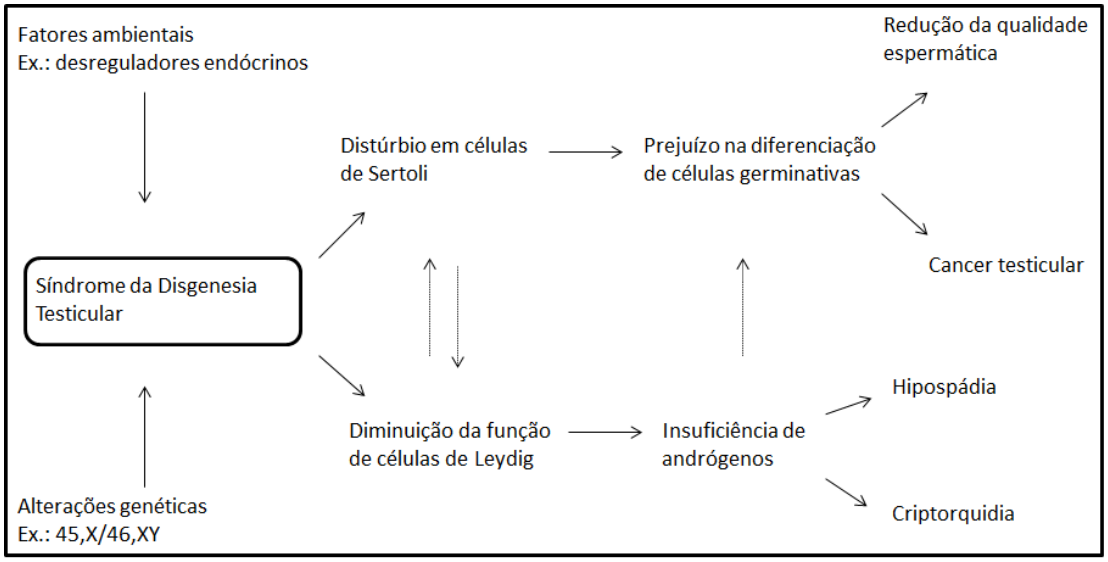

FIGURA 1. Representação esquemática das ligações patogênicas entre componentes e manifestações clínicas da Síndrome de Disgenesia Testicular (SDT). Adaptado de Skakkebaek et al., 2001.

Exposição ambiental

Atenção especial tem sido dada aos desreguladores endócrinos, definidos como substâncias ou misturas de substâncias exógenas naturais ou sintéticas que alteram órgãos e funções associadas ao sistema endócrino e, consequentemente, causam efeitos adversos ao organismo ou a seus descendentes (WHO, 2002; Wuttke et al., 2010). A maior incidência de desordens que compõem a SDT na Dinamarca, quando comparada à Finlândia, sugere que fatores ambientais locais possam contribuir para a maior frequência dessa ocorrência (Ekbom et al., 2003; Myrup et al., 2008; Sharpe and Skakkebaek, 2008; Gilbert et al., 2011).

O aumento de malformações genitais e da incidência de TTCG em filhos de trabalhadores expostos a contaminantes ambientais sugere que os gonócitos possam ser comprometidos durante a vida intra-uterina e que, após a puberdade, sofram a ação promotora de anti-andrógenos e de outros fatores ambientais, evoluindo para má qualidade espermática e/ou tumores (Skakkebaek et al., 2001; Thayer and Foster, 2007). Embora contaminantes ambientais sejam os prováveis responsáveis pelo aumento das incidências da SDT, o modo de ação pelo qual atuam não está esclarecido.

Ftalatos representam um dos grupos químicos mais produzidos no mundo (450 mil ton/ano), sendo utilizados, entre outras finalidades, para conferir

maleabilidade, transparência, durabilidade e longevidade a produtos plásticos (Crinnion, 2010). Os ftalatos são ésteres de ácido ftálico, cuja estrutura comum é o ácido diester de 1,2-benzeno-dicarboxílico. Eles são líquidos viscosos, incolores e inodoros; possuem baixa solubilidade em água, alta solubilidade em óleo e baixa volatilidade (National Research Council Committee on the Health Risks of Phthalates, 2008).

Em humanos, a relação entre exposição a ftalatos e disfunções testiculares ainda necessita de mais estudos. Entretanto, é relatado que essa exposição está associada à diminuição da distância anogenital (DAG) (Swan et al., 2005) - parâmetro pelo qual pode ser avaliado o desenvolvimento sexual masculino (Gallavan et al., 1999) - e alteração hormonal (Main et al., 2006), corroborando a hipótese de que o testículo possa ser vulnerável à exposição a ftalatos durante a fase de desenvolvimento (Main et al., 2006). Em ratos, a exposição *in utero* a ftalatos resulta em anormalidades na prole masculina, como criptorquidia, hipospadia e infertilidade (características presentes na SDT humana) (Fisher et al., 2003). Estas alterações pós-natais são precedidas por alterações das células de Leydig, como baixos níveis de testosterona testicular (Fisher et al., 2003), o que documenta o potencial antiandrogênico dos ftalatos (Crinnion, 2010; Boekelheide et al., 2009). Ainda, a disfunção da célula de Leydig fetal está diretamente associada com diminuição da DAG, uma vez que se trata de um parâmetro andrógeno-dependente. Essa redução da DAG em ratos machos foi observada após exposição *in utero* a doses de di-n-butil-ftalato (DBP) iguais ou superiores a 500 mg/kg (FIGURA 2) (Ema et al., 1998).

Em ratos Sprague-Dawley expostos *in utero* a 500 mg/kg de DBP durante a segunda metade do período gestacional, foram observados hiperplasia de células de Leydig e aumento do número de células germinativas multinucleadas (Foster et al., 2001). Esses efeitos parecem resultar de interferência do ftalato na ação da testosterona e di-hidrotestosterona, o que leva também à redução do número de células de Sertoli e a malformações do epidídimo e genitália externa (Foster et al., 2001; Scott et al., 2008).

O modo de ação dos ftalatos na célula de Leydig de ratos depende do período e da dose à qual o animal foi exposto (Ge et al., 2007). A exposição *in utero* a 500 mg/kg de DBP ou 10mg/kg de di(2-etilhexil)ftalato (DEHP) promove

agregação (*clusters*) das células de Leydig fetais, fenômeno que não ocorre em animais adultos expostos às mesmas doses (Ge et al., 2007). Ainda, uma das características da exposição aos ftalatos é o efeito bifásico na população de célula de Leydig adulta: em baixas doses (menores que 50 mg/kg), ftalatos aumentam a síntese de testosterona devido ao aumento no número de células de Leydig e/ou estimulação direta da sua produção; em altas doses (a partir de 500 mg/kg), esses químicos inibem a produção de testosterona, porém o mecanismo ainda não foi elucidado (Ge et al., 2007; Hu et al., 2009). Ftalatos mostram pouca ou limitada atividade estrogênica (Harris et al., 1997), e há um crescente consenso de que são substâncias antiandrogênicas. No entanto, ftalatos e seus metabolitos não se ligam ao receptor de andrógeno (AR) (Parks et al., 2000), indicando que não são antagonistas diretos de AR (Hu et al., 2009). Uma vez que os modelos experimentais evidenciam o potencial antiandrogênico dos ftalatos, a avaliação de enzimas relacionadas com a esteroidogênese pode ser adequada para detectar os efeitos mediados por essas substâncias nos testículos de ratos (Hu et al., 2009).

A acrilamida (ACR) é um polímero sintético com vasta gama de utilidades, como floculante em tratamento de água e selante em barragens (FIGURA 2). Também pode ocorrer espontaneamente durante o cozimento sob alta temperatura de alimentos ricos em carboidratos. A ACR foi classificada como "provavelmente cancerígeno para humano" (IARC 2A) pela Agência Internacional para Pesquisas de Câncer (IARC) em 1994 (Pelucchi et al., 2011). Possui baixo peso molecular e alta solubilidade em água, o que possibilita sua passagem pelas membranas biológicas. Poucas informações existem a respeito dos efeitos da exposição à ACR em humanos, porém ela está associada a danos no sistema nervoso e à provável carcinogenicidade de vários sítios topográficos (renal, pancreático, gástrico, mamário) (Pelucchi et al., 2011). O seu potencial genotóxico não está claro, mas há indicações de clastogenicidade aumentada em células germinativas de ratos e camundongos, efeito adverso que aparentemente depende da dose e do protocolo de exposição (Adler et al., 2000; Gamboa da Costa et al., 2003). Em camundongos C3H/E1, o teste do dominante letal mostrou aumento de implantes mortos após exposição dos machos à 125 mg/kg de ACR,

evidenciando o potencial mutagênico desta substância para as células germinativas parentais (Adler et al., 2000). Exposição oral aguda de camundongos ddY a 150mg/kg de ACR provocou danos no epitélio germinativo testicular como vacuolização, aumento no volume das espermátides redondas e exfoliação celular no lúmen um dia após o tratamento (Sakamoto et al., 1988).

Os efeitos da ACR nas células de Leydig de roedores são controversos. Ratos expostos a 10mg/kg de ACR na água de beber por 2 semanas apresentaram redução do volume total e citoplasmático de células de Leydig, sem alteração do seu número total, e redução nos níveis séricos de testosterona e aumento de LH (Camacho et al., 2012). Por outro lado, ratos expostos à mesma dose de ACR na água de beber por 8 semanas apresentaram hiperplasia de células de Leydig e aumento nos níveis séricos de testosterona (Wang et al., 2010).

Em resumo, há evidências de que a exposição *in utero* a essas substâncias, DBP e ACR, tem efeitos deltérios não só para as células germinativas testiculares, mas também para células de Leydig, de modo que elas podem ser entendidas como preferenciais para o estudo experimental da Síndrome de Disgenesia Testicular. Por outro lado, o estudo quantitativo e funcional das células de Leydig, devido sua função estratégica na produção da testosterona, pode auxiliar no melhor entendimento dos efeitos tóxicos destas substâncias no trato reprodutor masculino.

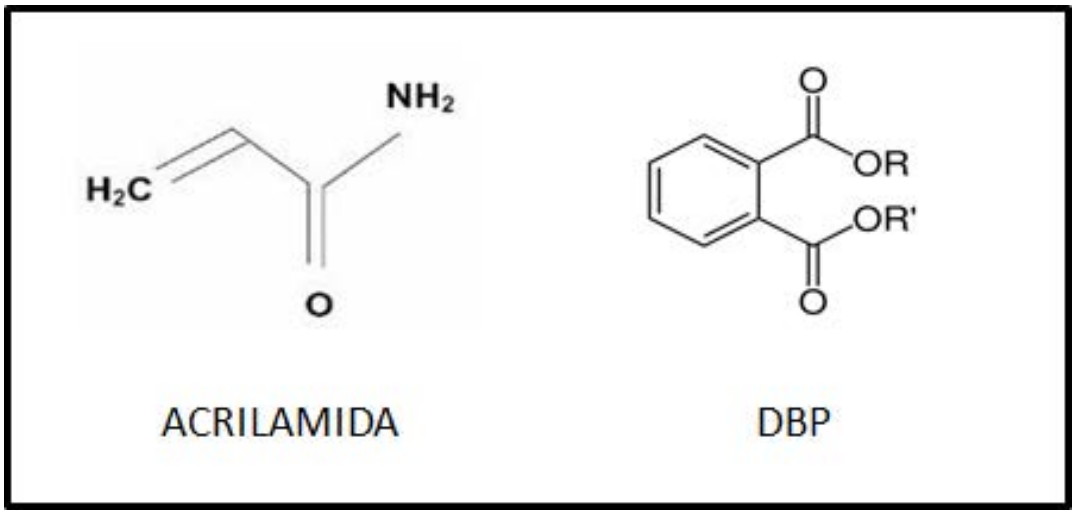

FIGURA 2. Estrutura química das substâncias acrilamida e di(n-butil) ftalato (DBP), tóxicas para células testiculares.

Criptorquidia e orquidopexia

A criptorquidia, descida incompleta do testículo para o escroto, é uma das anormalidades congênitas mais comuns (1 a 5%) em meninos recém-nascidos. A cirurgia para realojamento testicular (orquidopexia) é recomendada entre 6-18 meses de idade (Ritzen et al., 2007; Hutson et al., 2010). Há indícios do aumento da incidência desta malformação nos últimos anos (Watts et al., 2000).

A descida testicular nos homens ocorre em duas fases. A primeira, transabdominal, ocorre da 8ª à 15ª semana de gestação, sendo caracterizada pela descida dos testículos para o abdômen inferior. Essa descida é mediada pela substância anti-Mulleriana, secretada pelas células de Sertoli, e pelo Insl3, hormônio secretado pelas células de Leydig. Assim, alterações nas células de Leydig e/ou de Sertoli podem estar diretamente relacionadas com a maior incidência de criptorquidia (Hutson et al., 2010).

A segunda fase, inguinoescrotal, ocorre entre as 25ª e 35ª semanas de gestação e corresponde à descida do testículo através do canal inguinal para dentro do escroto. Esta fase é andrógeno-dependente e se relaciona com o produto de um gene relacionado à calcitonina (CGRP), um neurotransmissor fornecedor do gradiente quimiotático que guia a migração testicular (Hutson et al., 2010).

No homem, as alterações histológicas decorrentes da criptorquidia se iniciam aos dois anos de idade, sendo caracterizadas pela parada do desenvolvimento de células germinativas, espessamento e hialinização da lâmina basal, seguida de hipoplasia e atrofia dos túbulos seminíferos. Os túbulos passam a se apresentar como cordões fibro-hialinos densos, com suas lâminas basais proeminentes (Miliaras et al., 1997; Kumar et al., 2005). Em correspondência, há aumento ex-vacuo do interstício, onde as células de Leydig aparecem proeminentes, aparentemente hiperplásicas (Miliaras et al., 1997; Kumar et al., 2005). No final do processo, os testículos criptorquídicos apresentam tamanho diminuído e consistência firme, devido a fibrose e atrofia.

Criptorquidia e orquidopexia experimental

Em roedores, a criptorquidia experimental também resulta na redução da capacidade reprodutiva do animal. Nestas condições, o fluxo sanguíneo e a permeabilidade vascular se alteram, as células germinativas, particularmente espermatócitos e espermátides, morrem por apoptose, e as células de Sertoli e de Leydig sofrem alterações funcionais (Bergh and Soder, 2007). Em consequência, há interrupção do processo de maturação germinativa e diminuição do peso testicular (Rossi et al., 2005).

Tanto em humanos quanto em roedores, há indicativos de que a temperatura do testículo no escroto é menor do que no canal inguinal e no abdômen. Desse modo, as alterações histológicas no testículo criptorquídico possivelmente ocorram devido à temperatura mais elevada de seu sítio irregular (Zakaria et al., 1998). A extrapolação de dados de modelos animais de criptorquidia para humanos pode ser válida pois, apesar de algumas diferenças, o processo de descida testicular é semelhante entre mamíferos, com conservação de eventos-chave importantes (Hutson et al., 2010).

Os procedimentos para obter experimentalmente a criptorquidia são classificados em primários e secundários. Em roedores, a descida testicular ocorre entre os dias pós-natal (DPN) 18 e 21, e pode ser prevenida primariamente pela intervenção no gubernáculo ou no canal inguinal. A criptorquidia experimental secundária, mais comum, compreende o realojamento cirúrgico dos testículos, que já se acham no escroto, na cavidade abdominal. Ambos os procedimentos podem ser uni ou bilaterais (Quinn et al., 1991; Bergh and Soder, 2007). Já a orquidopexia pode ser realizada pelo corte da sutura que fixa o testículo na parede abdominal na criptorquidia experimental. O testículo é, então, realocado no escroto onde é suturado pela base para fixação (Jegou et al., 1983).

