UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E BIOTECNOLOGIA

BÁRBARA CAMPOS JORGE

EXPOSIÇÃO AO BENZO(A)PIRENO EM RATOS MACHOS DO PERÍODO JUVENIL ATÉ A PERIPUBERDADE: REPERCUSSÕES NA VIDA ADULTA EM PARÂMETROS REPRODUTIVOS E IMPACTOS NA PROLE

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Orientadora: **Profa. Dra. Arielle Cristina Arena**

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Resumo

Entre as substâncias com potencial de desregulação endócrina, destaca-se o Benzo(a)pireno (BaP), um poluente orgânico persistente e amplamente difundido no ambiente. É gerado pela combustão incompleta de compostos orgânicos e está presente na fumaça de cigarro, na exaustão de automóveis, em alimentos e água contaminados. Estudos demonstram que o BaP se acumula em órgãos vitais e reprodutores, incluindo o testículo, e pode interferir no processo de esteroidogênese, através da interação com a proteína StAR. Sabe-se que substâncias que atuam como desreguladores endócrinos (DEs) podem gerar prejuízos não somente no indivíduo exposto, mas também nas gerações subsequentes, via células germinativas. A exposição aos DEs torna-se mais relevante em períodos hormônio-dependentes, como a gestação, a infância e a peripuberdade, denominados de janelas críticas do desenvolvimento. Assim, torna-se fundamental a investigação da exposição ao BaP durante uma janela crítica do desenvolvimento (juvenil e peripuberdade) e avaliar quais são as repercussões disto na vida reprodutiva, bem como os possíveis impactos no desenvolvimento e reprodução da prole, via paterna. Para tal, 40 ratos machos Wistar no período juvenil (23 dias de idade) foram distribuídos em quatro grupos experimentais, sendo um controle (óleo de milho + DMSO); e três grupos que receberam diferentes doses de BaP: 0,1, 1,0 ou 10 μg/kg/dia. A exposição ao poluente ocorreu durante 31 dias consecutivos, do dia pós-natal (DPN) 23 ao 53, via oral (gavage). Durante o tratamento, foram avaliados sinais clínicos de toxicidade e a instalação da puberdade, e na vida adulta (DPN 90) foram realizados o comportamento sexual, teste de fertilidade e acasalamento natural com fêmeas não tratadas (gerando a prole de interesse). Posteriormente, os órgãos vitais, reprodutores e sangue foram coletados para as seguintes análises: parâmetros espermáticos (morfologia, motilidade e contagem), hematológicos, histológicos do testículo (dinâmica espermatogênica, contagem e volume das células de Leydig) e quantificação da proteína StAR. Na prole feminina, foram avaliados: parâmetros inicias do desenvolvimento sexual, instalação da puberdade, ciclo estral, teste de fertilidade e histologia do ovário. Na prole masculina, foram avaliados: parâmetros iniciais do desenvolvimento sexual, descida testicular, separação prepucial e parâmetros espermáticos na vida adulta. A exposição ao BaP causou prejuízos nos animais expostos (geração parental-F0), comprometendo parâmetros espermáticos, hematológicos e histológicos do testículo, principalmente no grupo de menor dose. Além disso, o BaP

demonstrou ter um efeito multigeracional. A prole feminina e masculina (F1) apresentaram alterações nos parâmetros do desenvolvimento sexual e na fertilidade na vida adulta (fêmeas), indicando que a exposição ao BaP acarretou em uma programação do desenvolvimento de origem paterna para alterações reprodutivas, em ambos os sexos. O padrão de resposta observada nesse estudo foi uma curva dose-resposta não-monotônica, característica de substâncias que se comportam como DEs. Assim, o benzo(a)pireno, neste modelo experimental, é capaz de causar impactos negativos na reprodução na vida adulta dos animais expostos durante o período juvenil até a peripuberdade, bem como de causar efeitos multigeracionais, comprometendo o desenvolvimento reprodutivo de seus descendentes. Aprovado pelo comitê de ética 958/2017 (CEUA/IBB).

Palavras-chaves: peripuberdade, desregulador endócrino, benzo(a)pireno, fertilidade, programação paterna.

Abstract

Among substances with potential for endocrine disruptor, benzo(a)pyrene (BaP), a persistent organic pollutant widely distributed in the environment, stands out. It is generated by the incomplete combustion of organic compounds and is present in cigarette smoke, exhaust from cars, contaminated food and water. Studies have shown that BaP accumulates in vital and reproductive organs, including the testis; and may interfere with the steroidogenesis process through interaction with the StAR protein. It is known that substances that act as endocrine-disrupting chemicals (EDCs) can generate damages not only in the exposed individual, but also in subsequent generations by germ cells. Exposure to EDCs becomes more relevant in hormone-dependent periods, such as gestation, childhood and peripuberty, called critical windows of development. Thus, it is essential to investigate exposure to BaP during the critical development window (juvenile and peripuberty) and to investigate the repercussions of this on reproductive life and the possible impacts on the development and reproduction of the offspring, paternal way. For this, 40 male Wistar rats were used in the juvenile period (23 days of age) and distributed into four experimental groups, one control (corn oil + DMSO); and three groups receiving different doses of BaP: 0.1, 1.0 or 10 μg/kg/ day. The exposure to pollutant occurred during 31 consecutive days, from the postnatal day (PND) 23 to 53, via oral (gavage). During the treatment, clinical signs of toxicity and preputial separation were evaluated, and sexual behavior, fertility testing and natural mating with untreated females (generating offspring of interest) were performed in adulthood. Subsequently, vital organs, breeding and blood were collected for the following analyzes: sperm parameters (morphology, motility and counting), hematological, histological of the testis (spermatogenic dynamics, counting and volume of Leydig cells) and StAR protein quantification. In the female offspring, the following parameters were evaluated: initial reproductive parameters of the development, puberty, estrous cycle, fertility test and ovary histology. In the male offspring, the following were evaluated: initial reproductive parameters of development, testicular descent, preputial separation and sperm parameters in adult life. From the parental generation (F0), the spermatic, hematological and histological parameters of the testis were altered, mainly in the lower dose group, showing losses in sperm quality of these animals. In addition, BaP has been shown to have a multigenerational effect. Female and male offspring (F1) presented changes in the parameters of sexual development and fertility

in adult life (females), indicating that the exposure to BaP resulted in a developmental programming from paternal origins to reproductive alterations in both sexes. The response pattern observed in this study was a non-monotonic dose-response curve, characteristic of substances that behave as EDCs. Thus, benzo(a)pyrene in this experimental model can cause negative impacts on reproduction in adult life of exposed animals during the juvenile period until peripuberty, as well as causing multigenerational effects, compromising the reproductive development of their offspring. Approved by ethics committee, nº 958/2017 (CEUA/IBB).

Keywords: peripuberty, endocrine disruptor, benzo(a)pyrene, fertility, paternal programming.

Sumário

Lista de abreviaturas (português)

AhR – receptor de hidrocarboneto de arila

ARNT – translocador nuclear do hidrocarboneto de arila

BaP – benzo(a)pireno

BHT – barreira hemato-testicular

BPDE – benzo(a)pireno-diol-épóxido

CG – células da granulosa

CGP – células germinativas primordiais

CL - células de Leydig

CS – células de Sertoli

CT – células da teca

DDT – diclorodifeniltricloroetano

DE – Desreguladores endócrinos

DG – dia gestacional

DPN – dia pós-natal

FSH – Hormônio folículo estimulante

HAP – hidrocarboneto aromático policíclico

HHG – hipotálamo-hipófise-gônada

LDL – lipoproteínas de baixa densidade

LH – Hormônio luteinizante

LHRH – Hormônio liberador de gonadotrofinas

OP – oócitos primários

SC – espermatócito

SF1 – fator esteroidogênico 1

SG – espermatogônia

StAR – proteína reguladora aguda esteroidogênica

SZ - espermatozoide

T – testosterona

VLDL – lipoproteínas de muito baixa densidade

Lista de abreviaturas (inglês)

AGD – anogenital distance

APA – adequate for pregnancy age

BaP – benzo(a)pyrene

DOHaD – development origins of health and disease

DSP – daily sperm production

EDC – endocrine disrupting chemical

GD – gestational day

LC – Leydig cell

LPA – large for pregnancy age

NMDR – non-monotonic dose response

PAH – polycyclic aromatic hydrocarbon

PND – post-natal day

SD – standard desviation

SEM – standard error mean

SPA – small for pregnancy age

StAR – Steroidogenic acute regulatory protein

INTRODUÇÃO

O ciclo reprodutivo masculino envolve processos complexos e sensíveis, os quais são controlados pelo sistema endócrino. Estes processos podem ser perturbados após exposição a compostos tóxicos exógenos em diferentes estágios do ciclo e por diversos modos de ação (Moore et al., 2016), acarretando em alterações genéticas e anomalias reprodutivas, que muitas vezes só são detectadas mais tarde, até mesmo em gerações futuras.

Os mecanismos envolvidos na toxicidade reprodutiva dependem do estágio do desenvolvimento em que a exposição ocorre, seja na vida intrauterina, na infância, na puberdade ou na vida adulta (Mantovani e Fucic, 2014; Perobelli, 2014). As alterações mais frequentes incluem criptorquidia, hipospadia, anomalias dos canais excretores e de glândulas acessórias, tumores testiculares e espermatogênese alterada (Sousa et al., 2017).

Para se identificar as alterações causadas por agentes externos, é necessário que se conheça a formação e a fisiologia normal dos órgãos de interesse no modelo experimental escolhido e, assim, pode-se identificar e quantificar as possíveis alterações desencadeadas por substâncias tóxicas neste grupo de indivíduos.

1. Desenvolvimento ontogenético do sistema genital

A determinação sexual de um indivíduo refere-se a um processo complexo que abrange a fertilização, diferenciação gonadal e a diferenciação sexual no cérebro. Nos mamíferos, o sexo genético é determinado por complementos cromossômicos na fertilização (XX para fêmeas ou XY para machos) (She e Yang, 2016). Tanto os ovários quanto os testículos derivam de uma estrutura denominada gônada bipotente, e o seu desenvolvimento em direção a um destes órgãos dá-se através do arranjo entre a ativação ou repressão de vias de sinalização na linhagem celular de suporte (células de Sertoli – XY ou células da granulosa - XX) (Svingen e Koopman, 2013).

Nos mamíferos, o sistema genital surge a partir do mesoderme intermediário. Logo após a formação dos ductos mesonéfricos, inicia-se a formação adjacente dos ductos paramesonéfricos (Arrotia et al., 2012). Nos machos, a diferenciação sexual gonadal se caracteriza através da regressão dos ductos paramesonéfricos e o desenvolvimento dos ductos mesonéfricos em órgãos reprodutivos característicos do

sexo masculino, como o epidídimo, os ductos deferentes e as glândulas seminais. Já nas fêmeas, os ductos mesonéfricos regridem e os ductos paramesonéfricos diferenciam-se para formar o útero, as tubas uterinas e a parte próximal da vagina (Hannema e Hughes, 2007).

1.1.Desenvolvimento pré-natal do testículo em ratos

Os mamíferos apresentam uma mesma sequência de eventos para o desenvolvimento testicular, diferindo-se somente no espaço de tempo. A sequência básica de maturação dos testículos é: (I) formação pré-natal de cordões testiculares contendo gonócitos e células de Sertoli (CS - células de sustentação do testículo); (II) transformação de gonócitos em espermatogônias (células germinativas masculinas); (III) proliferação das espermatogônias e das células de Sertoli; (IV) maturação das CS e formação da barreira das células de Sertoli; (V) desenvolvimento dos espermatócitos, marcando o início da meiose; e (VI) a espermiogênese (citodiferenciação das espermátides redondas para as alongadas) (Picut, Ziejewski, Stanislaus, 2018).

Após a fertilização, o indivíduo XY possui o gene *SRY* (gene determinante de testículo localizado no cromossomo Y) que é capaz de induzir a diferenciação da linhagem das células de suporte em células de Sertoli (CS) e, consequentemente, a transformação da gônada bipotencial em testículo (Gubbay et al., 1990). Em roedores, do DG 10,5 ao 12,5 (dia gestacional), o gene *SRY* inicia a indução da expressão do *Sox9* (Sekido et al., 2008), no qual promove a organogênese do testículo através da ativação de genes pró-testículo e repressão dos genes pró-ovário. A expressão de Sox9 também desencadeia a cascata de genes essenciais à diferenciação das CS, atuando sinergicamente com SF1 (fator de transcrição) para interagir com o promotor *Amh* para iniciar a expressão de Amh (produz o hormônio antimülleriano) (Arango, Lovell-badge, Behringer, 1999). Esta expressão é mantida ao longo do desenvolvimento dos testículos por meio de vários *loopings* de feedback positivo, estimulando assim a via da determinação masculina gonadal (She e Yang, 2016).

Em conjunto, o *Sox9* é essencial para o início da expressão de Amh nas células de Sertoli e o *Sox8* reforça a função do *Sox9* na regulação positiva dos transcritos do *Amh* (Lovell-badge, Canning, Sekido, 2002). A expressão de *Sox9* então orquestra a proliferação de CS, a expansão das células somáticas, a migração e diferenciação necessárias para o desenvolvimento, a regressão dos primórdios femininos (hormônio

anti-Mülleriano) e a virilização somática (secreção de testosterona pelas células de Leydig).

Outros fatores importantes, o Fgf9 e Wnt4, são inicialmente expressos em células de suporte sexualmente indiferenciadas em ambas as gônadas XX e XY, e então sua expressão dimórfica (com alta Fgf9/baixa Wnt4 na gônada XY e baixa Fgf9/alta Wnt4 na gônada XX) torna-se aparente na gônada após DG 12,5 (Hiramatsu et al., 2009). Assim, o papel crucial da sinalização de *Fgf9* é suprimir a expressão de *Wnt4* e outros genes específicos de fêmeas e, assim, inibir a via feminina (Jameson, Lin, Capel, 2012). O *Dmrt1* antagoniza *Foxl2* (gene específico de fêmea) para manter o desenvolvimento do testículo pós-natal e o *Foxl2* também reciprocamente suprime o *Dmrt1* para garantir o desenvolvimento do ovário adulto feminino (Uhlenhaut et al., 2009).

Imediatamente após este início da expressão do *Sry*, o hormônio luteinizante (LH) já pode ser detectado no cérebro fetal (Aubert et al., 1985), e o primeiro aspecto morfológico do testículo é identificado no DG 13,5 (Jobling et al., 2011). O testículo fetal torna-se sensível a testosterona entre o DG 14,5 ao 15,5, período em que as células de Leydig fetais produzem testosterona (T) (Habert e Picon, 1984; Scott et al., 2009). Os níveis de testosterona aumentam rapidamente no feto, com um pico no DG 18 a 19 e declina-se imediatamente após o nascimento (Habert e Picon, 1984; Baum et al., 1991; Scott et al., 2009).

Morfologicamente, o testículo fetal é composto de cordões seminíferos, formados no DG 13,5 no rato, inseridos no estroma mesenquimal. Dentro destes cordões, temos duas populações celulares: os gonócitos (células germinativas primordiais) e as células de Sertoli, com características proliferativas. Dentro do estroma mesenquimal, existe uma população proeminente de células de Leydig fetais, sendo responsáveis por produzir testosterona, necessária para virilizar fenotipicamente o embrião em um macho, e mais especificamente, para virilizar o ducto mesonéfrico em epidídimo, ducto deferente e glândula seminal (Huhtaniemi e Pelliniemi, 1992).

1.2. Desenvolvimento pós-natal do testículo em ratos

O desenvolvimento sexual pós-natal de ratos ocorre em quatro estágios de desenvolvimento (Tabela 1) (Ojeda et al., 1980; Picut et al., 2015; Picut e Remick, 2017):

Tabela 1. Comparação cronológica dos estágios de desenvolvimento dos testículos em ratos e humanos.

Período rato/humano	Ratos	Humanos
Neonatal/Recém-nascido	DPN 0 – 7	0 – 28 dias
Infantil/Bebê	DPN 8 – 20	28 dias – 2 anos
Juvenil/Infância	DPN 21 – 32	2 – 11 anos
Peripubere/ Adolescente	DPN 33 – 60	11 – 14 anos

DPN: dia pós-natal. Adaptada de Picut e Remick, 2017.

Período neonatal: é caracterizado morfologicamente por pequenos túbulos seminíferos contendo gonócitos que estão se diferenciando em espermatogônias, células de Sertoli em proliferação, assim como células de Leydig fetais sofrendo rápida regressão no estroma testicular (Picut et al., 2015).

Período infantil: é caracterizado por uma rápida proliferação das espermatogônias e das células de Sertoli e continuação da proliferação e maturação de células de Leydig definitivas. O final deste período é caracterizado pela formação da barreira das células de Sertoli e do lúmen tubular (Picut et al., 2015).

Período juvenil: é caracterizado pelo início da espermatogênese com o desenvolvimento dos espermatócitos e a diferenciação das células das linhagens germinativas dos túbulos seminíferos e o início da espermiogênese. A produção de testosterona aumenta rapidamente após o DPN 28, relacionando-se com a maturação das células de Leydig adultas (Picut et al., 2015).

Período peripubere: há um aumento rápido e contínuo de testosterona, acompanhado de um aumento acentuado do diâmetro dos túbulos seminíferos e o fim da diferenciação das células germinativas, formando os espermatozoides (Tabela 2) (Picut et al., 2015).

1.3. Desenvolvimento pré-natal do epidídimo em ratos.

Sob a influência do hormônio antimulliriano (produzido pelas SC imaturas) e da testosterona (produzida pelas células de Leydig fetais), a porção cranial dos ductos mesonéfricos se diferencia para formar o epidídimo pós-natal (Arrotia et al., 2012).

1.4.Desenvolvimento pós-natal do epidídimo em ratos.

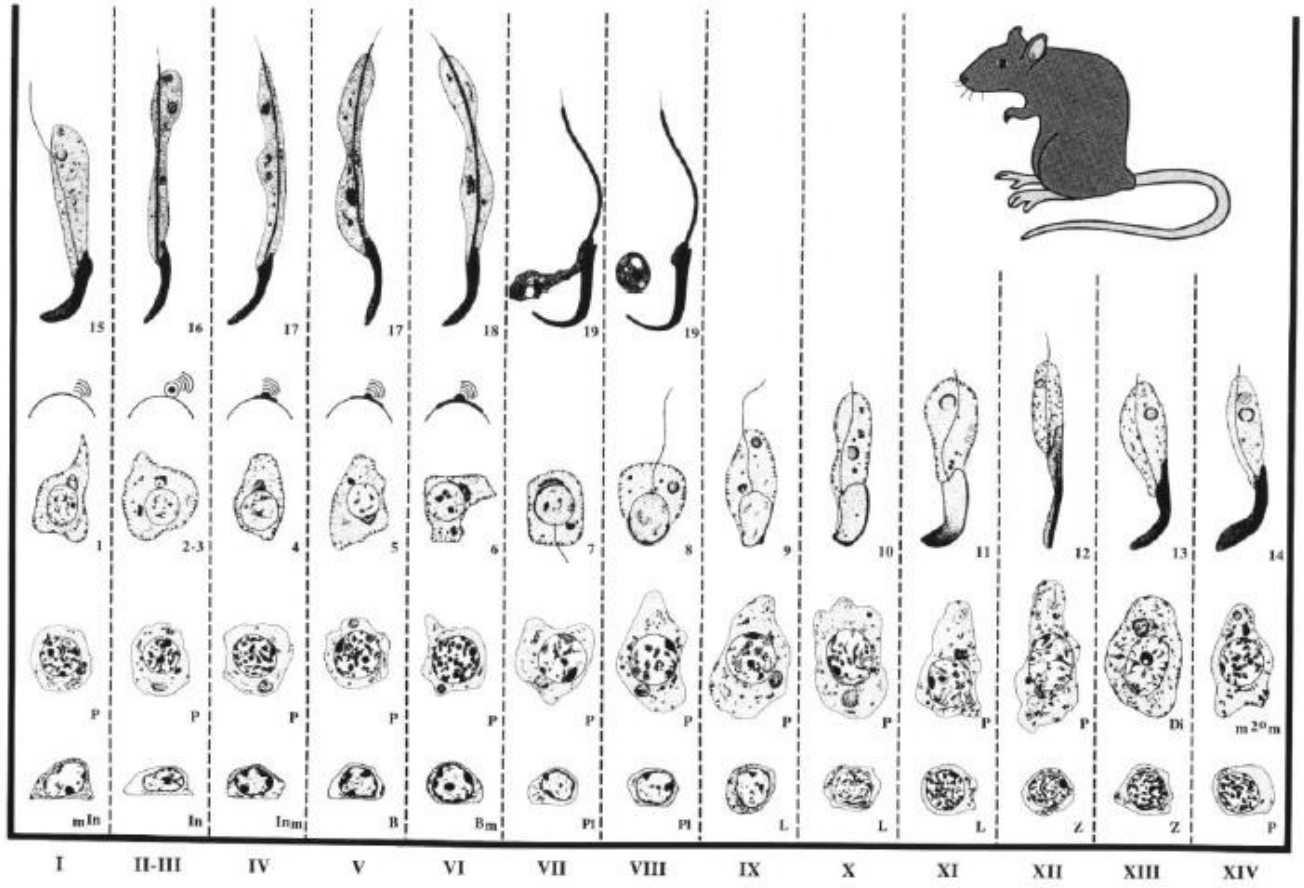
Enquanto a proliferação celular do ducto mesonéfrico parece ser dependente de andrógenos e fatores mesenquimais durante o desenvolvimento pré-natal, alguns fatores produzidos pelos testículos desempenham um papel adicional durante o desenvolvimento pós-natal (Hinton et al., 2011). Estes fatores são moléculas como androgênios, fatores de crescimento e enzimas que atuam na atividade secretora das células epiteliais do epidídimo e, posteriormente, nos espermatozoides que participam diretamente do processo de maturação epididimal (Robaire, Hinton, Orgebin-Crist, 2015). O desenvolvimento pós-natal do epidídimo de mamíferos pode ser dividido em três períodos (Sun e Flickinger, 1979): indiferenciação (do nascimento ao DPN 15), período de diferenciação (DPN 16 - 44) e período de expansão (> DPN 44).

Período de indiferenciação (do nascimento ao DPN 15): é caracterizada pela ausência de especialização das células epiteliais, crescimento e enovelamento do ducto epididimal. As primeiras alterações histológicas evidentes é o aparecimento de células halo que marca o fim do período de indiferenciação para o período de diferenciação (Robaire, Hinton, Orgebin-Crist, 2015).

Período de diferenciação (DPN 16 - 44): há uma rápida proliferação celular e diferenciação das células em principais, estreita, apical, basal, clara e halo, apresentando um gradiente no sentido cabeça-cauda (Limanowski et al., 2001). Acredita-se que tanto a testosterona quanto a chegada de células germinativas e fluido testicular contribuam para a diferenciação epididimária (Pryor et al., 2000). Como o epidídimo passa pela maior diferenciação celular durante esta fase, torna-se um alvo sensível para substâncias tóxicas de ação direta ou para qualquer fator que interfira na maturação testicular e na produção de testosterona.

Período de expansão (> DPN 44): os túbulos aumentam de tamanho e são preenchidos com espermatozoides. O epidídimo depende de andrógenos para manter a arquitetura e as funções epiteliais normais.

Tabela 2. Aspectos morfológicos do ciclo espermatogênico dos ratos



1.5.Desenvolvimento pré-natal do sistema genital feminino em ratas.

Por muito tempo, acreditava-se que para as gônadas bipotenciais se desenvolverem em ovários, seria necessário apenas a ausência do gene *SRY*. Recentemente, foi demonstrado que o processo de desenvolvimento ovariano depende da ativação de genes pró-ovário e repressão de genes pró-testículo, similarmente como ocorre no desenvolvimento testicular (Nicol et al., 2015). O chamado gene *Z*, correspondente do sexo feminino ao gene *SRY*, inibe vias determinantes para testículo enquanto promove a diferenciação ovariana (Nicol e Yao, 2014). O gene *Z* ativa outros genes, como o *WNT4* (Biason-Lauber et al., 2004), *RSPO1* (Chassot et al., 2008) e *FOXL2* (Pailhoux et al., 2002).

Ambos os genes Wnt4 e Rspo1 produzem proteínas que são expressas na gonada bipotencial e a sua expressão torna-se ovário-específica no DG 11,5 (Parma et al., 2006). Modelos de ratos *knockout* revelam que os genes *WNT4* e *RSPO1* são partes de uma via de sinalização comum que atua através da β-catenina (Figura 1) (Tomizuka et al., 2008). Alvos adicionais da via *Wnt4/Rspo1/β-catenina* foram identificados, incluindo a *folistatina (Fst*) e *Bmp2* (Manuylov et al., 2008), sendo que o papel do gene *FST* é prevenir a formação de vasculatura específica de testículo por meio da inibição da activina B, e do *gene FOXL2* é inibir a expressão do gene *SOX9* e a ativação de genes pró-ovarianos (Yao, Aardema, Holthusen, 2006).

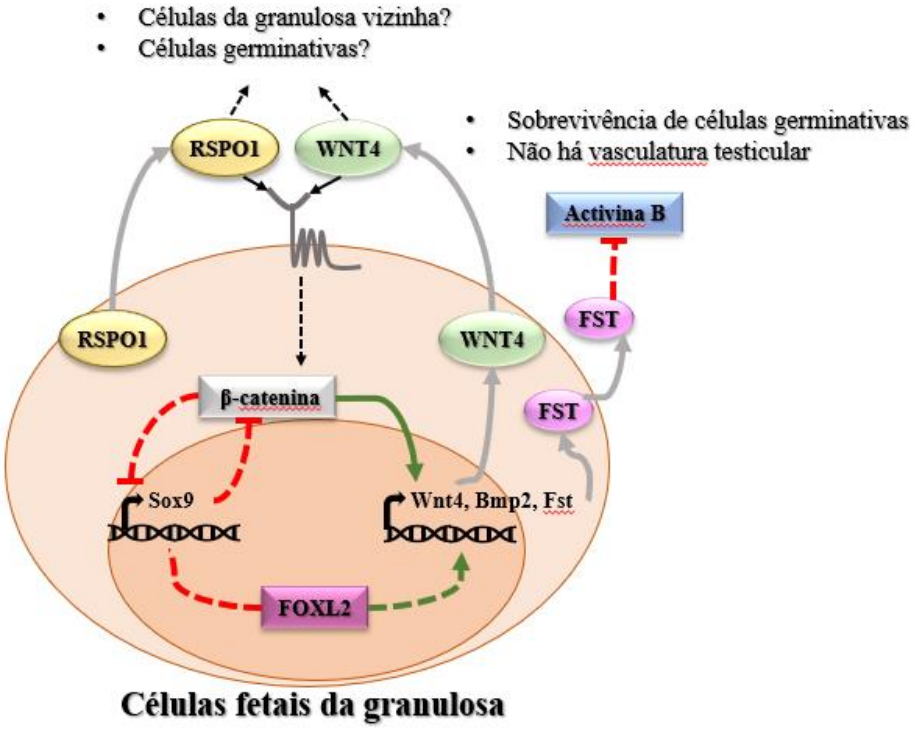


Figura 1. O desenvolvimento das células da granulosa fetal é promovido principalmente por duas proteínas, a Rspo1 e Wnt4, que atuam nas células pré-granulomatosas de maneira autócrina/parácrina. Com base na evidência de outros modelos, a via *Wnt4/Rspo1* ativa sinergicamente a β-catenina, que induz a transcrição de fatores ovarianos, como o *Fst, Bmp2* e o próprio *Wnt4*. O fator de transcrição FOXL2 participa na supressão de *Sox9* e ativação de outros genes ovarianos, como o *Fst.* Adaptada de Nicol e Yao (2014).