No protocolo discutido a seguir foi adotado o intervalo de 3 semanas entre os procedimentos cirúrgicos de criptorquidia e orquidopexia. Este intervalo foi selecionado para que abrangesse o período entre o surgimento das primeiras espermátides arredondadas e a maturação completa dessas células em espermátides alongadas maduras, observadas ao redor do DPN44 em ratos (Hermo et al., 2010; Russell et al., 1987). No dia DPN20, as células mais

desenvolvidas no testículo de rato são espermatócitos nas fases de paquíteno e diplóteno; após aproximadamente três semanas, quando o animal tem cerca de 44 dias de vida, já são observadas espermátides alongadas maduras margeando a luz tubular, ainda que quantitativamente de forma incompleta (Russell et al., 1987). Assim, o momento proposto para a indução cirúrgica do criptorquidismo (DPN21) corresponde a uma fase de grande atividade testicular.

A maioria dos modelos experimentais de criptorquidia existentes foi estabelecido para o estudo do impacto desta condição sobre a fertilidade. Embora a criptorquidia seja um dos componentes bem reconhecidos da SDT, os modelos de criptorquidia em roedores não têm sido adequadamente explorados para o estudo da história natural dessa síndrome, incluindo a relação dela com tóxicos testiculares. Além disso, o estabelecimento experimental de criptorquidia/orquidopexia pode ser útil para aprofundar o entendimento dos efeitos biológicos destas condições nos grupamentos celulares do testículo, dentre eles as células de Leydig.

Desenvolvimento intrauterino dos testículos, com ênfase em células de Leydig

Os testículos de humanos e de ratos possuem estrutura anatômica semelhante. Trata-se de órgão par, situado no escroto, fora da cavidade abdominal, constituído por lóbulos que contém túbulos seminíferos, local onde se originam os espermatozóides. Os lóbulos são definidos por septação fibrosa que converge para o hilo testicular; o epitélio seminífero contém as células de Sertoli e as células germinativas, estas em diferentes fases de maturação em direção à luz dos túbulos (Moore and Persaud, 2004; Chang et al., 2011; Tarulli et al., 2012; Haschek and Rousseaux, 2013). Ainda, o testículo abriga no interstício, entre os túbulos seminíferos, as células de Leydig e as células mióides peritubulares (Tarulli et al., 2012).

Em humanos, as células germinativas primordiais (CGPs) são encontradas na endoderme do alantóide por volta do 22º dia gestacional (DG22), sendo reconhecidas pela grande quantidade de fosfatase alcalina. As CGPs migram para o mesentério dorsal, fazendo com que ocorra espessamento desse epitélio, o qual passa a se chamar crista gonadal. Em

seguida, na 4,5ª semana de gestação, ocorre a invaginação da crista gonadal e subsequente formação dos cordões sexuais. O sistema genital humano permanece indiferenciado até a 9ª semana de gestação (Haseltine and Ohno, 1981; De Felici et al., 2004). Em camundongos as CGPs são primeiramente identificadas por volta do DG7 no epiblasto, próximo ao alantóide. Essas células migram para o mesonefro no DG10,5, fazendo com que ocorra espessamento desse epitélio, originando a crista gonadal. Em seguida, ocorre invaginação e formação dos cordões sexuais. Após 4 dias (DG14,5), as CGPs passam a se chamar gonócitos, por diferirem morfologicamente das primeiras (Culty, 2009; Zogbi et al., 2012). Em ratos, a formação do testículo embrionário tem início no DG10. Durante a diferenciação testicular, CGP e células somáticas progenitoras presentes no mesonefro migram em direção às gônadas em desenvolvimento (DG11). As células somáticas progenitoras que expressam fator esteroidogênico 1 têm o potencial de se diferenciar em células de Sertoli e de Leydig fetais, sendo que essas últimas aparecem no interstício no DG12.

Após a migração celular e formação da gônada indiferenciada, ocorre a definição do sexo do embrião. Em mamíferos, a presença do gene determinante sexual (Sry), localizado no cromossomo Y, é responsável por fazer com que a gônada primitiva se diferencie em testículo ao invés de ovário. Esse gene atua na célula de Sertoli, a qual produz a substância anti-Mulleriana e estimula a produção de testosterona pelas células de Leydig, produtos necessários para o desenvolvimento testicular (Gubbay et al., 1990; Sinclair et al., 1990; Capel, 2000; Fisher, 2004).

Duas populações distintas de células de Leydig foram identificadas: a fetal e a adulta (Chen et al., 2010). Ambas descendem de células tronco testiculares semelhantes às células mesenquimais (*mesenchymal-like stem cells*), sugerindo que suas ontogenias são idênticas (Byskov, 1986; O´Shaughnessy et al., 2005).

Em ratos, as células de Leydig fetais iniciam a síntese de testosterona no DG15 e atingem seu pico de produção no DG19, sendo que a testosterona secretada nessa fase é essencial para a diferenciação sexual secundária masculina (Huhtaniemi and Pelliniemi, 1992; Chen et al., 2010). A maior parte

das células de Leydig fetais rapidamente sofre apoptose após o nascimento, mas algumas delas permanecem no interstício mesmo no testículo adulto, podendo ser identificadas pelo seu perfil de expressão gênica (Codesal et al., 1990; Chen et al., 2010). No DPN11, células fusiformes, 3-β-hidroxiesteróide desidrogenase (3β-HSD) positivas, derivadas das *mesenchymal-like stem cells,* tornam-se aparentes no interstício (Ariyaratne et al., 2000). As células de Leydig adultas originam-se desse grupamento celular. A célula de Leydig adulta produz a testosterona necessária para manutenção da função reprodutiva masculina e, quando o rato atinge a maturidade sexual (DPN56), os testículos possuem aproximadamente 25 milhões desse tipo celular (Chen et.al., 2009).

Desenvolvimento das células de Leydig adultas em ratos

A célula de Leydig adulta possui cinco estágios distintos de diferenciação no período pós-natal (FIGURA 3):

1) no DPN7, são encontradas células fusiformes no interstício, as quais se caracterizam por sua localização peritubular e ausência de marcadores específicos, como a 3β-HSD e o LHR (receptor do hormônio luteinzante) (Ge et al., 2005; Chen et al., 2009). Esse tipo celular é classificado como *mesenchymal-like stem cells* (célula tronco de Leydig - CTL).

2) Entre os DPN10 e DPN14, essas células passam a ser classificadas como células de Leydig progenitoras (CLP), caracterizadas por alta taxa de proliferação (Chen et al., 2009). As CLP são pequenas e fusiformes, semelhantes às CTL, mas, já apresentam o LHR e possuem retículo endoplasmático liso que abriga enzimas esteroidogênicas como o CYP11A1, 3β-HSD, CYP17 (Shan et al., 1993; Chen et al., 2009).

3) A célula de Leydig adulta (CLA) recém-formada tem como principal característica a alteração da morfologia de fusiforme para poligonal, com citoplasma escasso e núcleo proeminente. Além disso, a CLA recém-formada começa a migrar da região peritubular para o centro do interstício (Mendis-Handagama and Ariyaratne, 2001).

4) A CLA imatura é caracterizada pelo aumento gradativo do citoplasma, assumindo aspecto arredondado, com redução de sua capacidade proliferativa.

Do DPN28 ao DPN56, elas adquirem gotículas lipídicas e têm o seu retículo endoplasmático liso expandido (Mendis-Handagama and Ariyaratne, 2001).

5) As CLA imaturas proliferam apenas uma vez entre os DPN28 e DPN56 e produzem a população final de CLA maduras que, no DPN90, totalizam 25 milhões de células por testículo. As CLA maduras caracterizam-se por não proliferarem (Keeney et al., 1988), mas podem se regenerar através da diferenciação das CTL, se sua população original for eliminada.

O processo de maturação das células de Leydig é semelhante entre ratos e humanos, o que favorece a extrapolação de achados entre estas espécies.

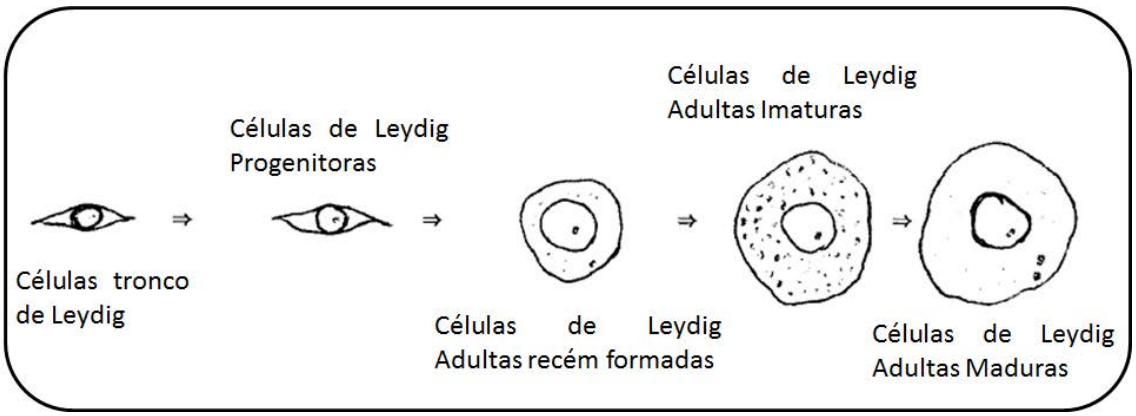

FIGURA 3. Proliferação e diferenciação de células de Leydig. Adaptado de Mendis-Handagama and Ariyaratne (2001).

Função da célula de Leydig

As células de Leydig são células esteroidogênicas que sintetizam testosterona a partir do colesterol livre. O colesterol é transferido para a membrana mitocondrial externa e, então, para a membrana interna, o que é dependente da proteína reguladora aguda esteroidogênica (StAR). Através da enzima CYP11A1, o colesterol é convertido em pregnenolona, sendo esta processada pelas enzimas 3β-HSD e CYP17 em progesterona, 17-hidroxiprogesterona e androstenediona. A androstenediona é, então, convertida em testosterona pela 17β-hidroxiesteroide desidrogenase (17β-HSD tipo 3) (FIGURA 4).

A testosterona produzida pelas células de Leydig tem diversos destinos e ações. Trata-se de um hormônio lipofílico que se difunde facilmente pelos túbulos seminíferos (Koeppen and Stanton, 2009). A proteína ligadora de

andrógenos (ABP), secretada pelas células de Sertoli, se liga à testosterona tornando-a menos lipofílica e possibilitando, portanto, sua concentração no interior dos túbulos. Assim sendo, os níveis de testosterona verificados nos túbulos seminíferos são cerca de 100 vezes superiores aos níveis circulantes (Creasy, 2001; Koeppen and Stanton, 2009).

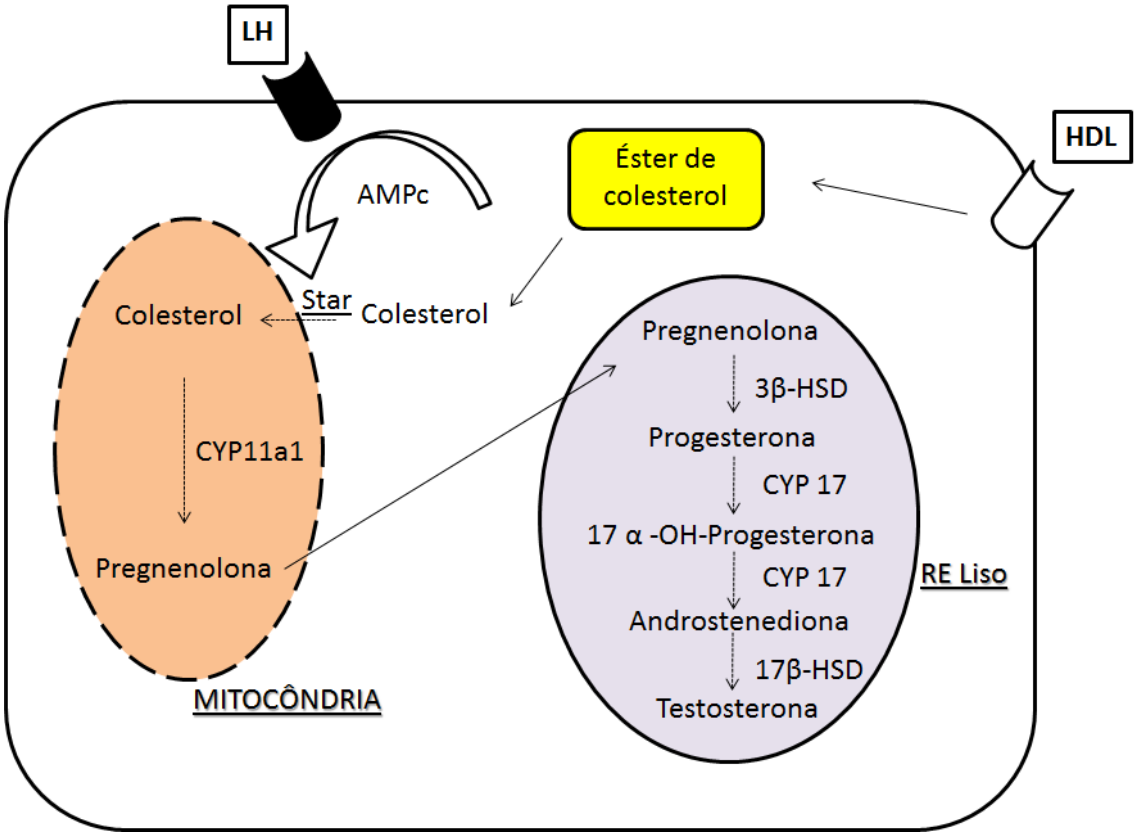

FIGURA 4. Representação esquemática do processo de esteroidogênese que ocorre na célula de Leydig, com a testosterona sendo o produto final principal (Amaral et al., 2013).

O testículo é regulado pelo eixo endócrino constituído por neurônios hipotalâmicos produtores de hormônio liberador de gonadotrofina (GnRH) que, por sua vez, estimulam tanto a produção de hormônio luteinizante (LH), que se liga às células de Leydig via LHR, quanto de hormônio folículo estimulante (FSH), que se liga ao receptores de FSH e é responsável pela maturação das células germinativas. A principal função do LH é estimular a hidrólise dos ésteres de colesterol e expressão de StAR, proteína importante na síntese de testosterona e que retroalimenta negativamente a produção do próprio LH. O LH é essencial nas fases mais tardias da linhagem de células de Leydig, por induzir a sua proliferação celular e hipertrofia (alteração morfológica pela qual

as células de Leydig passam durante processo de diferenciação), e estabelecer aumento gradual do volume do retículo endoplasmático liso, proporcionando maior atividade esteroidogênica. Ainda, a testosterona inibe a diferenciação de células de Leydig precursoras. Desse modo, a testosterona produzida pelas CLA maduras parece ser responsável por manter constante o número desse grupo celular (Mendis-Handagama and Ariyaratne, 2001). A intensidade da coloração imunohistoquímica do LHR tem sido utilizada como marcadora do funcionamento das células de Leydig, uma vez que a diminuição de expressão deste marcador pode estar relacionada com menor estimulo das células de Leydig pelo LH, comprometendo a síntese de testosterona.

Referências

Adami H., Bergstrom R., Mohner M. et al. Testicular cancer in nine northern European countries. Int. J. Cancer 1994; 59: 33-38.

Adler ID, Baumgartner A, Gonda H, Friedman MA, Skerhut M. 1-Aminobenzotriazole inhibits acrylamide-induced dominant lethal effects in spermatids of male mice. Mutagenesis 2000; 15(2): 133-6.

Amaral S, Amaral A, Ramalho-Santos J. Aging and male reproductive function: a mitochondrial perspective. Front Biosci (Schol Ed) 2013; 1;5:181-97.

Andersen AG, Jensen TK, Carlsen E, Jørgensen N, Andersson AM, Krarup T, Keiding N, Skakkebaek NE. High frequency of sub-optimal sêmen quality in a unselected population of Young men. Human Reprod 2000; 15(2): 366-372.

Ariyaratne HB, Mendis-Handagama SM, Hales DB, Mason IJ. Studies of the onset of Leydig cell differentiation in the prepubertal rat testis. Biol Reprod 2000; 63:165–71.

Bergh A and Soder O. Studies of cryptorchidism in experimental models. Acta Paediatr 2007;. 96(5):617-21.