Morfologicamente, há uma intensa proliferação das células germinativas primordiais após a migração para as gônadas. A gametogênese feminina inicia-se na vida pré-natal, sendo que as células de suporte feminina (células da granulosa) se arranjam em torno dos oócitos, formando os folículos primordiais (Tingen, Kim, Woodruff, 2009). Os oócitos avançam através da prófase I e são mantidos no diplóteno, onde permanecem quiescentes aguardando a indução da maturação dependente de gonadotrofina na puberdade, quando se transformaram em oócitos primários (Jones, 2008).

1.6.Desenvolvimento pós-natal do sistema genital feminino em ratas.

Nas fêmeas, há cinco períodos do desenvolvimento identificáveis, com alterações microscópicas. Esses estágios incluem os períodos neonatal (DPN 0–7), infantil (DPN 8–20), juvenil (DPN 21–32), peripubere (DPN 33–37) e púbere (DPN 38–46) (Tabela 3) (Ojeda, Advis, Andrews, 1980; Ojeda e Skinner, 2006).

Estes estágios de desenvolvimento dependem principalmente de alterações fisiológicas do desenvolvimento e não são definidos apenas por alterações reprodutivas, exceto nos períodos peripubere e púbere. O período peripubere é definido como os 3 a 5 dias anteriores à primeira ovocitação, quando o hormônio luteinizante (LH) exibe diferenças nos surtos matinal e vespertino e quando o líquido intrauterino aparece pela primeira vez; a puberdade é marcada pela abertura vaginal e a primeira ovocitação nas ratas (Picut e Remick, 2017).

Tabela 3. Comparação cronológica dos estágios de desenvolvimento dos ovários em ratas e humanos.

Período rato/humano	**Ratos**	**Humanos**
Neonatal/Recém-nascido	DPN 0 - 7	0 – 28 dias
Infantil/Bebê	DPN 8 - 20	28 dias – 2 anos
Juvenil/Infância	DPN 21 - 32	2 – 12 anos

Peripuberdade	DPN 33 - 37	ND
Pubere/Adolescente	DPN 38 – 46	12 – 16 anos

ND – Não identificável. Adaptado de Picut e Remick (2017).

Período neonatal: é marcado pelo desenvolvimento de folículos primordiais e secundários estritamente controlado por fatores de crescimento (como FGF-2, BMP-4 e FGF-7) parácrinos e autócrinos produzidos pelo oócito (Skinner, 2008), pelas células da granulosa, ou por células estromais. As células da granulosa dos folículos primários começam a expressar os receptores LH e FSH após o DPN 5 (Ojeda e Skinner, 2006). Alguns folículos primários imaturos começam a se diferenciar e se encaminham para a medula ovariana.

Período infantil: no DPN 10, o desenvolvimento antral precoce ocorre, e a formação do antro é a característica morfológica marcante que marca a mudança do ovário da independência hipofisária (período neonatal) para a dependência pituitária (período infantil) (Skinner, 2008). Durante o período infantil, há um aumento nos níveis séricos de LH e FSH, atingindo um máximo no DPN 12 a 14 (Ojeda e Skinner, 2006). Após este período, inicia a produção de estrógeno e ocorre assim um feedback negativo, acarretando em uma queda súbita de LH e FSH (Ojeda e Skinner, 2006).

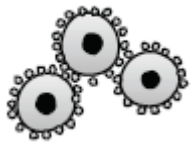
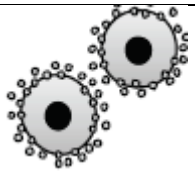
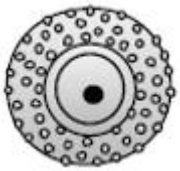
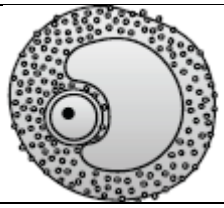
Período juvenil: a característica mais proeminente do período juvenil é a apoptose das células da granulosa e a atresia dos folículos. Os níveis séricos de FSH estão continuamente diminuindo, atingindo seu nível mínimo no DPN 30 (Ojeda e Skinner, 2006). Os níveis de LH diminuem durante a primeira metade do período juvenil, mas ao contrário dos níveis de FSH, começa a ser produzido em um padrão pulsátil e bimodal, com um pequeno surto de manhã e tarde. A maturação do padrão de produção de LH pulsátil e bimodal é devida ao início da retroalimentação positiva do estrógeno na produção de LH por volta do DPN 24 (Davis, Travlos, McShane, 2001). Os folículos corticais selecionados continuam a desenvolver-se devido, em parte, ao fato de os receptores FSH/LH atingirem um nível máximo de adultos no DPN 30, resultando em maior sensibilidade de certos folículos a níveis relativamente baixos de FSH sérica (Nicol e Yao 2015).

Período peripubere: se dá a partir do aparecimento do folículo ovulatório (0,9 a 1,0 mm de diâmetro) no córtex externo (Picut, 2017). Esse período dura de 3 a 5 dias e é definido como o momento em que há a secreção de LH em surtos, facilitando o

desenvolvimento de folículos ovulatórios. Os níveis de FSH aumentam somente após o DPN 35 (Ojeda e Skinner, 2006).

Período púbere: é marcado como a primeira ovocitação de folículos ovulatórios (0,9 a 1,0 mm de diâmetro), formando os corpos lúteos. Ocorre um mini-surto nos níveis de LH, sendo dependente da maturação do eixo hipotálamo-hipófise-gonadal (HHG), tendo assim níveis suficientes de estradiol sérico (Picut, 2017). O estradiol atua de maneira positiva tanto no hipotálamo quanto na liberação de LHRH (hormônio liberador do hormônio luteinizante) e na hipófise anterior, sensibilizando as células secretoras de LH ao efeito estimulatório da LHRH (Davis, Travlos e McShane, 2001). Durante esse estágio tardio do desenvolvimento folicular, os oócitos primários (após a obtenção dos cromossomos 4N) entram em sua primeira divisão meiótica e expelem um corpúsculo polar. Portanto, na ovocitação, um oócito secundário diploide é lançado para o oviduto. O processo de ovogênese está resumido na Tabela 4.

Tabela 4. Aspectos estruturais e morfológicos da ovogênese.

Folículo primordial	**Folículo primário**	**Folículo secundário/ crescimento**	**Folículo de Graff/ antral**
			
Estrutura folicular			
OP cercados por uma única camada achatada de CG.	OP cercado pela zona pelúcida (secretada pelas CG) e uma camada de GC com o formato cuboide.	OP rodeado por múltiplas camadas de GC com formato cuboide, mais as camadas internas e externas de CT.	A CG prolifera e produzem o fluido ovariano, formando o antro. As CGs que circundam o agora OS se diferenciam em coroa radiata e o cumulus oophorus mantém a ligação entre o oócito e o folículo.
Aspectos funcionais			
Formado na vida pré-natal e o OP está parado na meiose I.	Estágio independente de gonadotrofina: deslocamento cubóide por estímulos intra-ovarianos.	Estágio dependente de gonadotrofina: expressão aumentada dos receptores de FSH e de estrogênio nas CG. CT interna sintetiza	Funcionalmente preparado para a ovulação e transformação do OP para OS parados em metáfase II. A

		andrógenos que podem ser então aromatizados por CG.	corona radiata e oophorus cumulus são expelidos junto ao oócito durante a oocitação.

Adaptado de Nicol e Yao (2015). OP: oócitos primários; OS: oócitos secundários; CG: células da granulosa; CT: células da teca

Como todos os processos de formação dos órgãos reprodutores são altamente dependentes de hormônios e de uma complexa rede de ativação/repressão gênica que necessita ocorrer no intervalo de tempo correto, qualquer interferência neste processo pode gerar alterações significativas na fertilidade destes animais na vida adulta (Picut, Ziejewski, Stanislaus, 2018). Estes períodos sensíveis a modificações permanentes nos órgãos são chamados de janelas críticas do desenvolvimento, e são de grande importância para os estudos toxicológicos (Mantovani e Fucic, 2014). No entanto, apesar da importância destas fases, há uma escassez na literatura sobre os impactos de substâncias tóxicas durante o período juvenil até a peripuberdade, tornando-se relevantes estudos nesta área (Perobelli, 2014).

2. Peripuberdade como janela susceptível do desenvolvimento em estudos toxicológicos

A "puberdade" é um período complexo e contínuo de transição entre as fases juvenil até a vida adulta, e engloba várias alterações quando comparadas ao período infantil, com mudanças anatômicas (separação prepucial e abertura vaginal/primeiro estro nos ratos), histológicas (presença de espermatozoides no epidídimo e presença de folículos antrais) e hormonais (Wistuba et al. 2003; Ojeda e Skinner, 2006). O período peripubere abrange desde o início da puberdade até a maturidade sexual, sendo que o início da puberdade ocorre nos ratos machos no momento em que as espermátides no estágio 19 são vistas pela primeira vez nos túbulos do estágio VII (Marty et al., 2009; Picut et al., 2015), enquanto que nas fêmeas se dá com a primeira ovocitação, aproximadamente no DPN 40 (Picut et al., 2015), altamente regulados pelo sistema endócrino.

Em estudos toxicológicos, a peripuberdade merece atenção especial como já descrito anteriormente. Os indivíduos nessa fase do desenvolvimento são mais

susceptíveis aos desreguladores endócrinos (DEs), possivelmente devido ao aumento da produção hormonal responsável pela maturação dos órgãos reprodutivos (Chapin et al., 1997). Os DEs podem gerar efeitos adversos que podem ser visíveis durante a sua exposição em janelas críticas do desenvolvimento (gestação, lactação, infância e peripuberdade) ou se manifestar somente na vida adulta (Mantovani e Fucic, 2014); isto ocorre devido à capacidade dos DEs interferirem na programação do desenvolvimento (Mantovani e Fucic, 2014).

Durante estes períodos, sinais hormonais causam mudanças celulares e moleculares (expressão de genes específicos e/ou epigenética) e controla ou modifica a organização funcional ou estrutural dos tecidos (Gore et al., 2015). Assim, o equilíbrio entre os estrógenos/andrógenos é o componente chave do amadurecimento dos órgãos reprodutivos na puberdade, interagindo com fatores de crescimento a fim de regular o a maturação de todos os órgãos e sistemas durante a puberdade (Kasper-Sonnenberg et al., 2017). Assim, os DEs que podem reprogramar a vias de sinalização/diferenciação e levar as consequências ao longo da vida (Collman, 2011).

3. Desreguladores endócrinos

Embora muitos fatores possam interferir com a função reprodutiva, estudos demonstram claramente uma forte correlação entre o aumento na incidência de distúrbios reprodutivos e o aumento de substâncias químicas presentes no ambiente (Scsukova et al., 2016). Muitas dessas substâncias possuem propriedades hormonais e são conhecidas como desreguladores endócrinos (DEs). Um desregulador endócrino é definido como uma substância química exógena, ou uma mistura de substâncias, que podem interferir com qualquer aspecto da ação hormonal (Zoeller et al., 2012). Esses químicos podem atuar por meio da ligação a receptores hormonais, interação com enzimas que sintetizam ou metabolizam hormônios, interferência com o eixo hipotálamo-hipófise-gônada e/ou alteração da transdução de sinais (Kavlock et al.,1996; US EPA, 2007).

Substâncias denominadas DEs podem ser de origem sintética ou subprodutos liberados no ambiente, tais como alguns pesticidas (por exemplo, diclorodifeniltricloroetano (DDT) e outros compostos clorados) ou produtos químicos industriais, como os bifenilos policlorados (por exemplo, dioxinas) e alguns produtos

farmacêuticos produzidos sinteticamente (como pílulas anticoncepcionais e drogas para o tratamento de cânceres hormônio-sensível). Além disso, alguns produtos químicos naturais produzidos por plantas e fungos, os fitoestrógenos, também são denominados de DEs, como a genisteína, coumestrol ou isoflavonas, cuja exposição ocorre em altas doses, através de alimentos ou suplementos (Safe, 1995). Entretanto, muitos compostos ainda não foram classificados como DEs, mas fortes evidências suspeitam deste potencial, o que aumenta a necessidade de mais estudos sobre esse efeito.