Boekelheide K, Kleymenova E, Liu K, Swanson C, Gaido KW. Dose-dependent effects on cell proliferation, seminiferous tubules, and male germ cells in the fetal rat testis following exposure to di(n-butyl) phthalate. Microsc Res Tech 2009; 72(8): 629-38.

Byskov AG. Differentiation of mammalian embryonic gonad. Physiol Rev 1986; 66(1):71-117.

Camacho L, Latendresse JR, Muskhelishvili L, Patton R, Bowyer JF, Thomas M, Doerge DR. Effects of acrylamide exposure on serum hormones, gene expression, cell proliferation, and histopathology in male reproductive tissues of Fischer 344 rats. Toxicol Lett 2012; 1;211(2):135-43.

Capel B. The battle of the sexes. Mech Dev 2000; 15;92(1):89-103.

Chang YF, Lee-Chang JS, Panneerdoss S, MacLean JA 2nd, Rao MK. Isolation of Sertoli, Leydig, and spermatogenic cells from the mouse testis. Biotechniques 2011; 51(5):341-2, 344.

Chen H, Ge RS, Zirkin BR. Leydig cells: From stem cells to aging. Mol Cell Endocrinol 2009; 306(1-2):9-16.

Chen H1, Stanley E, Jin S, Zirkin BR. Stem Leydig cells: from fetal to aged animals. Birth Defects Res C Embryo Today. 2010; 90(4):272-83.

Codesal J, Regadera J, Nistal M, Regadera-Sejas J, Paniagua R. Involution of human fetal Leydig cells. An immunohistochemical, ultrastructural and quantitative study. J Anat. 1990; 172:103-14.

Creasy DM. Pathogenesis of male reproductive toxicity. Toxicol Pathol 2001; 29(1):64-76.

Crinnion WJ. Toxic effects of the easily avoidable phthalates and parabens. Altern Med Rev. 2010; 15(3):190-6.

Culty M. Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res C Embryo Today 2009; 87:1–26.

Ekbom A, Richiardi L, Akre O, Montgomery SM, Sparén P. Age at immigration and duration of stay in relation to risk for testicular cancer among Finnish immigrants in Sweden. J Natl Cancer Inst 2003; 20;95(16):1238-40.

Ema M, Miyawaki E, Kawashima K. Further evaluation of developmental toxicity of di-n-butyl phthalate following administration during late pregnancy in rats. Toxicol Lett. 1998; 98:87–93.

De Felici M, Scaldaferri ML, Lobascio M, Iona S, Nazzicone V, Klinger FG, Farini D. Experimental approaches to the study of primordial germ cell lineage and proliferation. Hum Reprod Update. 2004; 10(3):197-206.

Ferguson L, Agoulnik AI. Testicular cancer and cryptorchidism. Front Endocrinol.2013; 20;4:32.

Ferrara D, Hallmark N, Scott H, Brown R, McKinnell C, Mahood IK, Sharpe RM. Acute and long-term effects of in utero exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. Endocrinology. 2006; 147(11):5352-62. .

Fisher JS, Macpherson S, Marchetti N, Sharpe RM. Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. Hum Reprod. 2003; 18(7):1383-94.

Fisher JS. Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. Reproduction. 2004; 127(3):305-15.

Foster PM, Mylchreest E, Gaido KW, Sar M. Effects of phthalate esters on the developing reproductive tract of male rats. Hum Reprod Update 2001; 7(3): 231-5.

Gallavan RH Jr, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol. 1999; 13(5):383-90.

Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM, Doerge DR. DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice. Chem Res Toxicol 2003; 16(10): 1328-37.

Ge RS, Dong Q, Sottas CM, Chen H, Zirkin BR, Hardy MP. Gene expression in rat leydig cells during development from the progenitor to adult stage: a cluster analysis. Biol Reprod. 2005; 72(6):1405-15.

Ge RS, Chen GR, Tanrikut C, Hardy MP. Phthalate ester toxicity in Leydig cells: developmental timing and dosage considerations. Reprod Toxicol. 2007; 23(3):366-73.

Gilbert D, Rapley E, Shipley J. Testicular germ cell tumours: predisposition genes and the male germ cell niche. Nat Rev Cancer. 2011; 11(4):278-88.

Giwercman A, Giwercman YL. Environmental factors and testicular function. Best Pract Res Clin Endocrinol Metab. 2011; 25(11): 391–402.

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Münsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature. 1990; 19;346(6281):245-50.

Haseltine FP, Ohno S. Mechanisms of gonadal differentiation. Science. 1981; 20;211(4488):1272-8.

Haschek and Rousseaux's Handbook of Toxicologic Pathology (2013) 3ª edition.

Harris CA1, Henttu P, Parker MG, Sumpter JP. The estrogenic activity of phthalate esters in vitro. Environ Health Perspect. 1997; 105(8):802-11.

Hermo L, Pelletier RM, Cyr DG, Smith CE. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. Microsc Res Tech. 2010; 73(4):241-78.

Hu GX, Lian QQ, Ge RS, Hardy DO, Li XK. Phthalate-induced testicular dysgenesis syndrome: Leydig cell influence. Trends Endocrinol Metab. 2009; 20(3):139-45.

Huhtaniemi I, Pelliniemi LJ. Fetal Leydig cells: cellular origin, morphology, life span, and special functional features. Proc Soc Exp Biol Med. 1992; 201(2):125-40.

Hutson JM, Balic A, Nation T, Southwell B. Cryptorchidism. Semin Pediatr Surg. 2010; 19(3):215-24.

INCA – Instituto Nacional de Câncer (homepage). Testículo (acessado em: 23.05.2011) Disponível http://www2.inca.gov.br/wps/wcm/connect/tiposdecancer/site/home/testiculo.

Jégou B, Laws AO, de Kretser DM. The effect of cryptorchidism and subsequent orchidopexy on testicular function in adult rats. J Reprod Fertil. 1983; 69(1):137-45.

Jørgensen N1, Rajpert-De Meyts E, Main KM, Skakkebaek NE. Testicular dysgenesis syndrome comprises some but not all cases of hypospadias and impaired spermatogenesis. Int J Androl. 2010; 33(2):298-303.

Keeney DS, Mendis-Handagama SM, Zirkin BR, Ewing LL. Effect of long term deprivation of luteinizing hormone on Leydig cell volume, Leydig cell number, and steroidogenic capacity of the rat testis. Endocrinology. 1988; 123(6):2906-15.

Koeppen BM, Stanton BA. Berne & Levy Fisiologia, 6ª edição. Eisevier 2009, p. 663; 765-774.

Kumar V, Abbas AK, Fausto N. Patologia – Bases Patológicas das Doenças, 7ed. Local: Elsevier, 2005, p. 1082-1083.

Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen DV, Andersson AM, Toppari J, Skakkebaek NE. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ Health Perspect. 2006; 114(2):270-6.

McKiernan JM, Goluboff ET, Liberson GL, Golden R, Fisch H. Rising risk of testicular cancer by birth cohort in the United States from 1973 to 1995. J Urol 1999; 162(2): 361-3.

Mendis-Handagama SM, Ariyaratne HB. Differentiation of the adult Leydig cell population in the postnatal testis. Biol Reprod. 2001; 65(3):660-71.

Miliaras D, Vlahakis-Miliaras E, Anagnostopoulos D, Koutsoumis G, Pergamalis G, Miliaras S.Gross morphologic variations and histologic changes in cryptorchid testes. Pediatr Surg Int. 1997; 12(2-3):158-62.

Moore and Persaud (2004). Embriologia básica. 7ª edição.

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

Myrup C, Westergaard T, Schnack T, Oudin A, Ritz C, Wohlfahrt J, Melbye M. Testicular cancer risk in first- and second-generation immigrants to Denmark. Natl Cancer Inst. 2008; 100(1):41-7.

National Research Council (US) Committee on the Health Risks of Phthalates (2008). Phthalates and Cumulative Risk Assessment: The Task Ahead. 1st ed. Washington. National Academies Press (US).

O'Shaughnessy PJ1, Baker PJ, Johnston H. Neuroendocrine regulation of Leydig cell development. Ann N Y Acad Sci. 2005; 1061:109-19.

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LE Jr. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol Sci. 2000; 58(2):339-49.

Paulozzi LJ, Erickson JD, Jackson RJ. Hypospadias trends in two US surveillance systems. Pediatrics 1997; 100: 831-834.

Pelucchi C, La Vecchia C, Bosetti C, Boyle P, Boffetta P. Exposure to acrylamide and human cancer--a review and meta-analysis of epidemiologic studies. Ann Oncol. 2011; 22(7):1487-99.

Quinn FM, Crockard AD, Brown S. Reversal of degenerative changes in the scrotal testis after orchidopexy in experimental unilateral cryptorchidism. J Pediatr Surg. 1991; 26: 451–4.

Rajpert-De Meyts E, Hanstein R, Jørgensen N, Graem N, Vogt PH, Skakkebaek NE. Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. Hum Reprod. 2004; 19(6):1338-44.

Richiardi L, Pettersson A, Akre O. Genetic and environmental risk factors for testicular cancer. Int J Androl. 2007; 30(4): 230-40; discussion 240-1.

Ritzen EM, Bergh A, Bjerknes R, Christiansen P, Cortes D,Haugen SE, Jorgensen N, Kollin C, Lindahl S, Lackgren G, Main KM, Nordenskjold A, Rajpert-De Meyts E, Soder O, Taskinen S, Thorsson A, Thorup J, Toppari J, Virtanen H. Nordic consensus on treatment of undescended testes. Acta Paediatrica. 2007; 96:638–643.

Rossi LM, Pereira LA, de Santis L, Pasqualotto FF, Iaconelli A Jr, Ortiz V, Borges E Jr. Sperm retrieval techniques in rats with suppressed spermatogenesis by experimental cryptorchidism. Hum Reprod. 2005; 20(2):443-7.

Russell LD, Alger LE, Nequin LG. Hormonal control of pubertal spermatogenesis. Endocrinology. 1987; 120(4):1615-32.

Sakamoto J, Kurosaka Y, Hashimoto K. Histological changes of acrylamide-induced testicular lesions in mice. Exp Mol Pathol 1988; 48(3): 324-34.

Scott HM, Hutchison GR, Jobling MS, McKinnell C, Drake AJ, Sharpe RM. Relationship between androgen action in the "male programming window," fetal sertoli cell number, and adult testis size in the rat. Endocrinology 2008; 149(10): 5280-7.

Shan LX, Phillips DM, Bardin CW, Hardy MP. Differential regulation of steroidogenic enzymes during differentiation optimizes testosterone production by adult rat Leydig cells. Endocrinology. 1993; 133(5):2277-83.

Sharpe RM, Skakkebaek NE. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. Fertil Steril. 2008; 89(2 Suppl):e33-8.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature. 1990; 346, 240±244.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 2001; 16(5): 972-8.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S, Teague JL; Study for Future Families Research Team. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect. 2005; 113(8):1056-61.

Tarulli GA, Stanton PG, Meachem SJ. Is the Adult Sertoli Cell Terminally Differentiated? Biol Reprod. 2012; 1;87(1):13.

Thayer KA, Foster PM. Workgroup report: National Toxicology Program workshop on Hormonally Induced Reproductive Tumors - Relevance of Rodent Bioassays. Environ Health Perspect 2007; 115(9): 1351-6.

Ulbright TM, Emerson RE. In: Bostwick DG, Cheng L. (2008). Neoplasms of the testis. Urological Surgical Pathology. 2nd ed. China:Elsevier.

Wang H, Huang P, Lie T, Li J, Hutz RJ, Li K, Shi F. Reproductive toxicity of acrylamide-treated male rats. Reprod Toxicol 2010; 29(2): 225-30.

Watts LM, Hasthorpe S, Farmer PJ, Hutson JM. Apoptotic cell death and fertility in three unilateral cryptorchid rat models. Urol Res 2000; 28(5): 332-7.

WHO - World Health Organization. Global assessment of the state-of-the-science of endocrine disruptors. In: Damstra T, Brlow S, Bergman A, Kavlock R, Der Kraak Van, editors. An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. 2002.

Wuttke W, Jarry H, Seidlova-Wuttke D. Definition, classification and mechanism of action of endocrine disrupting chemicals. Hormones (Athens) 2010; 9(1): 9-15.

Zakaria O, Shono T, Imajima T, Suita S. Fertility and histological studies on the contralateral testes in two diferente intre- and extra-abdominal rat models of unilateral cryptorchidism. Br J Urol. 1998; 82(4):574-7.

Zogbi C, Tesser RB, Encinas G, Miraglia SM, Stumpp T. Gonocyte development in rats: proliferation, distribution;;n and death revisited. Histochem Cell Biol. 2012; 138(2):305-22.

Capítulo II

Cryptorchidism and in utero and postnatal exposure to di(n-butyl) phthalate or acrylamide - morphological evaluation of rat testicular damage*

Nathália P. Souza[a], Ana Paula F. Cardoso[a], Lígia Maria M. Gomide[a], Merielen G. N. Pontes[a], João Lauro V. de Camargo[a]

[a] UNESP- São Paulo State University, Botucatu Medical School, Department of Pathology, Botucatu, São Paulo, Brazil

*** to be submitted to Experimental and Toxicologic Pathology (www.elsevier.de/etp)**

ABSTRACT

The testicular dysgenesis syndrome (TDS) encompasses conditions such reduced semen quality, hypospadia, cryptorchidism and testicular germ cell tumors (TGCT), which may occur isolated or in association. The influence of environmental factors on TDS occurrence has become increasingly evident, special attention being paid to exogenous chemicals such phthalates and acrylamide (AA), well known testicular toxicants. Experimentally induced cryptorchidism can be an useful experimental tool to understand TDS pathogenesis. It has been reported that the testicular microenvironment is critically modified when rodents are experimentally exposed to dibutyl-phthalate (DBP), to AA or to cryptorchidism. However, the combined influence of cryptorchidism and testicular toxicants has not been explored. This study aimed to evaluate the morphological changes and the immunoexpression of the transcription factor binding octamer ¾ (OCT¾) and the luteinizing hormone receptor (LHR) in the testes of rats submitted to a model of testicular damage that associated chemicals (DBP or AA) and surgical (cryptorchidism/orchidopexy) treatments. *In utero* and postnatal DBP or AA exposures associated with surgically-established cryptorchidism induced disruption of spermatogenesis and increased the expression of OCT¾ in germ cells. Immunoreactivity of LHR was qualitatively decreased in the cryptorchid group also exposed to DBP. Exposures to DBP or AA did not alter the anogenital distance. All experimental groups showed decreased in the epididymis weights. Relative ventral prostate weights was decreased in cryptorchid animals and relative liver weights was decreased in DBP-exposed animals. The orchidopexy performed 3 weeks after cryptorchidism was effective to reestablish fairly the testes structure and reproductive organs weights. Our results indicate that the model used, which associated chemical exposures to surgical interventions, may be useful to understand TDS pathogenesis. Alterations of germ cell differentiation and immunoexpression suggest similarities with transformed cells related to human testicular cancer.

INTRODUCTION

Disorders of male reproductive health, including low sperm counts, hypospadia, cryptorchidism and testicular germ cell tumor (TGCT) are increasing in incidence in the Western world (Sharpe and Skakkebaek, 2008). These conditions may have common origin in fetal life, resulting from disruption of embryonic programing and gonadal development and, isolated or combined, they constitute the Testicular Dysgenesis Syndrome (TDS) (Skakkebaek et al., 2001). The current understanding of TDS includes both the hypothesis of a common environmental cause (Sharpe and Skakkebaek, 1993; Sharpe, 2003), as well as the existence of a common genetic factor responsible for all four well identified abnormalities (Ferguson and Agoulnik, 2013).

The increased incidence of genital malformations and of TGCT in children of workers exposed to environmental contaminants suggest that gonocytes may be affected during intrauterine life by those exogenous agents, eventually leading to poor sperm quality and or tumors late in the life (Skakkebaek et al., 2001; Thayer and Foster, 2007). Although environmental contaminants are likely responsible for the increased incidences of TDS, their mode of action is unclear.