4. Hidrocarbonetos Aromáticos Policíclicos

Entre os potenciais Des, há um importante grupo chamado de hidrocarbonetos aromáticos policíclicos (HAP), de característica lipofílica, que são rapidamente absorvidos pelo pulmão, trato gastrointestinal e pele após inalação, ingestão e exposição dérmica, respectivamente (ATSDR, 1990). Os HAP abrangem aproximadamente 100 componentes com conformações químicas diferentes, compostos apenas por átomos de carbono e hidrogênio e formados por dois ou mais anéis benzênicos, com arranjos lineares, aglomerado (cluster) ou angular (Arey e Atkinson, 2003). Podem ser produzidos durante processos biológicos naturais, como queima de floresta ou vulcão em atividade, ou em atividades antropogênicas, gerados pela combustão incompleta de substâncias orgânicas. Sendo assim, os HAP podem ser detectados no ar, no solo e na água (Arey e Atkinson, 2003).

As principais fontes de exposição aos HAP incluem a inalação de fumaça de cigarro, fumaça de lenha e ar ambiente contaminado. A ingestão de HAPs se dá em alimentos como cereais, pão, comidas grelhadas, alimentos processados, bem como frutas e vegetais cultivados em solo contaminado. Outras fontes de HAPs incluem escapamentos de veículos, estradas de asfalto e incineração de resíduos. A exposição ocupacional pode ocorrer em trabalhadores que inalam exaustão do motor, como mecânicos, vendedores ambulantes e motoristas, bem como aqueles que trabalham em indústrias como mineração, refino de petróleo e metalurgia (ATSDR, 2009).

A exposição aos HAPs pela dieta humana é limitada e apresenta dificuldade na padronização. Para Domingo e Nadal (2015), a ingestão alimentar média estimada de 16 tipos de HAPs para um adulto masculino padrão (70 kg de peso corporal) foi de aproximadamente 6,72 μg/dia (Martorell et al., 2010), um valor mais baixo do que o

encontrado em 2000 (8,42 µg/dia) (Falcó et al., 2003). Essas ingestões são muito diferentes do que o encontrado em outro estudo, de 59,2 µg/dia (Martorell et al., 2012), demostrando que esses valores dependem da metodologia empregada nestes estudos. Ramesh e colaboradores (2011) mostraram uma estimativa de ingestão diária de HAP em vários países, sendo maiores em países em desenvolvimento, como Brasil (2,90 µg/pessoa/dia), China (3,56 µg/pessoa/dia) e Índia (11 µg/pessoa/dia), quando comparados a países desenvolvidos, como Grã-Bretanha (0,06 µg/pessoa/dia) e Estados Unidos (0,05 µg/pessoa/dia).

5. Benzo(a)pireno

O HAP mais importante e o mais estudado é o benzo(a)pireno (BaP). O BaP está extensivamente difundido no ar, como um poluente ambiental gerado pela combustão incompleta de matérias orgânicas, nos processamentos industriais, nos combustíveis fósseis, incluindo a exaustão dos automóveis, e em comidas grelhadas (Sagredo et al., 2006; Wu et al., 2012), assim como na fumaça do cigarro (WHO, 1997; IARC, 2004). Este composto é capaz de acumular-se em um curto espaço de tempo em níveis considerados tóxicos em vários tecidos humanos (ATSDR, 1995), como pulmão, rim, cérebro e testículos (Ramesh et al., 2001), sendo classificado como um agente carcinogênico (na dose oral de 10 mg/kg/dia em cânceres de faringe e esofâgo) e mutagênico (IARC, 2004; US-EPA, 2017). Em estudo conduzido por Ramesh e colaboradores (2001), foi administrada uma dose aguda de 100 mg/kg de BaP em ratos machos adultos e, após 24 horas, cerca de 10% dessa dose inicial de BaP não metabolizado foi identificada em tecidos reprodutivos. O ser humano está em contato com o BaP por diferentes vias de exposição, que englobam a ingestão de água e alimentos contaminados, além da inalação de partículas presente no ar e com a fumaça de cigarro (ATSDR, 1995).

No ambiente, estima-se que os níveis de BaP no ar variam de 0,1 a 66 ng/m³ (ATSDR, 1995); nas exposições ocupacionais (atividades industriais) esses níveis sobem para 49 mg/m³ (Szczeklik et al., 1994). Já nos alimentos, a mediana do consumo de comida contaminada com BaP em todos os países da Europa é de aproximadamente 0,2 µg/kg/dia (EFSA, 2008), muito além do nível máximo da ingestão diária tolerada de 6-8 ng/kg/dia (EFSA, 2008); enquanto que, na água potável, esta quantidade é de

aproximadamente 0,2 parte por bilhão (ppb) (ATSDR, 2009). Fumar um maço de cigarros não filtrados por dia expõe o fumante ativo a 0,7 µg/dia de BaP (ATSDR, 2009).

5.1.Toxicocinética

O benzo(a)pireno possui um peso molecular de 252,32, com a fórmula molecular de $C_{20}H_{12}$. O tempo de meia-vida dessa substância é de 875 dias (água e ar) e de 290 dias (solo). A toxicidade aguda desta substância é baixa, e a administração oral a camundongos não causou morte na dose de 1,6 mg/kg (ATSDR, 2009).

O BaP é distribuído no fígado, rim e bexiga (Prough et at., 1979; Yamazaki et al., 1987) e atravessa facilmente a placenta após administração oral, intravenosa ou subcutânea (Kelman e Springer, 1982). Entre todos os órgãos, o fígado contém a maioria das enzimas necessárias para a bioativação de BaP (Ekstrom et al., 1982). Por ser lipofílico, o BaP favorece a sua distribuição e armazenamento no tecido adiposo e está associado no sangue a lipoproteínas de muito baixa densidade (VLDLs) e lipoproteínas de baixa densidade (LDLs) (Busbee et al., 1990). BaP também pode acumular-se em gotículas de lipídios plasmáticas (Plant et al., 1985), assim como nas mitocôndrias, no retículo endoplasmático, no complexo de Golgi, na membrana plasmática, envoltório nuclear, na matriz mitocondrial e no núcleo celular (Barhoumi et al., 2000; Holder et al., 1981). O metabolismo do BaP é complexo, envolvendo a geração de um grande número de metabólitos e a atividade das enzimas da fase I e II (Verma et al., 2012), e os mecanismos de ação propostos pelos quais o BaP e seus metabólitos agem no organismo são diversos, causando seus efeitos tóxicos.

5.2. Possíveis modos de ação do BaP

A elucidação dos modos de ação em que o BaP exibe sua toxicidade são limitados, e dentre as hipóteses temos: genotoxicidade e mutagenicidade, sinalização celular alterada (através principalmente dos receptores de hidrocarboneto de arila), citotoxicidade e estresse oxidativo.

O BaP é um ligante dos receptores citosólico de hidrocarboneto de arila (AhR) – um fator de transcrição que regula a expressão gênica, incluindo a indução de enzimas do citocromo P450 (Fujisawa-Sehara et al., 1988), importantes para a conversão dos metabólitos ativos (Schmidt e Braadfield, 1996). A ligação de BaP ao AhR induz a translocação do complexo AhR-BaP para o núcleo, onde interage com o translocador

nuclear de hidrocarboneto de arila (ARNT), formando o heterodímero AhR-ARNT. Este heterodímero liga-se aos elementos de resposta de hidrocarboneto de arila nas regiões reguladoras do citocromo P450, que induzem a produção de CYP1A1 e CYP1B1. A aceleração na produção destas enzimas que participam diretamente da metabolização do BaP desencadeia um aumento na toxicidade, pois seus metabólitos (como o BPDE), são mais citotóxicos do que o BaP intacto (Hakura et al., 1998; Levin et al., 1978). O BaP também pode provocar toxicidade produzindo oxidação do DNA, danos proteicos induzidos pelos metabólitos reativos e diminuindo as moléculas antioxidantes (US-EPA, 2017).

Com relação aos mecanismos de ação tóxicas do BaP, um dos alvos é o sistema reprodutor. Os danos no DNA provocados nas células germinativas podem levar a citotoxicidade, apoptose e diminuição da viabilidade embrionária pós-fertilização, comprometendo também a função das células de Sertoli e Leydig, ou gerando estresse oxidativo e alteração da regulação da proteína StAR (proteína reguladora aguda esteroidogênica) (US-EPA, 2017). Em estudo conduzido por Xu et al (2014), foi demostrado mutações nas células germinativas, com dose entre 50 – 200 mg/kg de BaP, assim como a interferência direta na esteroidogênese pela ligação com a StAR, em estudo estrutural de proteínas (Reddy et al., 2015), comprometendo assim a síntese de andrógenos. Em estudo *in vitro*, foi identificado que o BaP estimula a apoptose dos folículos ovarianos, altera o balanço hormonal e provoca genotoxicidade via formação de aductos de DNA no tecido ovariano (dose de 5 mg/kg) (US-EPA, 2017). Os aductos de DNA se formam pela ligação dos metabólitos ativos de BaP covalentemente ao DNA, como o BPDE (benzo(a)pireno diol-epóxido), impedindo que a tradução dos genes atingidos ocorra de forma correta (Piberger et al., 2018).

O mecanismo carcinogênico envolve diretamente a ação do BaP como agente mutagênico. As possíveis vias de sinalização já elucidadas são (Figura 3): 1 – bioativação do BaP para metabólitos reativos do DNA, através de três possíveis vias de ativação: uma via do epóxido-diol, uma via do radical cátion, e uma via o-quinona e estresse oxidativo; 2 – danos diretos ao DNA por metabólitos ativos, incluindo formação de aductos de DNA; 3 – mutações no DNA, especialmente nos genes supressores tumorais ou iniciadores de tumor e; 4 – expansão clonal das células mutadas

durante a promoção e progresso do desenvolvimento de cânceres (US-EPA 2017).

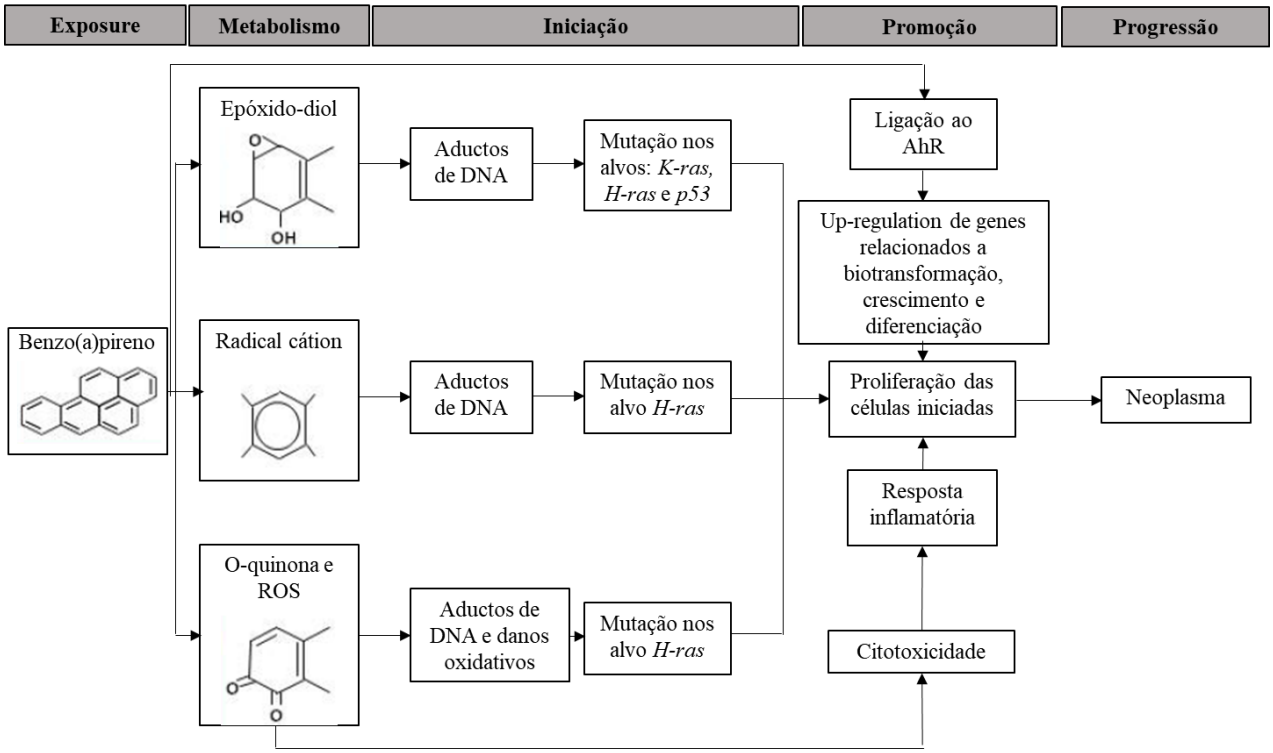


Figura 3. Possíveis modos de ação carcinogênica do BaP. Adaptada de US-EPA (2017).