In humans, it is reported that exposure to di(butyl)-phthalate (DBP), an widely used plasticizer, is associated with hormonal alterations (Main et al., 2006) and with reduced anogenital distance (AGD) (Swan et al., 2005) - one of the parameters adopted to evaluate environmentally-induced alterations of external genital organs differentiation (USEPA 1998a; USEPA 1998b; Gallavan et al., 1999). The “phthalate syndrome” is seen in rats exposed to DBP during

the perinatal period and it is the most accepted experimental model for TDS until the moment (Foster, 2006; Martino-Andrade et al., 2009).

Acrylamide (AA) is mainly formed during the cooking process of many common starchy foods. Its genotoxic potential has not been defined, but there are indications of increased clastogenicity in germ cells of rats and mice, adverse effect which apparently depends on the dose and exposure protocol (Adler et al., 2000; Gamboa da Costa et al., 2003; Zhang et al., 2009). Testicular toxicity has been reported in rodents exposed to AA but the mechanism(s) involved still requires studies (Sakamoto et al., 1988; Wang et al., 2010). Acute oral exposure of ddY mice to 150 mg/kg AA induced testicular germ cell damage as indicated by vacuolation of Sertoli cells, increased volume of round spermatids and cell exfoliation one day after exposure (Sakamoto et al., 1988).

Cryptorchidism or failure of testicular descent is a common anomaly that affects 2-4% of male newborns (Barthold and Gonzalez, 2003; Ferguson and Agoulnik, 2013). Most pubertal and adult cryptorchidic testes have anomalies in all testicular structures. Particularly, the seminiferous tubules have decreased diameters and deficient spermatogenesis; commonly, there are tubules with Sertoli cell and spermatogonia-only pattern, tubules only with Sertoli cells (dysgenetic tubules) and tubular hyalinization and atrophy. Areas with apparent Leydig cell hyperplasia are frequent (Bostwick and Cheng, 2008). In 0.3% of human cryptorchidic testes there is microlithiasis, an alteration characterized by calcified foci diffusely distributed throughout the testicular parenchyma. Finally, about 35% of cryptorchidic testes develop malignant tumors, particularly of germ cell origin (Bostwick and Cheng, 2008). Therefore, human cryptorchidism

is variably associated with impairment of germ cell maturation, infertility and TGCT (Bostwick & Cheng, 2008). In order to keep the fertility and prevent malignancy the surgical reallocation of the testes to the scrotum – orchidopexy – should be performed between the 6th and 18th months of age (Ritzen et al., 2007; Hutson et al., 2013). Different rodent experimental models have been used to better understand the etiology, physiopathology and potential treatments for cryptorchidism (Watts et al., 2000; Bergh and Soder, 2007). A recent National Toxicology Program (NTP) task force emphasized that presence of cryptorchidism and low sperm counts in rodents could be an early predictor of TGCT induction in these animals (Thayer and Foster, 2007).

During fetal life, it is essential that Leydig cells produce sufficient testosterone to ensure appropriate development of the male reproductive tract. Leydig cells are steroidogenic cells that synthesize testosterone from free cholesterol by the interaction between the luteinizing hormone (LH) and its receptor (LHR), located at the cell membrane (Mendis-Handagama and Ariyaratne, 2001). During adult life, LH is important to establish gradual increases of the Leydig cell smooth endoplasmic reticulum volume, resulting in steroidogenic activity enough to keep the basal synthesis of testosterone (Mendis-Handagama and Ariyaratne, 2001). Analyses of LHR gene expression have been used to evaluate the Leydig cell function (Heng et al., 2012; Kilcoyne et al., 2014). Immunohistochemistry is a technique that can also be used to study cell functions (Liang et al., 2014; Dzombeta et al., 2014; Okabe et al., 2015) and LHR immunoexpression might be an useful tool to document potential testicular damage.

All pathological conditions that characterize the TDS but not the TGCT have been reproduced in animal models (Sharpe and Skakkebaek, 2008). In humans, these tumors have their origin from premalignant germ cells or carcinoma in situ (CIS) cells (Sonne et al., 2008) which seems to result from a disturbance of the normal differentiation of gonocytes into prespermatogonia (Rajpert-De Meyts, 2006). These cells express the same range of specific proteins that are normally expressed by gonocytes during fetal life but that are not expressed by normal adult germ cells (Ferrara et al., 2006). Among these markers the octamer-binding transcription factor (OCT) ¾, involved with self-renewal and pluripotency of germ cells in the fetal and perinatal periods, gradually decreases after the 20th week of gestation in humans (Rajpert-De Meyts et al., 2004; Ferrara et al., 2006; Juul et al., 2014) and after the 17th embryonic day in rats (Ferrara et al., 2006) reaching lack of immunoreactivity in adult germ cells in both species (Dieckmann and Skakkebaek 1999). However, human germ cell tumors such as classical seminoma and embryonal carcinoma and their correspondent premalignant CIS cells retain a high expression of OCT ¾ (Rajpert-De Meyts, 2006).

In the present study the combined influence of exogenous chemicals and experimental cryptorchidism was evaluated in the testes of Sprague-Dawley rats in an attempt to reproduce some of the alterations that occur in the TDS. The reversibility of eventual alterations was evaluated by orchidopexy. The evaluation consisted of reproductive organs weights, testicular histology and immunoexpression of OCT ¾ and LHR in germ cells and Leydig cells, respectively. *In utero* and postnatal exposure to DBP or AA associated with cryptorchidism/orchidopexy established reversible morphologic alterations of

the testes. Even after almost complete reversibility there were frequent germ cells expressing immunohistochemically the OCT ¾, a human TCGT marker, suggesting that these cells could have been transformed by this rat model of testicular damage.

MATERIAL AND METHODS

Animals

This study was approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, SP, Brazil, protocol No. 926/2012. Sprague Dawley rats were obtained from the Multidisciplinary Center of Biological Investigations (CEMIB UNICAMP, Campinas, São Paulo, Brazil) and kept under a 12-h light/dark cycle and controlled temperature (22 ± 2 ºC). Standard pellet food (Purina) and water were provided *ad libitum*. The animals underwent a 2-week acclimatization period before the experiment. Adult female rats were mated overnight at the proportion of two females to each male. Vaginal smears were collected daily and the day of sperm detection was considered as day 0 of gestation.

Chemicals, dose selection and preparation of the experimental diets

Di(butyl)-phthalate 99% (DBP; CAS 84-74-2; Sigma-Aldrich, cat 524980) and acrylamide (AA; CAS 79-06-1; Sigma-Aldrich cat. A9099) were used. The dams were exposed by gavage to 10 mg/kg AA (2 g/L of distilled water) or to 500 mg/kg DBP (100 g/L of corn oil). These doses were chosen based on

reports indicating that exposure to 10 mg/kg AA diluted in drinking water during 8 consecutive days caused a reduction in the number of germ cells in the testis (Wang et al., 2010) and 500 mg/kg of DBP by gavage were sufficient to increase the incidences of hypospadia, cryptorchidism (Mylchreest et al., 1998), low sperm quality (Scott et al., 2008) and to compromise the function of fetal Leydig cells (Mahood et al., 2005; Scott et al., 2008). The control groups were treated with corn oil.

Pregnant dams allocated to three different experimental groups were treated daily by gavage from gestational days (GD) 12 to 21 with corn oil (vehicle control), 500 mg DBP/ kg bw/day or 10 mg AA/ kg bw/day, respectively. From the birth until the weaning, dams were exposed to DBP or AA through diet, the pups being exposed by maternal milk. After weaning, male pups were separated in the respective groups and exposed through the diet to those substances until the end of the study. For this, a mathematical estimate was performed in order to offer in the food the same amount of chemicals provided by gavage, resulting in 6000 ppm and 120 ppm of DBP and AA, respectively.

Experimental design

The male offspring were allocated to 4 experimental groups (Figure 1). The animals from both the cryptorchidic (CPT) and cryptorchidic-reverted (CPT-R) groups (n=10 each) were submitted to surgery for bilateral cryptorchidism in the 3rd week of life and euthanized at the 14th week. The CPT-R animals were submitted to the surgical reversion of cryptorchidism (orchidopexy) at the 6th week of age. The other two groups were constituted by male offspring whose

dams had been exposed by oral gavage to 500 mg/kg DBP (DBP/CPT-R; n=10) or 10 mg/kg AA (AA/CPT-R; n=11) between the gestational days (GD) 12th to the 21th. After birth these pups were exposed to 6000 ppm DBP or 120 ppm AA, firstly through the dam"s milk during lactation and after that through diet up to the end of the study. They were submitted to the same surgery procedures and at the same age of CPT and CPT-R animals: cryptorchidism at the 3rd week and orchidopexy at the 6th week. However, due to operational reasons they were euthanized at the 16th week of age. Each group had their respective control:the CPT and CPT-R control groups had 5 control animals each; the groups also exposed to DBP or AA had 4 control animals; these control animals received only corn oil through gavage and or were sham-operated at the same days as described for the experimental groups.

Surgical procedures

On the 3rd week of life, rats were anesthetized with ketamine (30mg/kg i.p.) and xylazine (4mg/kg i.p.). Abdominal anesthetic and analgesic effects were achieved with lidocaine injections (7mg/kg s.c.) and ketoprofen (10mg/kg s.c.), respectively. After asepsis, the abdominal cavity was opened by small incision in the midline and testes were translocated from the scrotum into the abdominal cavity through the inguinal rings. During this step care was taken to avoid twisting of the spermatic cord, which could lead to testicular damage. The testes were fixed to the dorsolateral abdominal wall with two stitches passing through the tunica albuginea in the cranial and caudal regions of the testes using a 5-0 blunt needle and non-absorbable suture material (NL50CR13 Nylon,

Bioline, BR) (Figure 2A, 2B). After that, the muscle and skin layers were closed and the animals were kept at 30°C for 30 minutes to minimize the deleterious effects of hypothermia induced by anesthesia.

In the orchidopexy surgery the anesthetic, analgesic and asepsis procedures were the same as for the cryptorchidism surgery. On the 6th week of life, a midline abdominal incision was made and the sutures which held the testes in the dorsolateral abdominal wall were carefully removed (Figure 2C, 2D). The scrotum wall was clamped and reversed to facilitate the manipulation; the testes were sutured through the tunica albuginea to the inner wall of the scrotum by a 5-0 blunt needle (NL50CR13 Nylon, Bioline, BR). Finally, the testes were guided into the scrotum. The muscular and skin layers were closed, cleaned and the animals left to recover under the same conditions described above. All the animals received antibiotic treatment (enrofloxacin 5 mg/kg sc) during the following three days (Figures 2C, 2D).

Control animals were submitted to sham surgeries under the same anesthesia, analgesia and aseptic conditions as described above. They had only the skin and muscle layers of the abdomen open and then sutured.

Anogenital distance (AGD)

At the postnatal day (PND) 1, long before the surgical intervention for cryptorchidism at the 3rd week of age, all male offspring from each litter of dams orally exposed to DBP or to AA had the distance between the anus and the genital tubercle (AGD) measured using calipers. To avoid possible errors caused by differences in body size, the AGD of each animal was divided by the

cube root of body weight (Gallavan et al., 1999). The total number of AA or DBP-exposed rats who had their AGD measured is greater than the total number of animals indicated by the experimental protocol because at that moment there were other similarly-treated animals available from a concurrent study.

Necropsy and sample collection

The animals were anesthetized between 8:00 and 10:00 a.m. with ketamine (30 mg / kg ip) and xylazine (4 mg / kg ip) and euthanasied by exsanguination via heart puncture. Immediately after the euthanasia the testes were removed, weighed, and placed in modified Davidson‟s fixative during 24h (Latendresse et al., 2002; Kittel et al., 2004) and embedded in paraffin. The liver, kidneys, adrenals, seminal vesicle, epididymis and ventral prostate were collected, weighed, fixed in 10% formalin and embedded in paraffin.

Histological examination

The paraffin blocks containing the testes were cut in histological sections of 5 µm thickness and stained by hematoxylin and eosin (H&E). The histological analyses were systematically and blindly performed following the criteria of Society of Toxicology Pathology (Lanning et al., 2002; Creasy et al., 2012).

Immunohistochemistry

Immunohistochemistry procedures for OCT ¾ and LHR were performed according to the following protocol. The slides were deparaffinized and rehydrated. Peroxidase and protein blocking was done followed by washing in TBST (Tris Buffered Saline Tween), and antigen retrieval was performed in pressurized camera with Trilogy solution (1:20). The slides were incubated overnight at 4°C with monoclonal primary antibody Prediluted anti-Mouse OCT 3/4 (Biocare, California, USA) or LHR monoclonal primary antibody 1: 300 (Bioss, Massachusetts, USA bs-6431R). Mach4 detection kit (MACH 4 Universal HRP-polymer, Biocare Medical) was used followed by DAB revelation system (DAB Substrate Kit, Vector Laboratories, Burlingame, CA). As a positive control of OCT ¾ and LHR, samples of human seminomas and control rat testis were used, respectively. As negative controls, the same material was processed without the application of primary antibody. OCT ¾ immunoexpression was evaluated as positive or negative for nuclear staining; immunoexpression of LHR was evaluated according to the intensity of the staining: weak (+), medium (++) and strong (+++).

Statistical analysis

The groups were compared each one with its control and, according to the results obtained in normality test, followed by the Student's t test (when parametric) or Mann-Whitney (when non-parametric). Differences were considered significant when $p < 0.05$.

RESULTS

Body weights at birth, anogenital distance (AGD) and chemicals intake

There were no differences between the groups regarding the body weights of pups at birth (Table 1). Although not statistically different, the AGD at the PND 1 was reduced in the pups whose mothers were exposed to DBP during the gestational period when compared with the control. The mean intake of each chemical during postnatal life was estimated based upon mean consumption of the diet after 10 weeks of experiment (DBP/CPT-R = 250.0 mg/kg/day; AA/CPT-R= 4.8 mg/kg/day).

Body and organ weights

At the end of the study, rats exposed to AA/CPT-R showed a significantly lower mean final body weight (18%) (Table 2) with no changes in food intake during the experiment (data not shown). DBP/CPT-R treatments did not alter the mean final body weight when compared to control (Table 2). Since the rat testicular weight is independent of body development, the absolute weight of this organ is more representative than the relative weight (Haschek and Rousseaux, 2013). Accordingly, all experimental groups (CPT, CPT-R, DBP/CPT-R and AA/CPT-R) showed lower absolute testicular weights when compared to their respective controls (Figure 3). Similarly, the epididymis relative weights were decreased in all groups (Table 2). The CPT group showed a significant reduction of the ventral prostate relative weights when compared to its control, and the groups submitted to orchidopexy did not (CPT-R, DBP/CPT-

R and AA/CPT-R groups) (Table 2). There were no significant differences of the seminal vesicle weights in the experimental groups (Table 2). The mean relative liver weights of the DBP/CPT-R group were higher when compared to control animals but no liver histological alterations were observed in these animals (Table 2).

Testicular histology

Animals submitted only to surgeries (CPT and CPT-R) presented homogeneous testicular lesions which were distributed similarly on all seminiferous tubules. However, in the groups also exposed to chemicals (DBP/CPT-R or AA/CPT-R) the picture was more heterogenous: areas with severe testicular damage were surrounded by apparently normal seminiferous tubules. Rats submitted only to cryptorchidism (CPT group) presented severe impairment of spermatogenesis: atrophy of the germinative epithelium, absence of sperm in the tubule lumen, presence of apoptotic bodies, multinucleated germ cells, germ cell exfoliation and vacuolation of Sertoli cells; some tubules presented a "Sertoli cell only" pattern (Figure 4B). After orchidopexy (CPT-R group), there was predominance of tubules with organized seminiferous epithelium with sperm in the lumen; however, some tubules still had vacuolation (Figure 4D). The interstitium presented “apparent” Leydig cell hyperplasia in the CPT group, what was not observed in the CPT-R group. Animals of DBP/CPT-R group had most of the seminiferous tubules with incomplete spermatogenesis, vacuolation of Sertoli cells and exfoliation of germ cells (Figures 5D, 5F, 5H). Since it was still possible to distinguish the different stages of maturation in the

seminiferous tubules and the germ cells presented similar morphology to normal. In some of these animals, foci of intratubular calcification and Leydig cell hyperplasia were apparent (Figure 5H). In the AA/CPT-R group, the testes presented some seminiferous tubules with “Sertoli cell only” pattern surrounded by tubules with germ cell differentiation until the stage of elongated spermatids, rare tubules with spermatozoa in the lumen, apoptotic cells, multinucleated giant cells and vacuolation. Some occurring germ cells presented altered morphology as hyperchromatic nuclei and pleomorphic cytoplasm which may indicate more severe testicular morphological changes than those that occurred in the DBP/CPT-R group. Also, as in the DBP/CPT-R group, some seminiferous tubules showed intraluminal calcification and “apparent” Leydig cell hyperplasia (Figures 5C, 5E, 5G).

Under H&E, adult normal rat Leydig cells are large eosinophilic cells, slightly rounded, with an abundant cytoplasm and few barely visible lipid droplets. The animals submitted to cryptorchidism (CPT) and orchidopexy (CPT-R) had the same Leydig cell morphology as the control. However, Leydig cells from the DBP/CPT-R treated animals animals had reduced volume and nuclei less prominent than the controls (Figure 6). The animals exposed to AA also showed Leydig cells with altered morphology, with predominance of cells with polygonal and fusiform cytoplasm (Figure 6).

Immunohistochemistry

Immunohistochemistry for OCT ¾ showed rare positive germ cells in the control group (Figure 7B). Cells expressing this antigen were close to the basal lamina of the seminiferous tubule and had morphological aspect of spermatogonias. There was no more than one positive cell/seminiferous tubule in the control animals. Testes of DBP/CPT-R animals showed rare OCT ¾ positive cells, even in seminiferous tubules with disgenetic morphology (Figure 7C). However, a greater number of labeled cells was observed in the testes of AA/CPT-R animals, the labeled cells occurring evenly in the majority of tubules present in histological sections; often, tubules with more than one positive cell were visualized (Figure 7D).

All groups except the DBP/CPT-R one presented Leydig cells with the same intensity pattern of LHR immunoexpression as their respective control. In the DBP/CPT-R group the LHR immunostaining was lighter than the respective control (Table 3, Figure 8), suggesting that LHR activity was less impressive in these animals.

DISCUSSION

In this study, experimental cryptorchidism and *in utero* and postnatal exposure to the testicular toxicants DBP or AA were combined in order to document the eventual occurrence and potential reversibility of testicular lesions in Sprague-Dawley rats.

At the end of the study, only the group exposed to AA/CPT-R showed reduced body weight. Neither the surgical procedures nor the DBP influenced negatively the weight gain of these rats despite damaging severely the testicular structure and probably its function. Similar body weight impairment was observed by Wang et al. (2010) after exposing rats to 10 mg/kg AA for 8 weeks through drinking water, suggesting that the Maximum Tolerated Dose (MTD) was reached and that the toxicity of AA in the present study was manifested systemically as decreased animal body weights.

Among the androgen-dependent reproductive organs, only the ventral prostate from animals submitted to cryptorchidism (CPT) presented a significantly decreased mean relative weight. This suggests that the orchidopexy was efficient to recover the organ weights of the CPT-R, DBP/CPT-R and AA/CPT-R groups. Besides the influence of a probable decrease of androgen production by the cryptorchidic and atrophic rat testes (Ren et al., 2006), several experimental observations (Grayhack et al., 1985; Darras et al., 1992; Ilio et al., 2000) have led to the hypothesis that the testes may secrete a nonsteroidal factor that promotes prostate growth; disruption of this pathway may have an important role in prostate growth (Ilio et al., 2000).

In rodents, some phthalates (including DBP) induce the activity of hepatic enzymes via activation of PPR-α receptors, what is followed by cellular adaptations consisting of hepatocellular hypertrophy and eventually hyperplasia leading to increased organ weight (Hardisty and Brix, 2005; Hall et al., 2012). In the present study, only the animals of the DBP/CPT-R group presented increased liver weights but no histological alterations of hepatocytes were observed. Therefore, it can be assumed that the increased mean relative liver

weight of DBP-treated animals resulted of association of mild alterations of body weight and enzymatic induction, both not enough to be significantly detected in this study.

Reduction of the epididymis weights was observed in all groups when compared to their respective controls what could be related to the lower production of fluid and sperm by the testes (Jegou et al., 1983). Likewise, testicular weights were reduced in all experimental groups and it was more evident in CPT group in which the mean organ weight was 72.2% lighter than the control. Morphological analyses of the testes showed severe impairment of spermatogenesis and "apparent" Leydig cell hyperplasia, as previously described. The frequently used term of "Leydig cell hyperplasia" may be incorrect, because even if the proportion of Leydig cells can appear increased in histological samples from dysgenetic testes, there is often no significant increase in total Leydig cell volume when testis size is taken into account (Holm et al., 2003). Animals which had the reversal surgery of cryptorchidism (CPT-R) still maintained the reduced testicular weight when compared to the control, however the difference between groups was smaller (19.9%) than for the animals submitted only to cryptorchidism (CPT). These data indicates that orchidopexy performed 3 weeks after cryptorchidism was adequate to reestablish the testes structure, being enough for partial recovery of testicular weight and architecture of the germinal epithelium accompanied by resumption of spermatogenesis. Thereby, also in the rat orchidopexy is potentially able to restore testicular function when performed at the appropriate moment.

At the end of the study the DBP/CPT-R and AA/CPT-R animals presented the testes weights 53.1% and 57.5% lower than the controls, respectively.

There is a series of evidences indicating that, in fact, phthalates in general and DBP, in particular, negatively affect the cellular structure of the seminiferous tubules with adverse effects on spermatogenesis (Kim et al., 2010; Kondo et al., 2006; Ryu et al., 2008; Jobling et al., 2011; Kay et al., 2014) It has been reported that AA induces incomplete spermatogenesis, vacuolar degeneration of Sertoli cells and multiple giant cell formations in rats exposed to 0,05% AA through drinking water during 21 days (Lebda et al., 2014). It is striking that no detailed AA-associated non-neoplastic alterations of the germinative epithelium had been described in rats submitted to long-term studies performed to evaluate chronic toxicity/carcinogenicity of AA (Johnson et al., 1986; Friedman et al., 1995; NTP, 2012), even when these rodents were exposed continuously for life since the 6th gestational day (Maronpot et al., 2014). The damage observed in our study on the seminiferous epithelium of the AA/CPT-R group as hyperchromatic nuclei and pleomorphic cytoplasm is in agreement with other reports that indicate the potential toxicity of this chemical agent for testicular germ cells (Yang et al., 2005; Zhang et al., 2009; Hamdy et al., 2012; Lebda et al., 2014). Calcified areas as confirmed with the special Von Kossa stain were observed in testis of animals exposed to either DBP/CPT-R or AA/CPT-R. The pathogenesis of these structures is uncertain, although it has been suggested that they result from cellular debris accumulation within the seminiferous tubules, followed by deposition of glycoproteins (Renshaw, 1998). In humans, intratubular calcification has been described in normal pre-pubertal testis in conjunction with a wide variety of clinical and pathological conditions, including infertility, cryptorchidic testis and atrophic seminiferous tubules at the periphery of primary germ cell tumors (Renshaw, 1998), all conditions seem in TDS.

Thereby, from the testicular histology analysis of the present study is possible verify some morphological changes induced by cryptorchidism even after orchidopexy (CPT-R). DBP/CPT-R group presented subtle morphological differences when compared with CPT-R being possible to recognize the stage of germ cells in each tubule, since the germ cells showed preserved morphology. The most evident morphological changes occurred in animals of AA/CPT-R group that presented hyperchromatic nuclei and pleomorphic cytoplasm on germ cells.

LH is responsible for stimulate the steroidogenesis process by binding to its transmembranous receptor (LHR) in the Leydig cells surface. The intensity of LHR immunostaining has been associated with normal steroidogenesis, and substances with anti-androgenic effect decrease this labeling (Haider, 2004). In the present study, animals exposed to DBP showed Leydig cells with lower labeling intensity than the other groups exposed to AA or submitted to cryptorchidism that did not differ from the controls. The mechanism of action of DBP remains uncertain, but it has been suggested that the metabolite ester-monobutyl phthalate (MBP) is responsible for this antiandrogenic activity (Drake et al., 2009; Shirai et al., 2013). LHR gene expression was unchanged in adults Wistar rat testes after *in utero* exposure to 500 mg/kg DBP (Kilcoyne et al., 2014). However, our study suggests that, although this gene could be expressed, the LHR are less functional in the testes of animals exposed to DBP when compared to control. This can result in reduction of steroidogenesis, lower testosterone synthesis in the adult animal (Kilcoyne et al., 2014) and, consequently, disruption of spermatogenesis, such as morphologically observed in the present animals.

The frequent occurrence of adult OCT ¾ positive germ cells under the conditions of this study - cryptorchidism and orchidopexy and exposure to environmental toxins -, particularly in the testes of AA-treated rats, is an unprecedented finding in the literature. Few OCT ¾ positive cells were already described in normal testes of adult control mice and were found to be spermatogonias A, a normal germ cell type with pluripotent characteristics (Pesce et al., 1998). In humans, OCT ¾ positive germ cells have been used as an immunohistochemical marker of human seminomas and embrional carcinomas, frequent testicular germ cell tumors. The finding of such cells indicates the possibility of potential neoplastic cell transformation under the severe experimental conditions that these animals were submitted, particularly in relation to DBP exposure (Kay et al., 2014).

No significant alterations were registered regarding the anogenital distance (AGD) in any of the experimental groups, although the DBP-treated animals presented absolute and relative AGD values numerically reduced when compared to the respective control. Reduced AGD values were registered on the PND1 in rats exposed *in utero*, during the period that includes the male reproductive window, to 500 mg/kg or 750 mg/kg of DBP (Mylchreest et al., 1998; Martino-Andrade et al., 2009). It can be estimated that in the present study the animals were exposed to a mean DBP ingestion of 250 mg/Kg/day, a half of the dose referred by those authors, what may explain the statistically non-significant findings on AGD.

In conclusion, our results confirm that the experimental protocol used, which combines surgical procedures and xenobiotics exposure, is useful to establish testicular damage and to increase expression of OCT ¾ by germ cells,

inducing a cell phenotype similar to the one occurring in preneoplastic and neoplastic germ cells in humans.

REFERENCES

Adler ID, Baumgartner A, Gonda H, Friedman MA, Skerhut M. (2000). 1-Aminobenzotriazole inhibits acrylamide-induced dominant lethal effects in spermatids of male mice. Mutagenesis 2000; 15(2): 133-6.

Barthold JS, Gonzalez R. The epidemiology of congenital cryptorchidism, testicular ascent and orchiopexy. J. Urol. 2003; 170, 2396–2401.

Bergh A, Soder O. Studies of cryptorchidism in experimental models. Acta Paediatr 2007; 96(5):617-21.

Bostwick DG, Cheng L. Urologic surgical pathology (2008) 2nd edition.

Creasy D, Bube A, de Rijk E, Kandori H, Kuwahara M, Masson R, Nolte T, Reams R, Regan K, Rehm S, Rogerson P, Whitney K. Proliferative and nonproliferative lesions of the rat and mouse male reproductive system. Toxicol Pathol 2012; 40(6 Suppl):40S-121S.

Darras FS, Lee C, Huprikar S, Rademaker AW, Grayhack JT. Evidence for a non-androgenic role of the testis and epididymis in androgensupported growth of the rat ventral prostate. J Urol 1992;148:432–440.

Dieckmann KP, Skakkebaek NE. Carcinoma in situ of the testis: review of biological and clinical features. Int J Cancer 1999; 83:815–822.

Drake AJ, van den Driesche S, Scott HM, Hutchison GR, Seckl JR, Sharpe RM. Glucocorticoids amplify dibutyl phthalate-induced disruption of testosterone production and male reproductive development. Endocrinology 2009; 150(11):5055-64.

Džombeta T, Kapuralin K, Ulamec M, Tomas D, Gajović S, Krušlin B. Immunohistochemical expression of STAM2 in gastrointestinal stromal tumors. Anticancer Res. 2014; 34(5):2291-6.

Ferguson L, Agoulnik AI. Testicular cancer and cryptorchidism. Front Endocrinol 2013; 20;4:32.

Ferrara D, Hallmark N, Scott H, Brown R, McKinnell C, Mahood IK, Sharpe RM. Acute and long-term effects of in utero exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. Endocrinology 2006; 147(11):5352-62.

Foster PM. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int J Androl 2006; 29:140-147.

Friedman MA, Dulak LH, Stedhamj MA. A Lifetime Oncogenicity Study in Rats with Acrylamide. Fund Appl Toxicol 1995; 27: 95-105.

Gallavan RH Jr, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 1999; 13(5):383-90.

Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM, Doerge DR. DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice. Chem Res Toxicol 2003; 16(10): 1328-37.

Grayhack JT, Lee C, Brand W. The effect of testicular irradiation on established BPH in the dog: evidence of a non-steroidal testicular factor for BPH maintenance. J Urol 1985;134:1276–1281.

Haider SG. Cell biology of Leydig cells in the testis. Int Rev Cytol 2004; 233:181-241.

Hall, A P, Elcombe CR, Foster JR, Harada T, Kaufmann W, Knippel A, Kuttler K, Malarkey DE, Maronpot RR, Nishikawa A, Nolte T, Schulte A, Strauss V, York MJ. Liver hypertrophy: a review of adaptative adverse and non-adverse changes – conclusions from the 3rd International ESTP Expert Workshop. Toxicol. Pathol 2012; 40: 971-994.

Hamdy SM, Bakeer HM, Eskander EF, Sayed ON. Effect of acrylamide on some hormones and endocrine tissues in male rats. Hum Exp Toxicol 2012; 31(5):483-91.

Hardisty, JF, Brix AE. Comparative hepatic toxicity: prechronic/chronic liver toxicity in rodents. Toxicol. Pathol 2005; 33: 35 - 40.

Haschek and Rousseaux's Handbook of Toxicologic Pathology (2013) 3ª edition.

Heng K, Anand-Ivell R, Teerds K, Ivell R. The endocrine disruptors dibutyl phthalate (DBP) and diethylstilbestrol (DES) influence Leydig cell regeneration following ethane dimethane sulphonate treatment of adult male rats. Int J Androl 2012; 35(3):353-63.

Holm M, Hoei-hansen CE, Rajpert-De Meyts, Skakkebaek NE. Increased risk of carcinoma in situ in patients with testicular germ cell cancer with ultrasonic microlithiasis in the contralateral testicle. J Urol 2003; 170: 1163-1167.

Hutson JM, Li R, Southwell BR, Petersen BL, Thorup J, Cortes D. Germ cell development in the postnatal testis: the key to prevent malignancy in cryptorchidism? Front Endocrinol (Lausanne) 2013; 3;3:176.

Ilio KY, Grayhack JT, Lee C. Experimental cryptorchidism inhibited growth of the rat ventral prostate. J Androl 2000; 21(3):438-43.

Jégou B, Laws AO, de Kretser DM. The effect of cryptorchidism and subsequent orchidopexy on testicular function in adult rats. J Reprod Fertil 1983; 69(1):137-45.

Jobling MS , Hutchison GR , van den Driesche S , Sharpe RM. Effects of di(n-butyl) phthalate exposure on foetal rat germ-cell number and diff erentiation: identifi cation of age-specifi c windows of vulnerability. Int J Androl 2011; 34: e386 – 96.

Johnson KA, Gorzinski SJ, Bodner KM, Campbell RA, Wolf CH, Friedman MA, Mast RW. Chronic Toxicity and Oncogenicity Study on Acrylamide Incorporated in the Drinking Water of Fischer 344 Rats. Toxicol Appl Pharmac 1986; 85, 154- 168.

Juul A, Almstrup K, Andersson AM, Jensen TK, Jørgensen N, Main KM, Rajpert-De Meyts E, Toppari J, Skakkebæk NE. Possible fetal determinants of male infertility. Nat Rev Endocrinol 2014; 10(9):553-62.

Kay VR, Bloom MS, Foster WG. Reproductive and developmental effects of phthalate diesters in males. Crit Rev Toxicol 2014; 44(6):467-98.