6. Benzo(a)pireno, sistema genital masculino e epigenética

Estudos têm demonstrado que a exposição ao BaP pode afetar também o sistema reprodutor de roedores machos, acarretando em importantes alterações reprodutivas, tais como, diminuição no peso do testículo e epidídimo, na contagem espermática e na síntese de DNA testicular (Revel et al., 2001; Mohammed et al., 2010). Além disso, Reddy e colaboradores (2015) demonstraram que o BaP, nas doses de 1, 10 e 100 µg/kg/dia, via intraperitoneal, em ratos adultos, diminui de forma significativa a produção espermática diária, a motilidade espermática e a fertilidade (aumento no número de perdas pré-implantação e reabsorção, assim como a diminuição no número de fetos vivos). Esses autores sugerem que há uma interação do BaP com a proteína StAR (proteína reguladora esteroidogênica aguda, responsável pela transferência do colesterol), afetando o transporte de colesterol, e assim, reduzindo a síntese androgênica. Em outro estudo, com as mesmas doses administradas por via oral em ratos adultos, foi demonstrado que uma exposição crônica provoca diminuição nos

níveis de testosterona de forma significativa, assim como redução da motilidade espermática e na expressão da proteína StAR. Assim, sugere-se que a exposição ao BaP gera diminuição na qualidade espermática devido à redução nos níveis de testosterona e, a StAR testicular pode ser um alvo-proteico do BaP e de outros HAP (Chung et al., 2011).

Apesar dos efeitos adversos reprodutivos causados pelo BaP serem bem estabelecidos, seu mecanismo de ação não está claro. Sabe-se que para ocorrer a espermatogênese, é necessária a síntese de testosterona pelas células de Leydig nos testículos, sendo que a velocidade da esteroidogênese é limitada pela entrada de colesterol nas mitocôndrias. Em roedores, o LH liga-se aos seus receptores nas células de Leydig e inicia-se a ativação da adenilil-ciclase, gerando um aumento continuo na produção de cAMP. É bem documentado que o espaço intermembranoso das mitocôndrias é impermeável aos compostos hidrofóbicos, incluindo o colesterol (Stocco e Clark, 1996), sendo assim necessária a interação entre a proteína StAR e o colesterol (Stocco, 2000). Após esse processo, a enzima de clivagem da cadeia lateral contendo citocromo P450 (P450scc) irá converter o colesterol em pregnenolona, no qual finalmente é transferido para o retículo endoplasmático liso, local de produção da testosterona pela ação das 3ß-hidroxiesteroide desidrogenase (3ß-HSD), 17α-hidroxilase e 17,20-lase (CYP17) e a 17ß-hidroxiesteroide desidrogenase (17ß-HSD) (Figura 4). Além de ser capaz de reduzir a expressão da STAR, o BaP possui similaridade estrutural com a molécula de colesterol, e há relatos na literatura que o metabolismo do BaP ocorre em mitocôndrias de diferentes tecidos, inclusive nos testículos (Ramesh et al., 2002). Dessa forma, esta substância poderia interferir na síntese de testosterona através da redução do transporte de colesterol dentro da mitocôndria testicular (Reddy et al., 2015), comprometendo a espermatogênese.

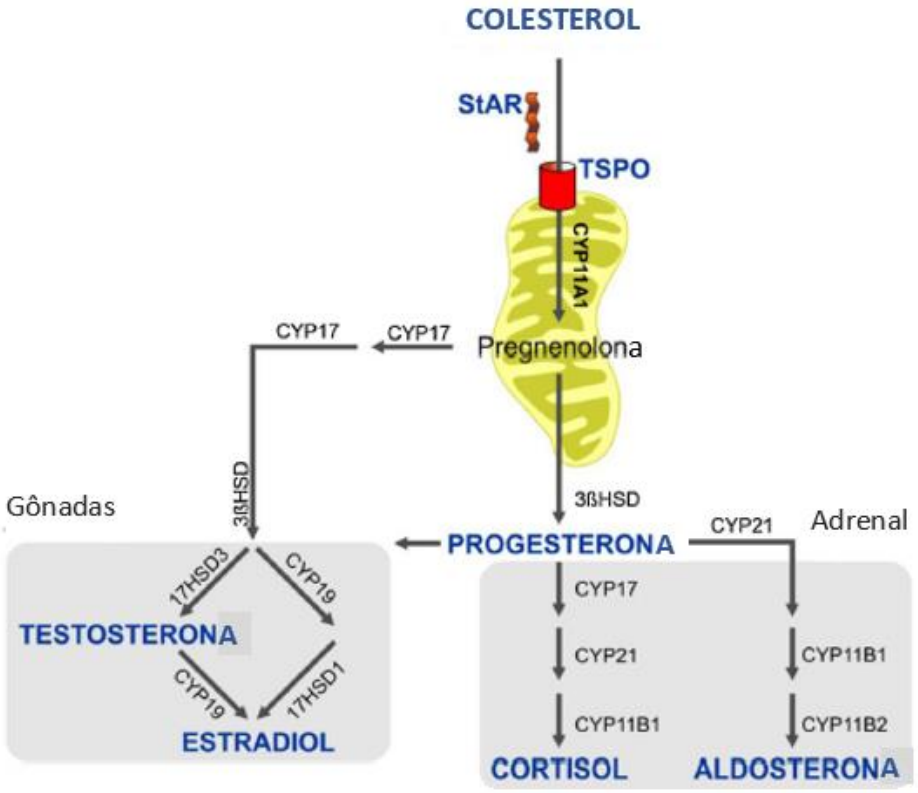


Figura 4. Esquema representando a esteroidogênese gonadal e da adrenal. Adaptada de Martinez et al. (2013).

O BaP também está presente na fumaça de cigarro e têm se tornado alvo de vários estudos toxicológicos envolvendo prejuízos ao sistema genital masculino (Kovac, Khanna, Lipshultz 2015; Esakky e Moley, 2016), pois essa substância é capaz de gerar aductos de DNA nos espermatozoides (Sipinen et al., 2010). Na Figura 5, há uma representação contendo esquemas dos mecanismos propostos de como a fumaça do cigarro poderia afetar as funções reprodutivas masculinas.

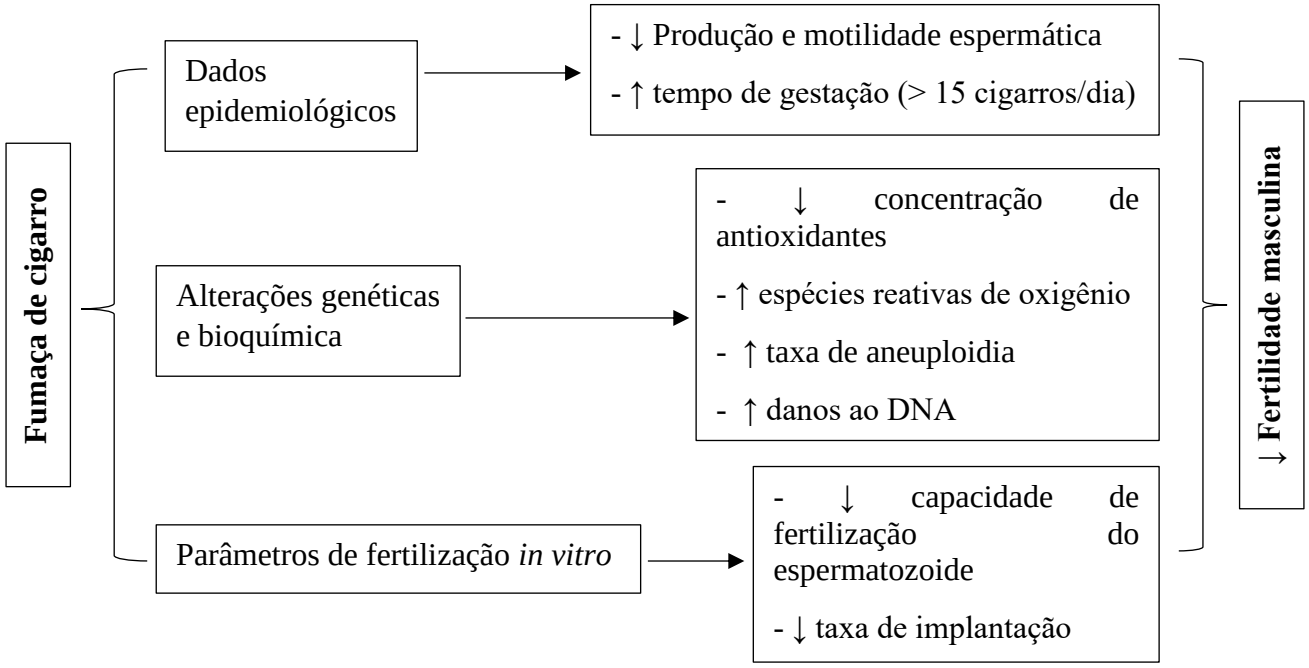


Figura 5. Possíveis mecanismos pelos quais a fumaça de cigarro pode afetar o sistema reprodutor masculino. Adaptada de Soares e Melo (2008).

7. Epigenética e os desreguladores endócrinos

Epigenética é um fenômeno descrito como modificações na expressão gênica sem que haja mudanças diretas nas sequências de DNA. Há vários mecanismos possíveis que geram mudanças epigenéticas, incluindo a metilação de resíduos de citosina de DNA, alterações na compactação da cromatina, modificações pós-traducionais das histonas e alteração na expressão de microRNA (Gore et al., 2016). Há substâncias que podem provocar tais alterações e levar a efeitos transgeracionais nos modelos experimentais, tendo os desreguladores endócrinos um papel relevante neste processo (Rissman e Adli, 2014).

Os DEs podem causar metilação do DNA, modificações nas histonas e alteração na expressão de microRNAs (Gore et al., 2014). Essas mudanças epigenéticas causam mudanças fenotípicas nos organismos, as quais podem aparecer imediatamente ou muito tempo após a exposição. Quando um indivíduo adulto é exposto aos DEs, pode gerar modificações nas células somáticas e, assim, provavelmente não serão transmitidas para as próximas gerações (Rissman e Adli, 2014). Para que um DE tenha efeitos

transgeracionais e multigeracionais, a exposição deve ocorrer durante uma janela crítica do desenvolvimento, e os efeitos adversos podem ser detectados até a geração F3 (Figura 6), sendo transmitidos via células germinativas (nos machos, as espermatogônias) (Gore et al., 2016).

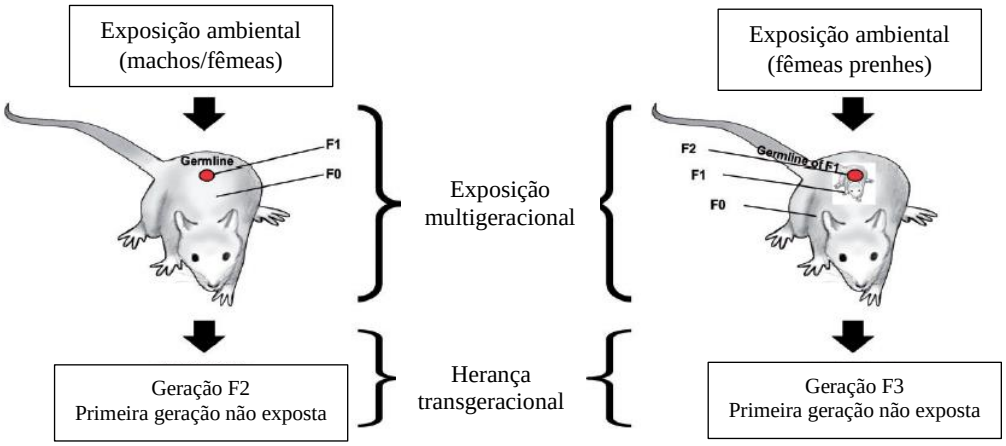


Figura 6. Esquema de exposições ambientais multigeracionais versus transgeracionais. Adaptado de Skinner MK (2008) e Nilsson e Skinner (2015).

Se as células somáticas forem expostas a um toxicante ambiental durante um período crítico do desenvolvimento, as consequências poderão se manifestar como alguma doença quando o indivíduo for adulto, porém não será transmitido para a próxima geração (Hanson e Gluckman, 2014). Já se este toxicante causar alguma injúria nas células germinativas, poderá transmitir essas alterações para as próximas gerações, mesmo que estas não tenham tido contato com a substância em questão (efeito transgeracional) (Surani, 2015; Hanson e Skinner, 2016).

8. Programação do desenvolvimento e contribuição paterna

O desenvolvimento do organismo pode ser considerado um caminho de sentido único e, portanto, qualquer substância de origem natural ou sintética (como DEs), que altere este processo unidirecional podem provocar consequências futuras. Essas perturbações podem induzir mudanças funcionais e/ou estruturais que podem desviar desta trajetória do desenvolvimento, muitas vezes levando as mudanças fenotípicas ao

longo da vida (como um aumento da propensão de doenças endócrinas) (Hanson and Gluckman 2008; Gore et al., 2016;).