Kilcoyne KR, Smith LB, Atanassova N, Macpherson S, McKinnell C, van den Driesche S, Jobling MS, Chambers TJ, De Gendt K, Verhoeven G, O'Hara L, Platts S, Renato de Franca L, Lara NL, Anderson RA, Sharpe RM. Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells. Proc Natl Acad Sci U S A. 2014; 6;111(18):E1924-32.

Kim TS, Jung KK, Kim SS, Kang IH, Baek JH, Nam HS, Hong SK, Lee BM, Hong JT, Oh KW, Kim HS, Han SY, Kang TS. Eff ects of in utero exposure to DI(n-Butyl) phthalate on development of male reproductive tracts in Sprague-Dawley rats. J Toxicol Environ Health A 2010; 73, 1544– 59.

Kittel B, Ruehl-Fehlert C, Morawietz G, Klapwijk J, Elwell MR, Lenz B, O'Sullivan MG, Roth DR, Wadsworth PF; RITA Group; NACAD Group. Revised guides for organ sampling and trimming in rats and mice--Part 2. A joint publication of the RITA and NACAD groups. Exp Toxicol Pathol 2004; 55(6):413-31.

Kondo T, Shono T, Suita S. Age-specific effect of phthalate ester on testicular development in rats . J Pediatr Surg 2006; 41: 1290 – 3.

Lanning LL, Creasy DM, Chapin RE, Mann PC, Barlow NJ, Regan KS, Goodman DG. Recommended approaches for the evaluation of testicular and epididymal toxicity. Toxicol Pathol 2002; 30(4):507-20.

Latendresse JR, Warbrittion AR, Jonassen H, Creasy DM. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. Toxicol Pathol 2002; 30(4):524-33.

Lebda M, Gad S, Gaafar H. Effects of lipoic acid on acrylamide induced testicular damage. Mater Sociomed 2014; 26(3):208-12.

Liang S, Zhou Y, Chen Y, Ke G, Wen H, Wu X. Decreased expression of EIF4A1 after preoperative brachytherapy predicts better tumor-specific survival in cervical cancer. Int J Gynecol Cancer 2014; 24(5):908-15.

Mahood IK, Hallmark N, McKinnell C, Walker M, Fisher JS, Sharpe RM. Abnormal Leydig Cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. Endocrinology 2005; 146(2):613-23.

Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen DV, Andersson AM, Toppari J, Skakkebaek NE. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ Health Perspect 2006; 114(2):270-6.

Maronpot RR, Thoolenb RJMM, Hansen B. Experimental Two-year carcinogenicity study of acrylamide in Wistar Han rats with in utero exposure. Exp Toxicol Pathol 2014, http://dx.doi.org/10.1016/j.etp.2014.11.009.

Martino-Andrade AJ, Morais RN, Botelho GG, Muller G, Grande SW, Carpentieri GB, Leão GM, Dalsenter PR. Coadministration of active phthalates results in disruption of foetal testicular function in rats. Int J Androl 2009; 32(6):704-12.

Mendis-Handagama SM, Ariyaratne HB. Differentiation of the adult Leydig cell population in the postnatal testis. Biol Reprod 2001; 65(3):660-71.

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

National Toxicology Program. Report On the Toxicology and carcinogenesis Studies of acrylamide (cas no. 79-06-1) In f344/N rats and b6c3f1 mice (feed and drinking water studies), 2012.

NTP – National Toxicology Program. Toxicology and carcinogenesis studies of acrylamide, 2012

Okabe N, Ezaki J, Yamaura T, Muto S, Osugi J, Tamura H, Imai J, Ito E, Yanagisawa Y, Honma R, Gotoh M, Watanabe S, Waguri S, Suzuki H. FAM83B is a novel biomarker for diagnosis and prognosis of lung squamous cell carcinoma. Int J Oncol 2015; 46(3):999-1006.

Pesce M, Wang X, Wolgemuth DJ, Schöler H. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech Dev 1998; 71(1-2):89-98.

Rajpert-De Meyts E, Hanstein R, Jørgensen N, Graem N, Vogt PH, Skakkebaek NE. Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. Hum Reprod 2004; 19(6):1338-44.

Rajpert-De Meyts E. Developmental model for the pathogenesis of testicular carcinoma in situ: genetic and environmental aspects. Hum Reprod Update 2006; 12:303–323.

Ren L, Medan MS, Ozu M, Li C, Watanabe G, Taya K. Effects of experimental cryptorchidism on sperm motility and testicular endocrinology in adult male rats. J Reprod Dev 2006; 52: 219-228)

Renshaw AA. Testicular calcifications: incidence, histology and proposed pathological criteria for testicular microlithiasis. J Urol 1998; 160(5):1625-8.

Ritzen EM, Bergh A, Bjerknes R, Christiansen P, Cortes D,Haugen SE, Jorgensen N, Kollin C, Lindahl S, Lackgren G, Main KM, Nordenskjold A, Rajpert-De Meyts E, Soder O, Taskinen S, Thorsson A, Thorup J, Toppari J, Virtanen H. Nordic consensus on treatment of undescended testes. Acta Paediatrica 2007; 96:638–643.

Ryu JY , Lee E , Kim TH , Lee YJ , Lee J , Lee BM , Kwack SJ, Jung KK, Han SY, Kim SH, Kacew S, Kim HS. Time response effects of testicular gene expression profi les in Sprague-Dawley male rats treated with di(n-butyl) phthalate. J Toxicol Environ Health A 2008; 71, 1542– 9.

Sakamoto J, Kurosaka Y, Hashimoto K. Histological changes of acrylamide-induced testicular lesions in mice. Exp Mol Pathol 1988; 48(3): 324-34.

Scott HM, Hutchison GR, Jobling MS, McKinnell C, Drake AJ, Sharpe RM. Relationship between androgen action in the "male programming window," fetal sertoli cell number, and adult testis size in the rat. Endocrinology 2008; 149(10): 5280-7.

Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993; 341, 1392–1395.

Sharpe RM, Skakkebaek NE. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. Fertil Steril 2008; 89(2 Suppl):e33-8.

Sharpe RM. The„oestrogen hypothesis"–where do we stand now? Int.J.Androl 2003; 26, 2–15.

Shirai M, Wakui S, Wempe MF, Mutou T, Oyama N, Motohashi M, Takahashi H, Kansaku N, Asari M, Hano H, Endou H. Male Sprague-Dawley rats exposed to in utero

di(n-butyl) phthalate: dose dependent and age-related morphological changes in Leydig cell smooth endoplasmic reticulum. Toxicol Pathol 2013; 41(7):984-91.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 2001; 16(5): 972-8.

Sonne SB, Kristensen DM, Novotny GW, Olesen IA, Nielsen JE, Skakkebaek NE, Rajpert-De Meyts E, Leffers H. Testicular dysgenesis syndrome and the origin of carcinoma in situ testis. Int J Androl 2008; 31(2):275-87.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S, Teague JL; Study for Future Families Research Team. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 2005; 113(8):1056-61.

Thayer KA, Foster PM. Workgroup report: National Toxicology Program workshop on Hormonally Induced Reproductive Tumors - Relevance of Rodent Bioassays. Environ Health Perspect 2007; 115(9): 1351-6.

U.S. EPA. EDSTAC final report. 1998a

U.S. EPA. Health effects test guidelines OPPTS 870.3800 reproduction and fertility effects. 1998b; EPA 712, C, 1–12.

Wang H, Huang P, Lie T, Li J, Hutz RJ, Li K, Shi F. Reproductive toxicity of acrylamide-treated male rats. Reprod Toxicol 2010; 29(2): 225-30.

Watts LM, Hasthorpe S, Farmer PJ, Hutson JM. Apoptotic cell death and fertility in three unilateral cryptorchid rat models. Urol Res 2000; 28(5): 332-7.

Yang H, Lee S, Jin Y, Choi J, Han D, Chae C, Lee M, Han C. Toxicological effects of acrylamide on rat testicular gene expression profile. Reprod Toxicol. 2005; 19: 527-534.

Zhang X, Chen F, Huang Z. Apoptosis induced by acrylamide is suppressed in a 21.5% fat diet through caspase-3-independent pathway in mice testis. Toxicol Mech Methods 2009; 19(3):219-24.

FIGURES

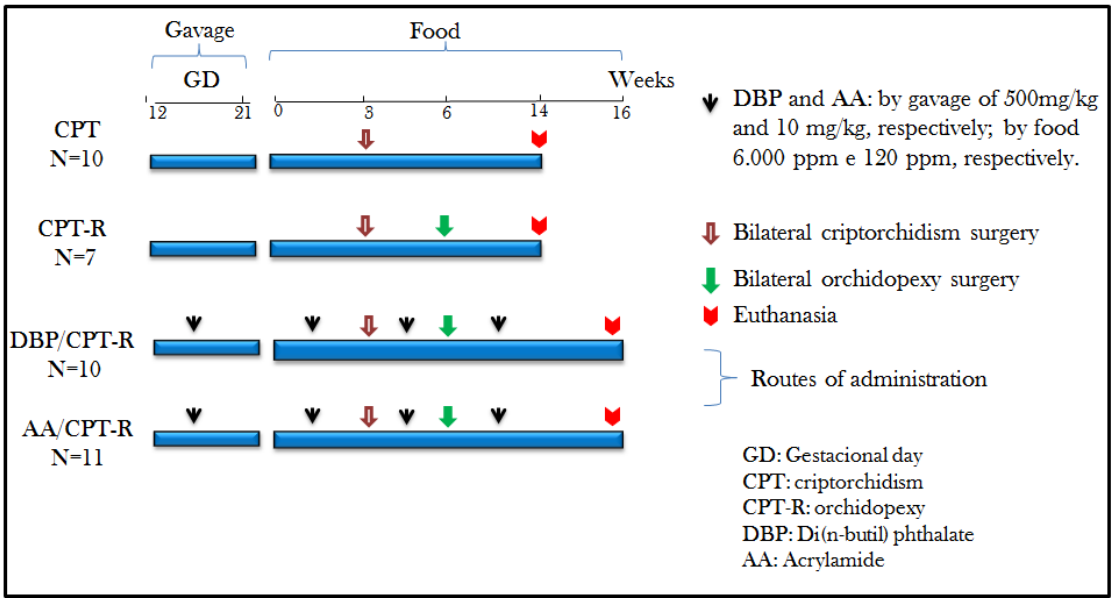

Figure 1. Experimental design. Cryptorchid (CPT) and cryptorchid-reverted (CPT-R) animals were submitted to surgery for bilateral cryptorchidism in the 3rd week of life and euthanized at the 14th week. The CPT-R animals were also submitted to the surgical reversion of cryptorchidism (orchidopexy) at the 6th week of age. Each control group were established with sham-operated animals, the sham surgeries done at the same days as described for the experimental groups. The DBP/CPT-R and AA/CPT-R groups were constituted by male offspring whose dams had been exposed by oral gavage to 500 mg/kg DBP or 10 mg/kg AA between the gestational day (GD) 12th-21th. After birth these pups were exposed to 6000 ppm DBP or 120 ppm AA, firstly through the dam"s milk during lactation and after that through diet up to the end of the study. They were also submitted to the same surgery procedures and at the same age as the CPT and CPT-R animals. Control animals received only corn oil through gavage.

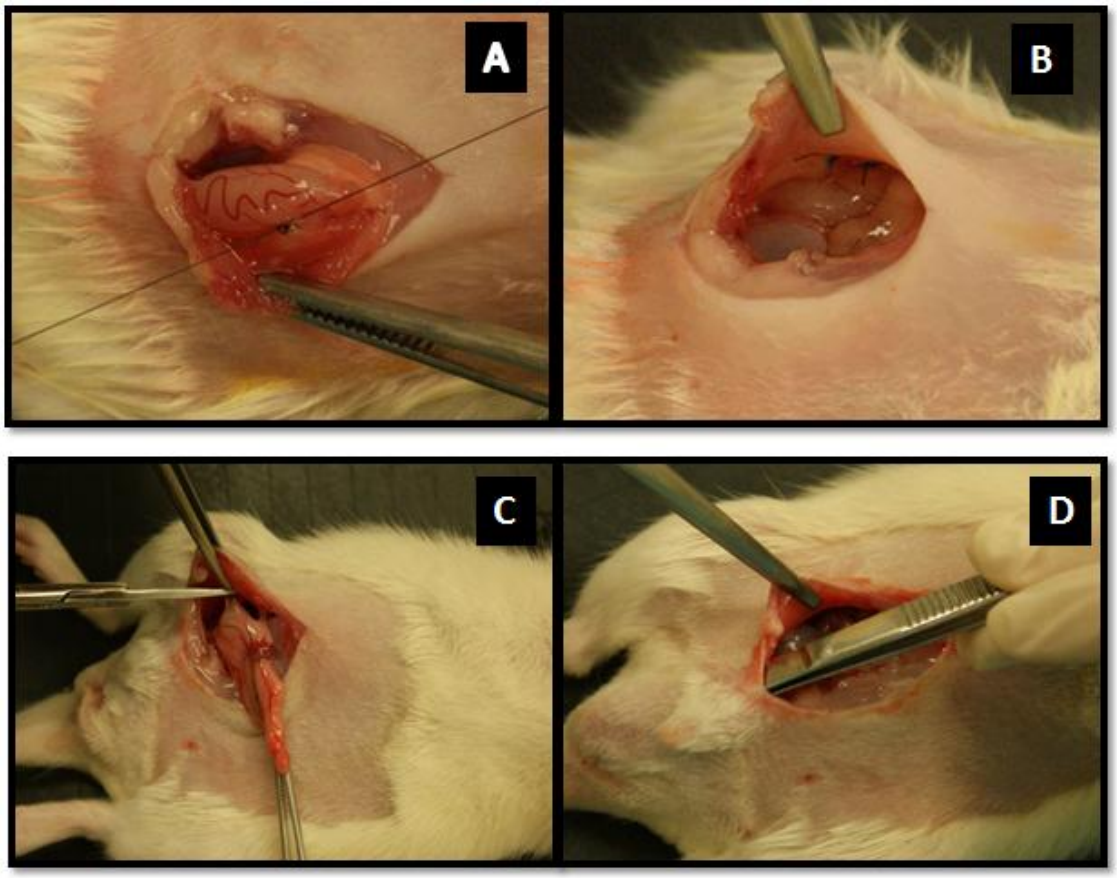

Figure 2. **(A,B)** Surgery for establishment of experimental cryptorchidism in 3-week old Sprague-Dawley rats. **(C,D)** Surgery for orchidopexy in 6-week old animals.

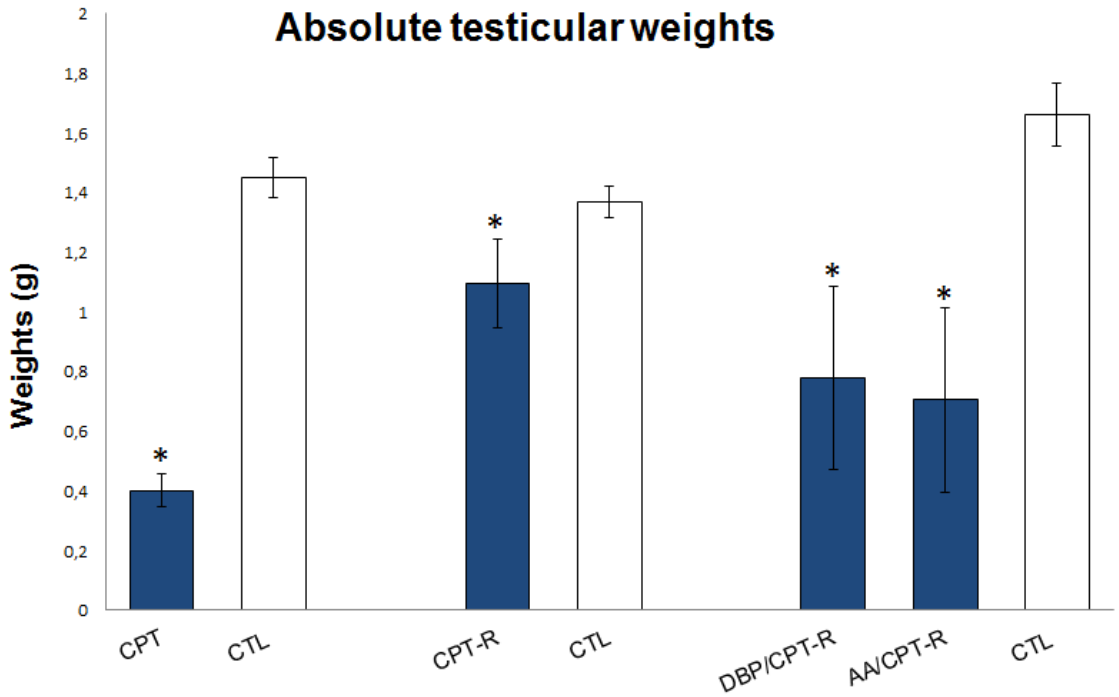

Figure 3. Absolute testicular weights (g). Data are expressed as mean ± standard deviation. The surgeries for cryptorchidism (CPT) were performed when the animals were 3-week old. The surgical reversion of cryptorchidism (orchidopexy, CPT-R) was done 3 weeks later, when the animals were 6-week old. Control (CTL) groups were submitted to sham operations at the same moments. Rats only submitted to surgical procedures were euthanized at the 14th week of age. Groups exposed to DBP or AA in *utero* and postnatally were also submitted to the surgeries, at the same age as the previous groups, but were euthanized at the 16th week of age. * Significant difference from the respective control group (Student's t test, $p < 0.05$).

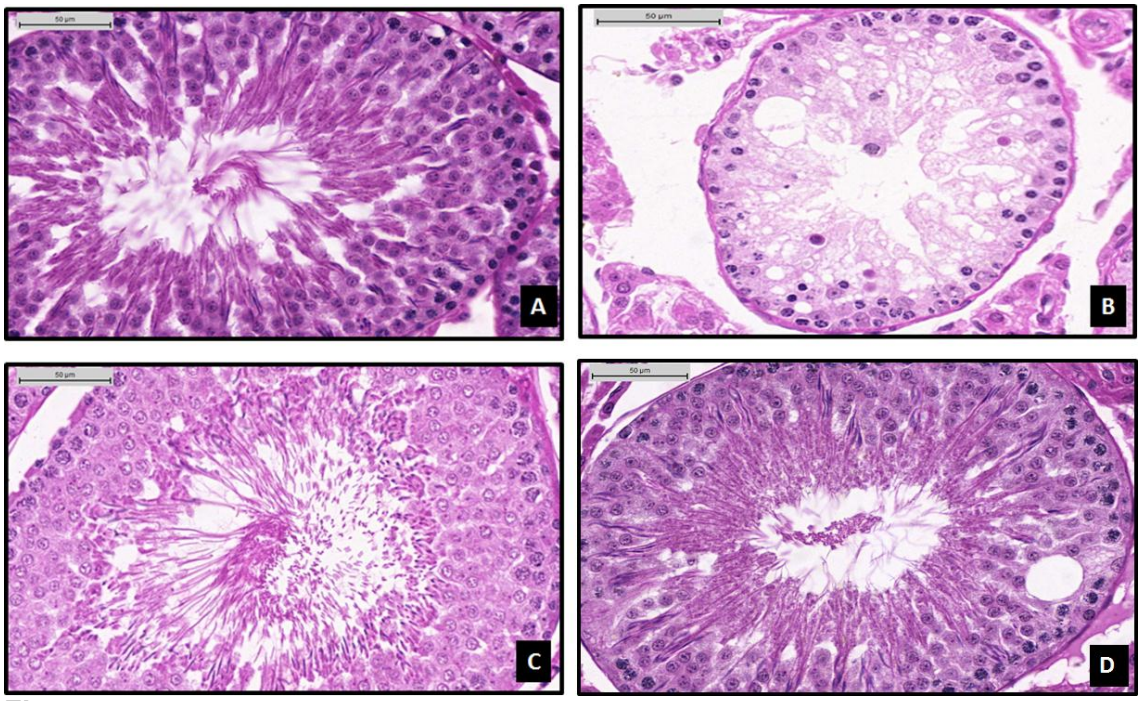

Figure 4. Testes of Sprague-Dawley rats (H&E) with regressive changes of the germinal epithelium. **(A)** and **(C)** Control groups submitted to sham surgeries for cryptorquidism and cryptorchidism-orchidopexy, respectively. **(B)** Cryptorquidic group (CPT) – surgery performed at the 3rd week and euthanasia at the 14th week: extremely altered seminiferous tubules showing almost absolute absence of germ cells, with few spermatogonia reaching the lumen of the tubule and vacuoles within the thickness of the germinal epithelium. **(D)** Reverted cryptorquidic group (CPT-R) – surgery for cryptorchidism at the 3rd week, orchidopexy at the 6th week and euthanasia at the 14th week: the germinal epithelium practically reverted to the normal.

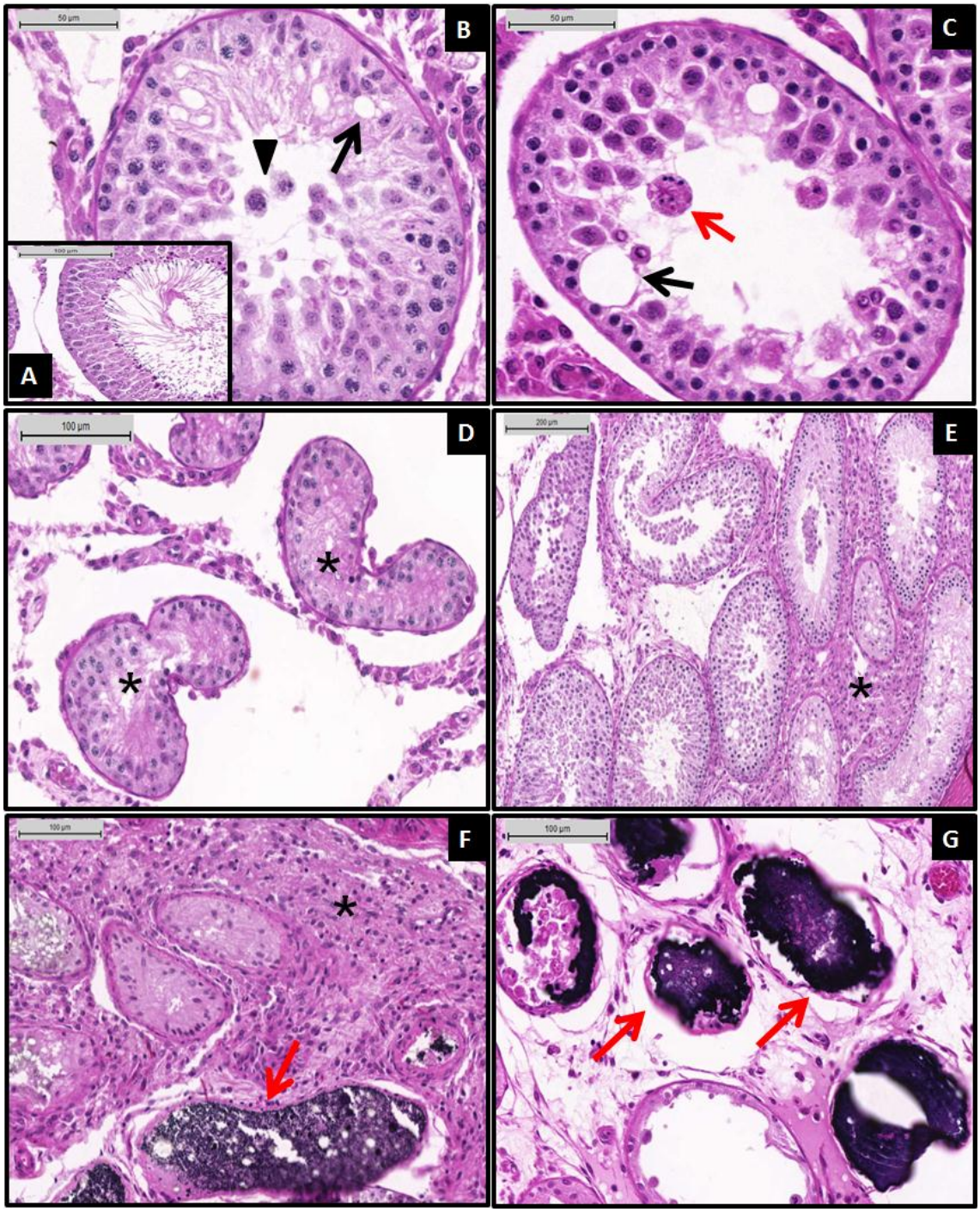

Figure 5. Testes from male Sprague-Dawley rats (H&E). **(A)** Control animal. **(B, D, F)** Exposure to DBP *in utero* and postnatally, cryptorchidism at the 3rd week, orchidopexy at the 6th week and euthanasia at the 16th week. **(C, E and G)** Exposure to AA *in utero* and postnatally, cryptorchidism at the 3rd week, orchidopexy at 6th week and euthanasia at the 16th week. **(B)** Epithelial vacuolation (black arrow) and germ cells exfoliation (arrowhead). **(D)** atrophic seminiferous tubules, spermatogenesis arrest, interstitial edema. **(F)** "Apparent" Leydig cell hyperplasia (*) and intratubular calcification (red arrow). **(C)** Vacuolization (black arrow), multinucleated germ cells undergoing apoptosis (red arrow) and hypercromatic nuclei. **(E)** **"**Apparent" Leydig cell hyperplasia (*). **(G)** Intratubular calcification (red arrows).

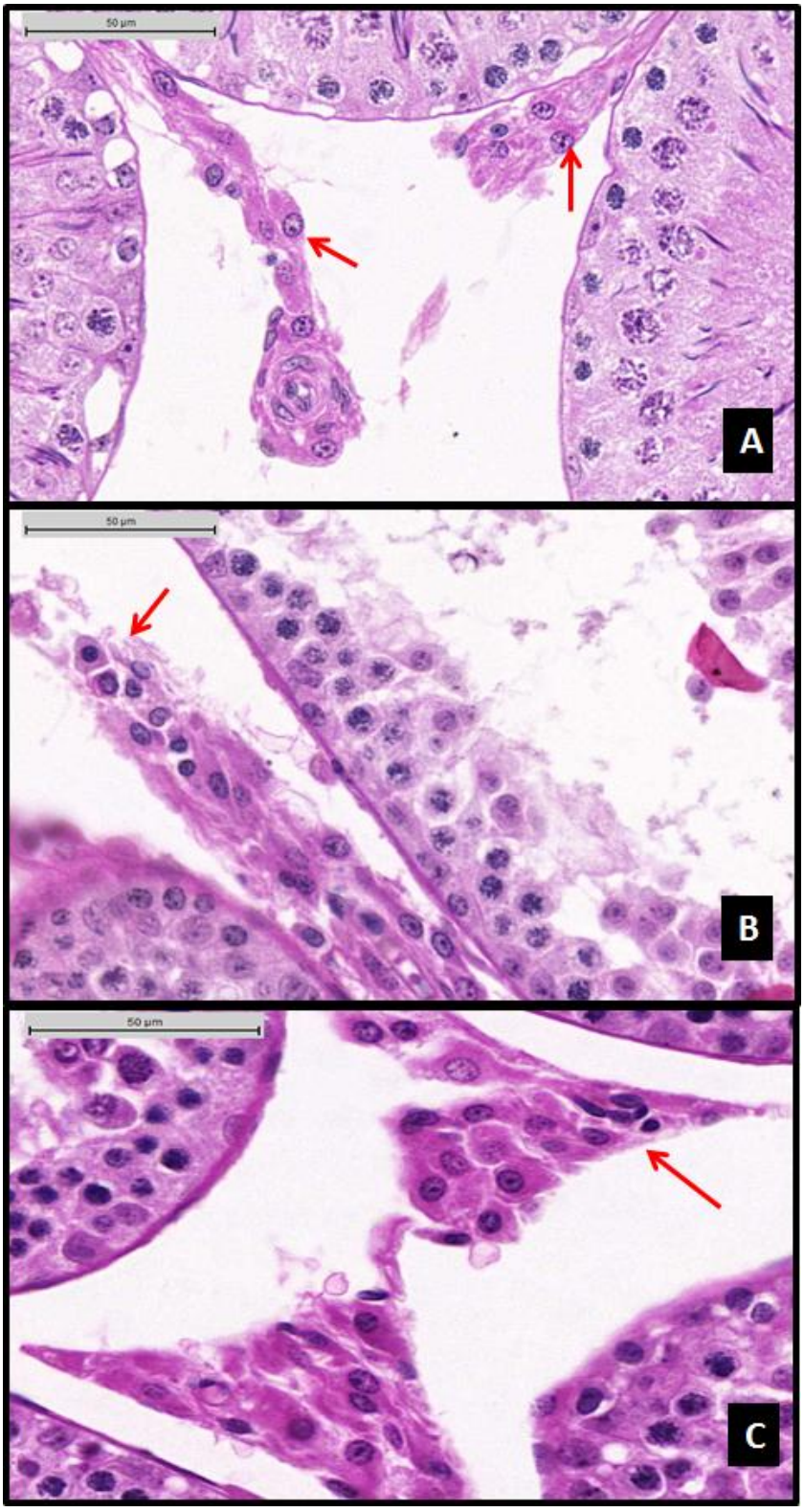

Figure 6. Sprague-Dawley rats Leydig cells (H&E). **(A)** Control: eosinophilic rounded cells grouped within the interstitium, near to small vessels. **(B)** Exposure to DBP *in utero* and postnatally, cryptorchidism at the 3rd week, orchidopexy at the 6th week and euthanasia at the 16th week. Cells with reduced cytoplasm and nucleus not always prominent. **(C)** Exposure to AA *in utero* and postnatally, cryptorchidism at the at 3rd week, orchidopexy at 6th week and euthanasia at the 16th week: polygonal and spindle cells.

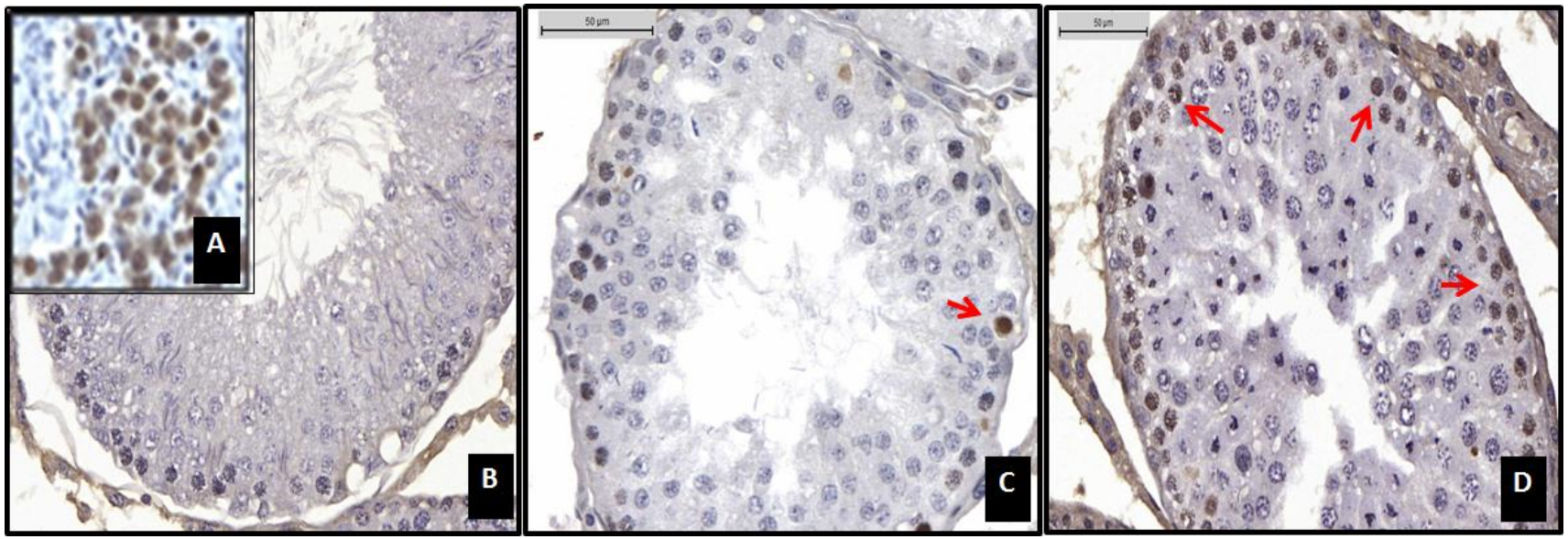

Figure 7. Immunohistochemistry for OCT 3/4 (400x). (A) Human seminoma used as positive control of the reaction; (B) Control Sprague-Dawley rat - no nuclear staining in germ cells; (C and D) Sprague-Dawley rats exposed to DBP and AA, respectively: Positive nuclear staining in germ cells (red arrows), with a predominance of labeled cells in the AA group (D).