Vários estudos demonstram a importância de se investigar a exposição aos DEs durante a gestação, considerando que o embrião/feto é submetido a exposição direta a essas substâncias via intrauterina e, posteriormente, as possíveis repercussões desta exposição no desenvolvimento da prole (Arruda et al., 2016; Sobinoff et al., 2014; Ramos et al., 2016). Somente nos últimos anos, aumentou-se a conscientização e a pesquisa sobre os efeitos adversos mediados pelos ratos machos sobre a sua prole (Davis et al. 1992; Anderson, Schmid, Baumgartner, 2014), porém os estudos ainda são limitados. A retenção e a modificação de histonas, a incorporação de protamina na cromatina, a metilação do DNA e transcritos de RNA de espermatozoides parecem desempenhar papéis importantes nos processos epigenéticos do espermatozoide maduro (Jenkins e Carrell, 2012). Sendo assim, torna-se relevante investigar a contribuição paterna (Figura 7) para o desenvolvimento físico e sexual da prole, elucidando novos mecanismos de ação.

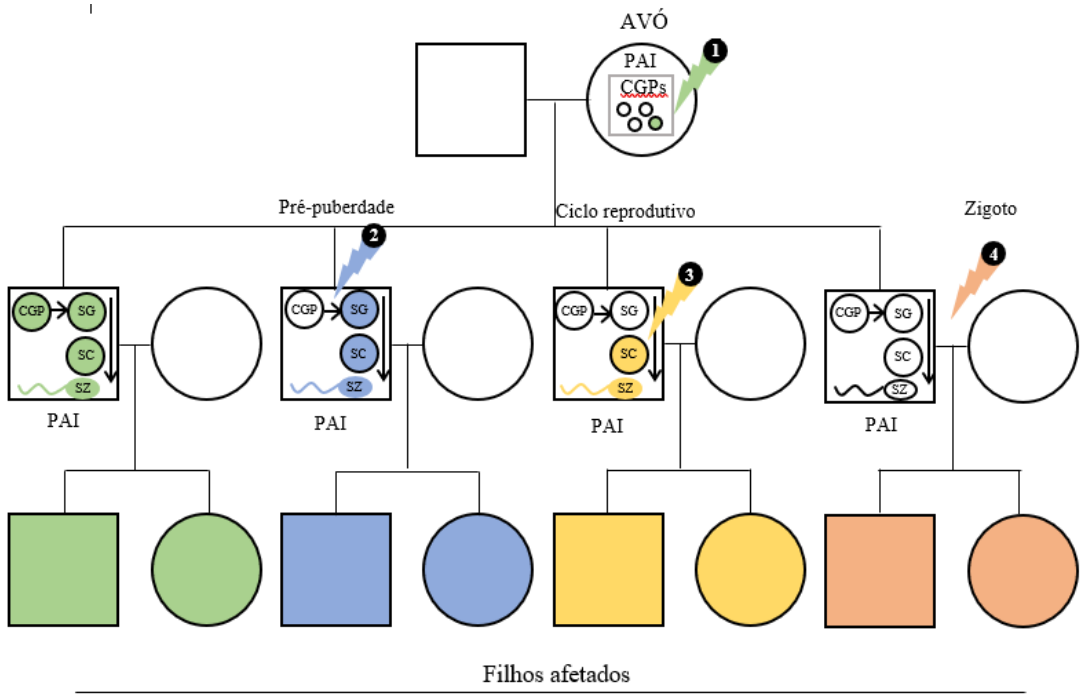


Figura 7. Janelas de suscetibilidade para mudanças epigenéticas induzidas ambientalmente através da linhagem germinativa paterna. Modificações nos perfis epigenéticos podem ter diferentes causas que variam de acordo com o tempo (representados pelos raios) e tipos de exposição, incluindo toxicantes ambientais, poluentes, desreguladores endócrinos, radiação ionizante, tabagismo, nutrição, etc. As

janelas que podem desencadear danos epigenéticos hereditários incluem: 1 - durante a migração de células germinativas primordiais (CGPs) para a crista genital; 2 - antes da puberdade, de CGPs para a espermatogônia (SG); 3 - durante cada ciclo reprodutivo, da espermatogônia (SG) ao espermatócito (SC) e finalmente ao espermatozoide (SZ); e 4 - no zigoto, quando as marcas de metilação adquiridas precisam resistir à reprogramação epigenética pós-zigótica. Figura adaptada de Soubry et al., (2014).

Justificativa

Assim, tendo em vista toda a problemática exposta acima e a relevância dos estudos toxicológicos envolvendo o período juvenil até a peripuberdade (importantes janelas susceptíveis) e a importância da mesma na maturação sexual, torna-se essencial a investigação da influência do benzo(a)pireno (BaP) na saúde reprodutiva do animal após exposição nesta fase da vida e suas possíveis repercussões na vida adulta, visto que este composto possui potencial de desregulação endócrina e está amplamente disseminado no ambiente. Torna-se relevante investigar também se o BaP pode provocar uma programação do desenvolvimento para alterações no sistema reprodutor nos machos expostos e, se essa programação pode afetar o desenvolvimento sexual da sua prole (masculina e feminina).

Hipótese

Na literatura é comprovado que o BaP interfere na esteroidogênese devido sua semelhança estrutural com o colesterol e sua interação com a proteína StAR, prejudicando assim, a síntese de testosterona. Como a peripuberdade é uma fase altamente dependente de andrógenos, qualquer alteração em sua síntese provocada pelo composto em estudo causará impactos negativos na saúde reprodutiva do animal na vida adulta, prejudicando a fertilidade e, possivelmente, sua prole.

Objetivo geral

Avaliar os efeitos da exposição ao benzo(a)pireno durante o período juvenil a peripuberdade sobre parâmetros reprodutivos de ratos machos na vida adulta e de sua prole masculina e feminina.

Objetivos específicos

Investigar se o BaP produz efeitos tóxicos durante sua exposição e se altera a instalação da puberdade;

Investigar se a exposição ao BaP no período juvenil a peripuberdade altera a qualidade espermática e/ou fertilidade na vida adulta destes animais;

Investigar se o BaP é capaz de alterar o comportamento sexual masculino e a produção hormonal;

Investigar a translocação do colesterol para as mitocôndrias via StAR;

Investigar se o BaP é capaz de alterar as células germinativas dos machos expostos ao BaP;

Avaliações reprodutivas da prole masculina e feminina em que os pais foram expostos ao BaP do período juvenil até a peripuberdade.

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Capítulo I – O manuscrito intitulado **"Exposure to low-doses of benzo(a)pyrene from juvenile period to peripuberty leads negative impacts on male reproduction in adult rats**" será submetido para publicação no periódico "Environmental Pollution". Fator de impacto: 4.358.

EXPOSURE TO LOW-DOSES OF BENZO(A)PYRENE FROM JUVENILE PERIOD TO PERIPUBERTY LEADS NEGATIVE IMPACTS ON MALE REPRODUCTION IN ADULT RATS

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ABSTRACT

Benzo(a)pyrene (BaP) is formed by the incomplete combustion of complex organic compounds. Studies have revealed that the BaP can interfere in steroidogenesis process through its interaction with the StAR protein. This study evaluated the effects of the exposure to BaP from juvenile period to peripuberty on reproductive parameters of male rats in adult life. Juvenile male (PND 23) rats were distributed into four experimental groups: a control group (corn oil + DMSO); and three groups treated with BaP: 0.1; 1 or 10 μg/kg/day, during 31 consecutive days (by gavage). During the treatment, clinical signs of toxicity, as well as the puberty installation were evaluated. On PND 90, reproductive and sperm parameters of BaP-treated rats were evaluated. There was an increase in the latency for the first ejaculation in the group treated with 0.1 μg/kg and this same group presented an increase in the pre-implantation loss. The sex ratio was altered in the all treated groups, as well as a decrease in the thyroid and seminal gland relative weights and sperm parameters. These results showed that BaP can act as an endocrine disrupter, interfering with the male reproductive system in the adult life of the males exposed from the juvenile period to peripuberty.

Keywords: benzo(a)pyrene, endocrine disruptor, peripuberty, StAR protein.

INTRODUCTION

Benzo(a)pyrene (BaP) is an important substance in the group of polycyclic aromatic hydrocarbons (PAH) and is widely diffused in the environment (ATSDR, 2009). It is generated by the incomplete combustion of organic compounds, including natural and anthropological activities, such as burning of fossil fuels, volcanism, automobile exhaust, contaminated water and food, cigarette smoke, and industrial activities (US-EPA 2017).

The median consumption of food contaminated with BaP is approximately 0.2 μg/kg/day in all European countries (EFSA et al. 2008), well beyond the maximum tolerated daily intake level of 6-8 ng/kg/day (EFSA et al. 2008). In drinking water, this amount is approximately 0.2 part per billion (ppb) (ATSDR, 2009) and an active smoker may be exposed to 0.7 μg/day of the BaP (ATSDR, 2009).

Because of its lipophilic characteristics, this compound can accumulate in the body (US-EPA 2017). Studies have shown that the BaP interferes with reproduction process through its interaction with the steroidogenic acute regulatory protein (StAR) (Reddy, Girish, and Reddy 2015), a protein complex present in the membrane of mitochondria and responsible for the transport of cholesterol to produce androgens in males (Miller 2007, 2013). It is known that substances with anti-androgenic potential affect the sperm quality (Chung et al. 2011; Perobelli et al. 2012; Sanabria et al. 2016) and the reproductive health of exposed individuals (Gore et al. 2016).

Thus, it is important to investigate the effects of this exposure in periods highly dependent on androgens, such as peripuberty (Gore et al. 2016; Perobelli 2014). Puberty is a critical period that requires androgenic action for the development of the male genital system (Ojeda and Skinner 2006). In this phase, important morphological

alterations and neuroendocrine interactions occurs (Stoker et al. 2000), promoting the maturation of secondary sexual characteristics and reproductive capacity. The male genital system is more susceptible to the action of chemical agents during the juvenile period and peripuberty, since spermatogenesis and steroidogenesis are not yet completely established (Favareto, Toledo, and Kempinas 2011), and any alteration in these processes may result in increased risk of disease in adulthood (Mantovani and Fucic 2014). Therefore, the exposure to endocrine disruptors may substantially alter the puberty process (Mantovani and Fucic 2014).

Thus, it is essential an investigation about the potential endocrine disruptors of BaP in environmentally relevant doses on periods highly androgen-dependent (juvenile and peripuberty). The aimed to study was evaluated the effects these exposition to BaP on reproductive parameters (sperm quality and fertility) of male rats in adult life.

MATERIALS AND METHODS

Animals

12 female Wistar (90 days old) and 12 Wistar males (90 days old) were obtained from the UNESP Central Animal Bioterium for natural mating. Male offspring (23 days old, weighing approximately 70 g, n= 10/group) was randomly divided into four experimental groups. The animals were kept under standard conditions (temperature of 22ºC, photoperiod of 12h light / 12h dark, humidity of the relative air in 50%), water and commercial food *ad libitium*. The experimental procedures are in accordance with the Ethical Principles of the National Council for the Control of Animal Experimentation (CONCEA) and were executed after approval by the Committee on

Ethics in Animal Use (CEUA), protocol no. 958/2017, of the Institute of Biosciences of UNESP of Botucatu.

Experimental design

Male rats were divided into four experimental groups: a control group, which received only the vehicle (corn oil + dimethylsulfoxide – DMSO (0,5%)); and three groups treated with three different doses of benzo(a)pyrene (BaP) (96% purity - Sigma-Aldrich Co. Ltd.) diluted in vehicle: 0.1; 1.0 or 10 μg/kg. The treatment was performed orally (gavage), daily, for 31 consecutive days, following the test protocol for male rats from juvenile period (21-32) to peripuberty (33-55), range from 23-53 days of animal life (US-EPA 2009). The period of treatment performed in the present study corresponds to the stages of child (2 - 12 years) and adolescents (12-16 years) in humans (Picut, Remick, et al. 2015). These doses are environmentally important because the estimated intake of BaP by humans living close to hazardous waste sites is about 0.8 μg/day (Lioy et al. 1988), in addition to the fact that the median consumption of food contaminated with BaP in Europe was 0.2 μg/kg (EFSA et al. 2008), a larger amount when compared to the lowest dose used in this study.

Clinical signs of toxicity during the treatment and puberty installation

During the treatment, clinical signs of toxicity (body weight, diarrhea, piloerection, bleeding, abnormal respiration, tremors, convulsions and gait change, posture and reaction to manipulation) were evaluated, as well as the water and food average intake. In this period, since the post-natal day (PND) 40, the date of the preputial separation of the animals exposed to BaP, an important indicator of puberty establishment (Marty et al. 2009), was evaluated through manual retraction of the foreskin.

Reproductive parameters in adult life

These animals were evaluated in relation to the male sexual behavior and fertility test on 90 days of age because they are considered sexually mature (Picut et al., 2015). After, sexually experienced males treated rested for approximately 30 days to recover the sperm reserves (Borges et al. 2016); thus, the collection of organs and blood of the same ones continued on PND 120.

Male sexual behavior

On PND 90, adult male rats (n= 10 animals/group) from each experimental group were individually placed in polycarbonate boxes, 5 minutes before the introduction of an untreated female (80 days old, in estrous), determined by vaginal lavage. The animals were observed in the dark period of the cycle, in a separate room under dark red light, and all tests of sexual behavior were performed 2-4 hours after the onset of the dark period. The following parameters were observed for 30 minutes: latency for first mating, intrusion and ejaculation; number of intrusions until the first ejaculation; latency of the first post-ejaculation intrusion; number of post-ejaculation intrusions; and total number of ejaculations (Ahlenius & Larsson, 1984; Ågmo, 1997). Males that did not show sexual behavior in the first 10 minutes were considered sexually inactive. After this process, males and females were separated and vaginal material was collected to confirm the presence of sperm and, consequently, pregnancy.

Fertility test

The day that sperm was found in the vaginal lavage and the female was in estrous, was considered gestational day 0 (GD0). Sperm-positive females were killed on the 20th day of pregnancy (n= 7 animals/group), by decapitation, to collect the uterus,

ovaries (for recording the number of corpora lutea), implantation sites, resorptions, live and dead fetuses, for further determination of the potential of fertility (implantation efficiency): implantation sites / number of corpora lutea x 100; gestation rate: number of pregnant females / number of females placed to mate x 100; pre-implantation loss rate: number of corpora lutea - number of implantations / number of corpora lutea x 100; post-implantation loss rate: number of implantations - number of live fetuses / number of implantations x 100; fetal sex ratio: number of male fetuses / number of female fetuses.

Collection of organs

After 30 days of the sexual behavior test (Borges et al., 2016), males exposed to BaP from juvenile period to peripuberty were killed after CO_2 inhalation for cardiac puncture (n = 10 animals/group). Blood was collected directly from the heart with a 10 ml syringe, for hormonal dosage, and organs (heart, lung, liver, kidney, spleen and thyroid) as well as the sex organs (testis, epididymis, seminal gland, ventral prostate and vas deferens) were collected and weighed and subsequently stored (reproductive) to measure the sperm parameters.

Hematological parameters

The counts of red cells, total leukocytes and platelets were performed manually with the aid of the Neubauer Chamber under the light microscope. For differential leukocyte identification, it was performed in a blood smear and then stained with the fast Panoptic kit (Laborclin, Pinhais, Pr). Hemoglobin was measured by the BIOPLUS 200 semi-automatic device.

Sperm parameters

Sperm morphology (n = 10 animals/ group): The right vas deferens of the animals were sectioned at the proximal and distal extremities, and washed with a needle and syringe, with 1 ml of 10% formalin solution. 20 µl of this duct lavage was deposited on histological slides for the qualitative evaluation of spermatozoa. Thus, 200 sperm per animal were evaluated under light microscopy (400X magnification). Morphological abnormalities found in spermatozoa were classified as head and tail abnormalities (Filler, 1993).

Sperm Count in the testis and epididymis (n = 10 animals/group): Spermatids resistant to homogenization (stage 19 of spermiogenesis) and spermatozoa on the caput/ corpus and cauda of the epididymis were counted as previously described by Robb et al. (1978), with adaptations adopted by Fernandes et al. (2007). Briefly, each right testis, decapsulated and weighted soon after collection, was homogenized in 5mL of 0.9% NaCl containing 0.5% TritonX100. After dilution of 10 times, the sample was transferred to Neubauer chambers (4 fields per animal), counting mature spermatids. For the calculation of the daily sperm production (DSP) the number of spermatids in stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium. Likewise, portions of the caput/corpus and epididymal cauda were cut into small fragments and homogenized, and sperm count as described as for the testis. The transit time of sperm through the epididymis was determined by dividing the number of sperm in each portion by the DSP.

Sperm motility (n = 5 animals/ group): was evaluated in the right cauda, described by Perobelli et al. (2010). For this, an aliquot of 10 ml of sperm suspensions was immediately transferred to a Makler chamber maintained at 34 ºC. Using a phase-contrast microscope (400X magnification), 100 sperm were counted and classified as

Type A (mobile with progressive movement) Type B (mobile without progressive movement) and Type C (immobile).

Histological Analysis of the Testis

The rights testis of the animals was embedded with Bouin solution for one day and subsequent changed in alcohol. It was paraplast embedded, sliced and stained with hematoxylin and eosin (H&E) for the following evaluations:

Stages of the seminiferous tubules (n = 5 animals/ group): the relative frequency of the spermatogenesis stages was quantified in order to evaluate the spermatogenic dynamics of the animals, classified them as: I-VI (two generations of spermatids - round and elongated), VII-VIII (spermiation), IX-XIII (only one generation of elongated spermatids), XIV (presence of secondary spermatocytes) in 100 sections of seminiferous tubules per animal.

Leydig cell nucleus count and volume (n = 5 animals/ group): Leydig cell nuclei will be counted in 10 random fields in each histological section of the testis. The mean core diameter of the Leydig cells will also be measured for the calculation of their volume. For this, 50 random nucleus (circular or elliptical) will be measured per animal (Mantovani & Fucic, 2014). The larger (D) and smaller (d) diameter of the cell nuclei will be obtained using a Nikon E-200 (X40) Microscope coupled to a digital camera and computer with NisElements software (version 4.20 for Windows). The mean diameter (M) will be calculated using the formula M = (D xd) / 2, the nuclear area (A) and the volume (V) will be obtained by the following formulas: $A = \pi \times ¼ \times M2$ and $V = \pi \times ⅙ \times M3$, respectively (Cury et al., 2006).

Western blot

Frozen sample testis of males treated with BaP at PND 90 were mechanically homogenized with RIPA extraction buffer, plus protease inhibitors (Sigma-Aldrich®, USA), in a Tureaux type homogenizer (Ultra Stirrer-Ultra80) in 3 cycles of 10 seconds at 4 °C. The homogenate was centrifuged at 14,000 rpm for 20 minutes at 4 °C, and the supernatant was collected. Protein concentration was determined by the Bradford method on 96-well polystyrene plates and reading of absorbance was performed on Biochrom microplate reader (Holliston, Massachusetts, USA).

A protein sample (50 μg) were separated on SDS-PAGE. Following the electrophoresis, the proteins were transferred to nitrocellulose membranes or Polyvinylidene Difluoride (PVDF). The nonspecific binding of proteins was blocked by incubating the membrane in 5% milk in TBST buffer for 90 min at room temperature. The membranes were incubated with the respective primary antibody in 5% milk diluted in TBST (1:350 - 1:1.000) overnight at 4 °C: StAR (D10H12 – Cell Signaling Technology®); B-Actin (sc-47778-Santa cruz® Biotechnology, Inc., USA).

The membranes were then incubated with a specific secondary antibody conjugated with peroxidase, which was diluted (1:10.00 -1:20.000) in TBST for 1 h (IgG anti-rabbit, ab97051 and IgG anti-mouse, ab97023, Abcam® Inc., USA). The immunoreactive components were revealed by GE Amersham ECL chemiluminescent substrate (GE Healthcare). Analyses were done in five different biological samples per group. To calculate the mean and SEM, the optic density of band was used as the unit of measure with software Image J (version 1.33u—National Institutes of Health, USA), and normalized by β-actin values and the results were normalized from fold change and expressed as median with interquartile range.

Statistical analysis

Values was expressed as mean ± SEM or median (Q1-Q3). For the comparison of the results between the experimental groups, statistical analysis of variance (ANOVA), followed by the Dunnet a posteriori test, or by non-parametric (Kruskal-Wallis) analysis of variance, followed by a posteriori test of Dunn, when necessary. The differences were considered significant at $p < 0.05$.

RESULTS

During the treatment period, the animals exposed to BaP did not presented clinical signs of toxicity (supplementary material – Table 1) and the date of preputial separation was not changed (supplementary material – Table 2). Concerning hematological parameters in adult life, the group of the highest dose had a reduction in the percentage of lymphocytes, in the platelet count (also found in the lower dose group) and in the amount of hemoglobin when compared to the control (supplementary material – Table 3). All groups BaP-treated had a reduction in the relative weights of the thyroid and full seminal vesicle when compared to the control group (Table 1).

On PND 90, the male sexual behavior was displayed in the group treated with the lowest dose (0.1 µg/kg) (Table 2). These animals showed an increase of the latency for the first ejaculation, presenting a delay when compared to the control group, as well as a biological increase in the latency for the first mount. In the fertility test, rats exposed to mid dose (1 µg/kg) showed a reduction in the sex ratio, presenting more fetuses female than male (Table 3).

All groups BaP-treated showed a reduction in the percentage of normal sperm when compared to the control group (Table 4) and increased of abnormal sperm in vas deferens. Also, there was decreased in the percentage of progressive sperm (type A) and

an increased in the percentage of type B (mobile with nonprogressive) and C (immotile) when compared to the control group (Figure 1).

The group of the lowest dose had a reduction in the DSP and in the number of mature spermatids in the testis. This group also showed a decrease in the relative number of sperms in the caput/corpus, and a biological increase of 27% in sperm transit through the epididymis, both in caput/corpus and cauda (Table 5).

The classification of the spermatogenic dynamics presented alteration in the groups treated with 0.1 and 1 μg/kg of BaP. There was a reduction in the percentage of seminiferous tubules in the I-VI (1 μg/kg) and VII-VIII (0.1 μg/kg) stages and an increased in the frequency of the IX-XIII stage in both groups when compared to control group (Figure 2). The mean Leydig cell count per unit of interstitial tissue was decreased in all treated groups, as well as the area and volume of these cells, indicating atrophy (Figure 3). Expression of the StAR protein in the testis on PND 120 was increased in the higher dose group and, although not significant, the lower dose group also showed increased expression of this protein that cannot be despised (Figure 4).

DISCUSSION

Studies have reported the interference of high doses of BaP on androgen-dependent reproductive parameters when administered chronically in adult life (Chung et al. 2011; Mohamed et al. 2010; Reddy, Girish, and Reddy 2015; US-EPA 2017). To our knowledge, this study represents by the first time that low and environmentally relevant doses of BaP leads to negative repercussions in sperm quality on adult animals exposed during juvenile period to peripuberty.

The onset of puberty can be altered by endocrine disruptors (Mantovani and Fucic, 2014), such as bisphenol A (BPA) and phthalates (Kasper-Sonnenberg et al., 2017). In the present study, treated-males did not showed clinical signs of toxicity and altered puberty during the exposure, indicating that BaP at the doses used in this study were not able to induce immediate systemic toxicity in this experimental model. However, studies show that endocrine disruptors can cause not only immediate but also long-term impacts that may manifest in adulthood, leading to the development of diseases (Gore et al. 2016).

To evaluating the direct toxic effects of BaP on the reproductive system, a systemic toxicological evaluation is important and hematological tests are of great relevance for the identification of this type of toxicity (Ruiz, Vassallo, and de Souza 1993). In the group exposed to the higher dose, the decrease in lymphocyte ratio, platelet count, and hemoglobin dosage are indicative of BaP-induced toxic effects. Studies have shown that exposure to aromatic hydrocarbons may lead to a significant decrease in circulating erythrocytes, hemoglobin, platelets, and total white blood cells (Ruiz, Vassallo, and de Souza 1993), and, this effects can be due to the medullary toxicity of benzene, that can conjugate metabolites formed in the biotransformation to important macromolecules such as DNA and proteins. This benzene property gives a radiomimetic action, which is responsible for the interference in bone marrow progenitor cells and damage to the medullary microenvironment (Ruiz, Vassallo, and de Souza 1993). This damage results in peripheral hematological alterations, which corroborates with the results found in this study.

The regulation and maintenance of the organism, as well as reproduction, relies on an important gland, the thyroid, which if altered can lead to adverse effects on the organism (Wang et al. 2018). It is known that thyroid is an important target for

endocrine disruptors and can induced dysfunction in this gland, such as atrophy (Calsolaro et al. 2017), similar result to found in our study. Androgen-dependent organs are also targeting of EDCs and may be altered by changes in their levels. In this study, the atrophy of the seminal vesicle (androgen-dependent organ) (US-EPA 1996) may be associated with changes in the testicular function and /or hormonal status of the animal. Analyzes of the testosterone levels of these animals are still ongoing.

In adult life, the exposure to low-doses of BaP compromised some important parameters related to male sexual behavior. It is known that male sexual behavior depends on the action of testicular hormones acting on the brain (Harding and McGinnis 2004) and that olfactory information influences the sexual behavior of males and females (Cavalcante, Bittencourt, and Elias 2006). Circulating testosterone levels are increased in adult male rats due to pheromones released by females in natural mating (Macrides, Bartke, and Dalterio 1975) and the onset of male sexual behavior occurs in response to the activation of neural pathways related to copulation (Halpern and Martínez-Marcos 2003). Thus, the delay in the first ejaculation presented in our study may be related to the decrease of the levels of circulating testosterone or to the inefficiency of the olfactory perception of the males for the beginning of the copulation.

The sex ratio between the birth of males and females is an important parameter used in experimental and epidemiological studies and this data is relatively stable and any alteration in this relation can measure damages to the reproductive health of a population (Bae et al. 2017; Terrell, Hartnett, and Marcus 2011). In our study, all groups treated with BaP showed decrease in sex ratio when compared to control, corroborating with other studies that demonstrated the relationship between exposure to endocrine disruptors, such as dioxins, and changes in the sex ratio (Terrell, Hartnett, and Marcus 2011).

Sperm quality can be measured by counting, morphology and motility of sperm and are of great importance in the evaluating the viability of sperm to fertilization of the oocyte and the possible signs of toxicity of the male reproductive system caused by exogenous substances, which may reflect the mutagenicity of spermatogonia (US-EPA 1996). The decrease in the percentage of normal spermatozoa of the animals exposed to BaP may be related to the decrease of the testosterone synthesis (Queiroz and Waissmann 2006). Alterations in the microenvironment of the epididymis resulting from an androgen deficiency may be a consequence of decreased ability of the epididymis epithelium to reabsorb abnormal sperm during the maturation process (Cruceño et al. 2013). In present study, the biological increase of pre-implantation loss may be related to sperm abnormalities (discussed below) (OECD 2008; Ribeiro and Pereira 2005).