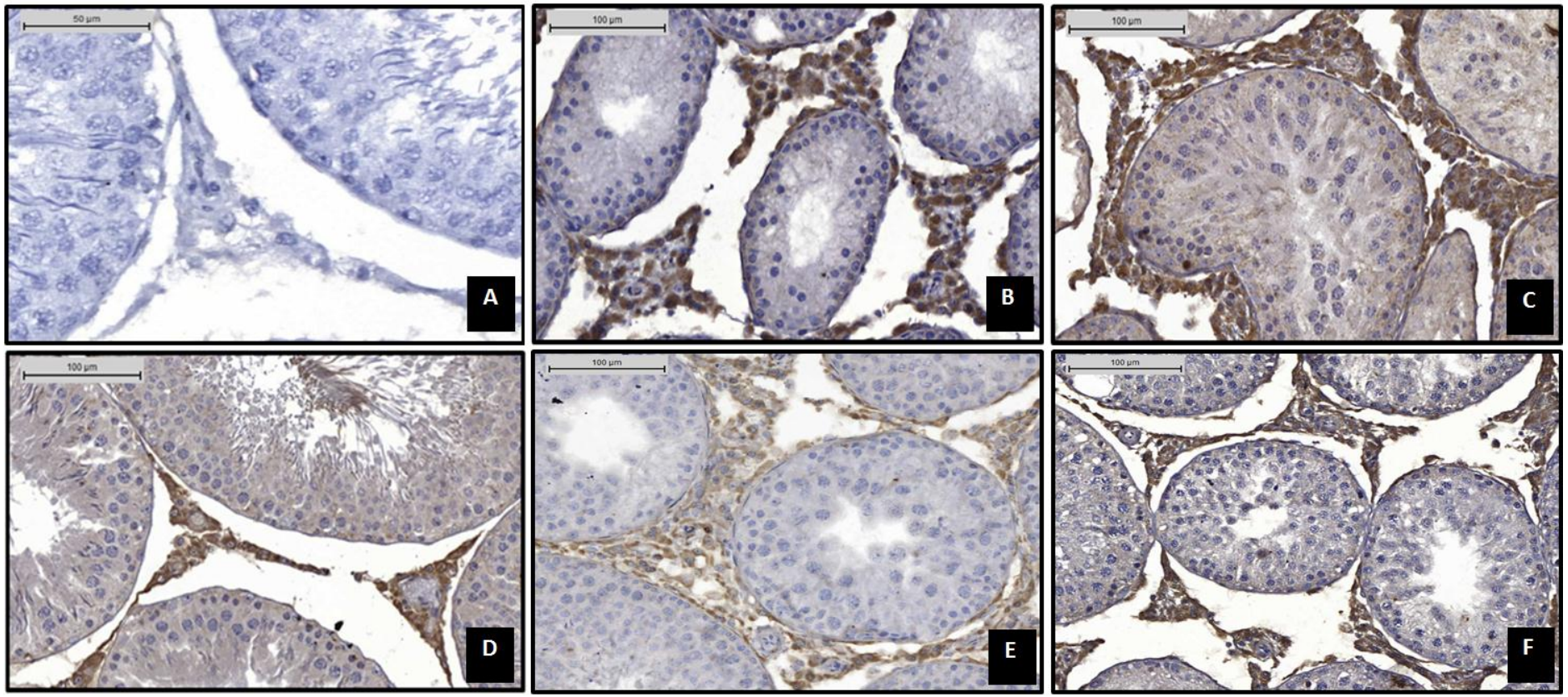

Figure 8. Immunohistochemistry for LHR. **(A)** Negative control – no primary antibody in the immunohistochemical reaction. **(D)** Control. **(B)** Cryptorchidism in 3rd week and euthanasia in the 14th week of life. **(C)** Cryptorchidism at the 3rd week, orchidopexy at the 6th week and euthanasia at the 14th week of life. **(E)** Rat exposed to DBP *in utero* and postnatally, cryptorchidism at 3rd week, orchidopexy at 6th week and euthanasia at the 16th week of life. **(F)** Rat exposed to AA *at utero* and postnatally, cryptorchidism at 3rd week, orchidopexy at 6th week and euthanasia at the 16th week of life. Weaker staining intensity in animals exposed to DBP when compared to control.

TABLES

Table 1. Body weights at birth and Anogenital distance (AGD) of the male offspring exposed to the testicular toxicants di-buthylphthalate (DBP) and acrylamide (AA) *in utero* at the postnatal day 1.

Experimental groups	N	Birth body weights (g)	Absolute AGD (mm/g)	Relative AGD (mm/g)[1]
DBP	36	7,0 [6,0-7,0]	3,5 [3,0-4,0]	1,830 [1,651-2,000]
AA	40	7,0 [7,0-7,0]	4,0 [4,0-4,0]	2,091 [2,000-2,091]
CTL	16	7,0 [6,0-7,0]	4,0 [3,0-4,0]	2,091 [1,568-2,091]

N = number of animals. Rat exposed either to DBP or to AA *in utero* and postnatally, cryptorchidism in 3rd week, orchidopexy in 6th week and euthanasia in the 16th week of life. Control (CLT) rats were not exposed to chemicals nor submitted to surgical procedures.. [1]Relative anogenital distance (AGD) = AGD/weights$^{1/3}$. AGD expressed as median [Q_1 – Q_3]. Statistical analysis by Mann-Whitney test.

Table 2. Final body weights and relative weights (%) of ventral prostate, seminal vesicle, epididymis and liver.

	Body weights (g)		Ventral prostate relative weights (%)		Seminal vesicle relative weights (%)		Epididymis relative weights (%)		Liver relative weights (%)	
	Experimental	**Control**	**Experimental**	**Control**	**Experimental**	**Control**	**Experimental**	**Control**	**Experimental**	**Control**
CPT	404 ± 40,5 (10)	399 ± 10,0 (5)	0,099 ± 0,014*	0,138 ± 0,052	0,226 ± 0,028	0,195 ± 0,070	0,044 ± 0,006*	0,117 ± 0,006	3,418 ± 0,214	3,431 ± 0,229
CPT-R	427 ± 20,8 (7)	411 ± 13,7 (5)	0,120 ± 0,022	0,125 ± 0,016	0,211 ± 0,029	0,239 ± 0,007	0,071 ± 0,009*	0,105 ± 0,008	3,301 ± 0,171	3,270 ± 0,179
DBP/ CPT-R	456 ± 22,5 (10)	456 ± 30,4 (6)	0,111 ± 0,014	0,124 ± 0,015	0,232 ± 0,038	0,213 ± 0,046	0,053 ± 0,024*	0,114 ± 0,008	3,811 ±0,247*	3,451 ± 0,199
AA/ CPT-R	374 ± 20,7 (11)*		0,137 ± 0,019		0,250 ± 0,043		0,070 ± 0,021*		3,632 ± 0,176	

Data expressed as mean ± standard deviation. Student's t test. CPT: cryptorchidism at 3rd week and euthanasia at the 14th week of life (CTL: false cryptorchidism surgery at 3rd week and euthanasia at the 14th week of life). CPT-R: cryptorchidism at 3rd week, orchidopexy at 6th week and euthanasia at the 14th week of life (CTL: false cryptorchidism surgery at 3rd week, false orchidopexy surgery at 6th week and euthanasia at the 14th week of life). DBP and AA: exposure to DBP or AA (*at utero* and postnatal), cryptorchidism at 3rd week, orchidopexy at 6th week and euthanasia at the 16th week of life (CTL: false cryptorchidism surgery at 3rd week, false orchidopexy surgery at 6th week, without exposure to chemical and euthanasia at the 16th week of life). * Significant difference from the respective control group ($p < 0.05$).

Table 3. LHR immunoreactivity in Leydig cells of male Sprague-Dawley rats exposed to criptorchidism/orchidopexy and testicular toxics[a]

	Grupos			Controle		
	+	++	+++	+	++	+++
CRPT	0	2	3	0	0	3
CRPT-R	0	2	3	0	0	3
DBP/CPT-R	3*	2	0	0	1	2
AA/CPT-R	0	0	5			

Mann-Whitney's t test. CPT: cryptorchidism in 3rd week and euthanasia in the 14th week of life (CTL: false cryptorchidism surgery in 3rd week and euthanasia in the 14th week of life). CPT-R: cryptorchidism in 3rd week, orchidopexy in 6th week and euthanasia in the 14th week of life (CTL: false cryptorchidism surgery in 3rd week, false orchidopexy surgery in 6th week and euthanasia in the 14th week of life). DBP and AA: exposure to DBP or AA (*in utero* and postnatal), cryptorchidism in 3rd week, orchidopexy in 6th week and euthanasia in the 16th week of life (CTL: false cryptorchidism surgery in 3rd week, false orchidopexy surgery in 6th week, without exposure to chemical and euthanasia in the 16th week of life). Asterisks (*) indicate significant difference compared to the respective control group ($p < 0.05$) .Immunoexpression was evaluated as weak (+), medium (++) and strong (+++).

Conclusões Finais do Trabalho

A criptorquidia experimental foi capaz de promover dano testicular e prejudicar a morfologia do epitélio seminífero, à semelhança dos achados na criptorquidia humana. Os efeitos foram revertidos pela cirurgia de orquidopexia.

A exposição *in utero* a 500 mg/kg DBP ou 10 mg/kg AA não resultou em alteração de distância anogenital em prole masculina. No entanto, a desregulação endócrina induzida por DBP (prejuízo na esteroidogênese) pode ser sugerida uma vez que foi observada mudança de imunoexpressão de LHR pelas células de Leydig.

Exposição *in utero* e pós-natal ao DBP ou à AA associado a criptorquidia/orquidopexia induziu prejuízo na morfologia do epitélio seminífero. Particularmente importante foi a descoberta de células germinativas com expressão imunohistoquímica positiva para um dos marcadores de tumor de células germinativas humanas (OCT ¾ antígeno positivo), o que pode preceder o desenvolvimento TGCT neste modelo de rato.

No geral, as nossas observações sugerem que o modelo utilizado que associa cirurgias e exposição a substâncias químicas, embora não seja uma tradução das condições naturais que ocorrem nos homens, pode ser útil para o estudo e compreensão dos fenômenos subjacentes a instalação de SDT e suas consequências imediatas e tardias.

Anexos

Universidade Estadual de Campinas - UNICAMP
Centro Multidisciplinar para Investigação Biológica na
Área da Ciência em Animais de Laboratório – CEMIB
International Council For Laboratory Animal Science
ICLAS Network Member for Promotion of Animal Quality in Research
www.cemib.unicamp.br

Prof. João Lauro Viana de Camargo

CPF 513.355.738-87

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: ração, 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.

Drª. Sônia Cano Montebelo Rachel
CRMV-SP: 4462 - Médica Veterinária
E-mail : soniacmr@cemib.unicamp.br

Prof.Dr.Rovilson Gilioli
Diretor do Centro Multidisciplinar para
Investigação Biológica
CEMIB/UNICAMP
E-mail: diretoria@cemib.unicamp.br

Rua Cinco de Junho, 230 – Cidade Universitária Zeferino Vaz – Barão Geraldo – CEP: 13.083-970 – Campinas - SP.
Fone/Fax: (19) 3521-7652 - Universidade Estadual de Campinas – CNPJ 46.068.425/0001-33

unesp

UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

FMB BOTUCATU

CEEA Ética
Comissão de Ética em Experimentação Animal

Criada através da Portaria DFM nº 30 de 28/04/99

Certificado

Certificamos que o (Protocolo CEEA 926/2012) Estabelecimento de modelo de tumores de células germinativas em testículos de ratos Sprague-Dawley, a ser conduzido por Merielen Garcia Nascimento, orientada pelo Prof. Dr. João Lauro Vianna de Camargo, com a colaboração de Ana Paula Ferragut Cardoso, Deilson Elgui de Oliveira, Samuel Cohen, Sohei Kitazawa e Wilma de Grava Kempinas, com o apoio técnico de Paulo César Georgete e Paulo Roberto Cardoso, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os "ratos" são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEEA em 29/03/2012.

Profª Drª Maria Rosa Bet Moraes Silva
Presidente do CEEA

Alberto Santos Capelluppi
Secretário da CEEA

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fone/Fax: (0xx14) 3811-6143 e-mail secretaria: capellup@fmb.unesp.br

unesp **Universidade Estadual Paulista**
Faculdade de Medicina de Botucatu

Distrito Rubião Junior, s/nº - Botucatu – S.P.
CEP: 18.618-970
Fone: (14) 3880-1608/3880-1609
e-mail secretaria: capellup@fmb.unesp.br
Instituída na Faculdade de Medicina através da Portaria do Diretor nº 30 de 26/04/99

Comissão de Ética no Uso de Animais

Botucatu, 06 de agosto de 2014. Of. 29/2014-CEUA

Ilustríssimo Senhor
Prof. Dr. João Lauro Vianna de Camargo
Departamento de Patologia da
Faculdade de Medicina de Botucatu

Prezado Prof. João Lauro,

Atendendo à solicitação de Vossa Senhoria, informo que o Sub-Projeto de Pesquisa intitulado: "**Isolamento e caracterização de células germinativas testiculares de ratos Sprague-Dawley**" que fazia parte do Projeto de Pesquisa **(Protocolo CEEA 926-2012)** Estabelecimento de modelo de tumores de células germinativas em testículos de ratos Sprague-Dawley, foi **cancelado** sua execução, haja vista, que a solicitação de auxílio junto ao órgão de fomento foi negada. Em substituição a este, abaixo especificamos como se configura a história do Protocolo em questão:

Projeto Maior: (Protocolo CEUA 926-2012) "Estabelecimento de modelo de tumores de células germinativas em testículos de ratos Sprague-Dawley" de autoria de Merielen Garcia Nascimento, orientada por Vossa Senhoria e colaboradores aprovado por este colegiado em 29/03/2012, conta com os seguintes **Sub-Projetos** a saber:

Sub-Projeto I: (Protocolo CEUA 926-2012-A) "Estudo da maturação e ultraestrutura de células de Sertoli em Modelo experimental de criptorquidia e orquidopexia" conduzido por **Ligia Maria Gomide**, orientada pelo Prof. Titular João Lauro Vianna de Camargo e Co-orientada por Merielen Garcia Nascimento, com objetivo de **Dissertação de Mestrado**, aprovado pela Presidência da CEUA em 22 de maio de 2.014, através do ofício 14/2014.

Sub-Projeto II: (Protocolo CEUA 926-2012-B) "CRIPTORQUIDIA E EXPOSIÇÃO IN ÚTERO AO DI(N-BUTIL) FTALATO e à ACRILAMIDA- AVALIAÇÃO DAS CÉLULAS DE LEYDIG" que será conduzido por **Nathália Pereira de Souza**, orientada pelo Prof. Titular João Lauro Vianna de Camargo e Co-orientada por Merielen Garcia Nascimento, e pesquisadores associados Dda Ana Paula Ferragut Cardoso, Mda Ligia Maria Gomide e Dr. Samuel Cohen, com objetivo de **Dissertação de Mestrado**, aprovado pela Presidência da CEUA em **05/08/2014.**

Atenciosamente,

Profª Adjunta Noeme Sousa Rocha
Vice-Presidente da CEUA