The sperm counts provide indications about the efficiency of spermatogenesis, because it depends of Sertoli cells functionality in creating an appropriate micro ambient. Changes in the number, structure and/or function of this cell type can result in damage to germinal epithelium and impairment of spermatogenesis (Perreault, Klinefelter, and Clegg 2007). The group exposed to the lowest dose of BaP presented a reduction in the daily sperm production in the testis, directly linked to the decrease in the frequency of the VII and VIII stages (characterized by spermiation), reflecting the decrease of sperm production. The reduction found in the sperm number in the caput/corpus of the epididymis is consistent with the reduction in the daily sperm production of the testis. In the caudal segment, this number remained unchanged, can be associated to the period of 30 days after the mating, that can be considered enough for these animals to recover the sperm reserves in the cauda. The increase in sperm transit time in the epididymis of approximately 27% was not significant, and considering our

results, the time transit still within the normal range for rats (10-12 days of complete transit through the epididymis) (Bernard Robaire, Hinton, and Orgebin-Crist 2015).

The differentiation of epididymal regions occurs during the juvenile period until peripuberty (PND 15 to 44) due to hormonal influences (Picut and Remick 2017; Bernard Robaire, Hinton, and Orgebin-Crist 2015). In the present study, BaP administration was from PND 23 to 55, into the period of differentiation of this important organ, and any hormonal imbalance (testosterone and estradiol) caused by exposure to substances may be detrimental to the development of the epididymis, thus compromising its function and, consequently, the maturation process. This possible functional modification caused by the Bap in the epididymis may be related to the decrease in the percentage of progressive-mobile sperm, since changes in the sperm maturation impairs sperm quality (Kempinas and Klinefelter 2018; Sullivan and Mieusset 2016).

The production of testosterone is essential for the maintenance of reproductive function in males. Leydig cells (LC) are the sites of testicular androgen production and are constituted of two different origins: the fetal and the adult. Fetal LCs are responsible for testosterone peaks production at moments essential for the constitution of the male reproductive system during the perinatal period in humans and rodents. In rodents, soon after birth, the fetal LC degenerate and about the PND 20 days begins the differentiation of adult LC responsible for testosterone production during the early reproductive system maturation and throughout adult life of the animal (Martinez-Arguelles et al. 2013; Picut, Ziejewski, and Stanislaus 2018). The decrease in LC numbers found in this study may be related to the onset of exposure to BaP (PND 23) in which adult LCs are still in the differentiation period, thus being more susceptible to interference in this process.

Mohamed et al. (2010) showed that BaP metabolites can induce apoptosis in LC, reinforcing the results in the present study.

The maintenance and homeostasis of the organism is highly regulated for several mechanism and if some toxic substance changes this balance, the organism searches for ways of compensation. The up-regulation of StAR protein can be associated to compensatory mechanism, probably due to interference of BaP, searching to increase the transport of cholesterol to inner mitochondria membrane since StAR is a limiting step of steroidogenesis (Miller, 2013). This result reinforces Leydig cell atrophy and strong evidence that testosterone is decreased explaining a possible mechanism by which StAR expression is increased in the groups with the greatest negative impact caused by anti-androgenic action.

The results obtained in the present study permit affirm that exposure to low-doses of benzo(a)pyrene from the juvenile period to peripuberty of male rats were detrimental to the reproduction of these animals mainly in relation to the decrease in sperm quality, with strong evidence that the substance behaves like a potential endocrine disruptor.

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Table 1 - Body weight and relative organ weight of adult males (120 days) exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty

Parameters	Experimental groups			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Final body weight (g)	470.6 ± 11.25	487.9 ± 16.05	462.1 ± 13.66	486.2 ± 14.79
Ventral prostate (g)	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
Full seminal vesicle (g)	0.33 ± 0.02	0.26 ± 0.02*	0.27 ± 0.01*	0.26 ± 0.01*
Testis (g)	0.37 ± 0.01	0.39 ± 0.01	0.37 ± 0.01	0.38 ± 0.01
Epididymis (g)	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00
Vas deferens (g)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Left kidney (g)	0.36 ± 0.02	0.38 ± 0.01	0.39 ± 0.01	0.38 ± 0.01
Left adrenal gland (mg)	8.03 ± 0.60	7.95 ± 0.62	7.21 ± 0.32	7.37 ± 0.51
Liver (g)	3.67 ± 0.12	3.47 ± 0.13	3.47 ± 0.14	3.55 ± 0.14
Lung (g)	0.39 ± 0.01	0.42 ± 0.02	0.46 ± 0.03	0.42 ± 0.02
Heart (g)	0.31 ± 0.01	0.29 ± 0.01	0.31 ± 0.01	0.30 ± 0.01
Thyroid (mg)	3.37 ± 0.47	2.34 ± 0.20*	2.26 ± 0.20*	1.94 ± 0.22**
Spleen (g)	0.17 ± 0.00	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.00

Values expressed as mean ± SEM, 9-10 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. * $p < 0.05$ and ** $p < 0.01$ compared to the control group.

Table 2– Male sexual behavior of adult rats (90 days) exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty.

Parameters	**Experimental groups**			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Latency of the first mount (s)	83.62 ± 15.39	172.28 ± 50.40	104.20 ± 18.84	86.22 ± 21.54
Number of mounts	6.14 ± 1.93	10.44 ± 2.12	11.11 ± 2.40	6.62 ± 1.39
Latency of the first intromission (s)	101.75 ± 18.13	186.33 ± 58.82	118.37 ± 20.69	135.55 ± 30.25
Number of intromissions	24.25 ± 2.60	29.78 ± 1.75	28.55 ± 2.89	22.89 ± 2.76
Latency of the first ejaculation (s)	751.00 ± 100.61	1162.44 ± 99.95**	916.12 ± 66.68	737.37 ± 65.99
Latency of the first mount post-ejaculation (s)	290.37 ± 10.84	320.90 ± 15.96	295.14 ± 14.39	315.75 ± 13.78
Number of mounts post-ejaculation	4.00 ± 1.03	2.40 ± 0.45	3.71 ± 0.94	3.00 ± 0.67
Latency of the first intromission post-ejaculation (s)	309.78 ± 18.12	321.30 ± 15.98	306.87 ± 21.60	313.78 ± 13.96
Number of intromission post-ejaculation	13.12 ± 1.37	12.33 ± 1.55	17.62 ± 1.86	12.77 ± 1.68
Number of ejaculations	2.50 ± 0.27	1,89 ± 0.20	1.75 ± 0.16	2.62 ± 0.18

Values expressed as mean ± standard error mean (SEM), 9-10 rats/group. Analysis of variance - ANOVA with Dunett's posterior test.
** $p < 0.01$ compared to the control group.

Table 3 – Fertility test realized by exploratory laparotomy of female rats untreated and naturally mated with of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty.

Parameters	**Experimental groups**			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
#Fertility potencial (%)	100 (92 - 100)	92 (83 - 100)	100 (83 - 100)	100 (69 - 100)
Maternal final body weight (g)	341.48 ± 12.35	348.03 ± 9.05	366.38 ± 14.61	355.19 ± 12.07
Uterus + fetuses (g)	61.23 ± 2.67	59.73 ± 2.88	69.44 ± 5.97	59.26 ± 5.38
Body weight of fetuses (g)	3.07 ± 0.06	3.24 ± 0.07	3.24 ± 0.10	3.17 ± 0.07
Male	3.08 ± 0.02	3.36 ± 0.08	3.36 ± 0.10	3.23 ± 0.09
Female	2.99 ± 0.07	3.15 ± 0.06	3.15 ± 0.11	3.09 ± 0.07
Weight of placentas (g)	0.56 ± 0.03	0.54 ± 0.02	0.56 ± 0.02	0.53 ± 0.02
Number of fetuses	12.00 ± 0.45	11.43 ± 0.61	13.00 ± 0.93	12.33 ± 0.76
Number of implantation	12.00 ± 0.45	11.86 ± 0.59	13.14 ± 0.88	12.57 ± 0.78
Number of corpora lutea	12.14 ± 0.34	12.57 ± 0.37	13.57 ± 0.84	13.57 ± 0.43
Number of resorptions	0.00 ± 0.00	0.43 ± 0.20	0.14 ± 0.14	1.14 ± 0.46
# Pre-implantation loss rate (%)	0 (0 - 8)	8 (0 - 17)	0 (0 - 6)	0 (0 - 31)
# Post-implantation loss rate (%)	0 (0 - 0)	0 (0 -10)	0 (0 - 8)	9 (0 - 33)
Sex ratio (M:F)	1.23 ± 0.19	0.80 ± 0.09	0.69 ± 0.08*	0.79 ± 0.10

Values expressed as mean ± SEM, 6-7 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. #Values expressed in median (Q1-Q3), 6-7 rats/group. Kruskal-Wallis with Dunn's posterior test. * $p < 0.05$ compared to the control group.

Table 4 - Sperm morphology of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty.

Parameters	**Experimental groups**			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Normal sperm (%)	99,00 (97,5-99,5)	95,75 (87–100)*	95,75 (89,5–99)*	95,00 (86–99,5)*
Abnormal sperm (%)	1,00 (0,5 – 2,5)	4,25 (0 – 13)*	4,25 (1 – 10,5)*	5,00 (0,5 – 14)*
Broken tail	0,00 (0 – 0,5)	0,50 (0 – 1,5)	0,00 (0 – 1,5)	0,50 (0 – 4)
Coiled tail	0,50 (0 – 1)	2,00 (0 – 10,5)	2,50 (0 – 7)	2,25 (0 – 10)
Isolated tail	0,25 (0 – 0,5)	0,00 (0 – 2)	0,50 (0 – 1)	0,00 (0 – 0,5)
Isolated head	0,25 (0 – 1)	0,75 (0 – 2,5)	1,00 (0 – 4)	0,75 (0 – 5)
Straight head	0,00 (0 – 0,5)	0,00 (0 – 2)	0,00 (0 – 2,5)	0,00 (0 – 1,5)

Values expressed in median (Q1-Q3), 9-10 rats/group. Kruskal-Wallis with Dunn's posterior test. * $p < 0.05$ compared to the control group.

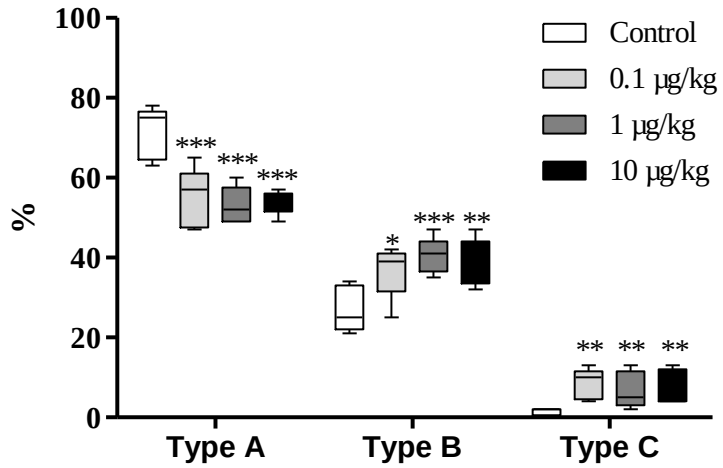

Figure 1 – Sperm motility - classification of sperm in type A, B or C according to their motility sperm of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty. Values expressed in median (Q1-Q3), 9-10 rats/group. Kruskal-Wallis with Dunn's posterior test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the control group.

Table 5 - Sperm count (PND 120) of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty.

Parameters	Experimental groups			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Testis				
Number of mature spermatids ($x10^6$)	230.06 ± 10.98	182.67 ± 11.26*	198.62 ± 16.47	225.52 ± 3.675
Relative number of mature spermatids ($x10^6$/g)	152.69 ± 5.18	119.73 ± 3.25**	132.21 ± 9.57	142.22 ± 4.99
Daily sperm production	37.71 ± 1.80	30.89 ± 1.90*	33.58 ± 2.62	37.67 ± 0.89
Epididymis – Caput/corpus				
Number of sperm ($x10^6$)	154.88 ± 12.18	136.29 ± 8.82	139.78 ± 14.34	154.70 ± 11.29
Relative number of sperm ($x10^6$/g)	492.34 ± 34.90	379.84 ± 21.55*	437.08 ± 35.91	472.64 ± 24.93
Transit (days)	3.86 ± 0.22	4.90 ± 0.48	4.32 ± 0.26	4.22 ± 0.27
Epididymis - Cauda				
Number of sperm ($x10^6$)	235.34 ± 18.85	205.27 ± 18.00	199.10 ± 11.11	228.94 ± 22.39
Relative number of sperm ($x10^6$/g)	1033.89 ± 54.70	916.11 ± 62.40	986.75 ± 70.89	975.55 ± 83.24
Transit (days)	5.95 ± 0.35	7.23 ± 0.40	6.46 ± 0.45	6.36 ± 0.61
Total transit (days)	9.81 ± 0.47	12.05 ± 0.84	10.92 ± 0.50	10.96 ± 0.75

Values expressed as mean ± SEM, 9-10 rats/group. Analysis of variance - ANOVA with Dunett's posterior test.
* $p < 0.05$ and ** $p < 0.01$ compared to the control group.

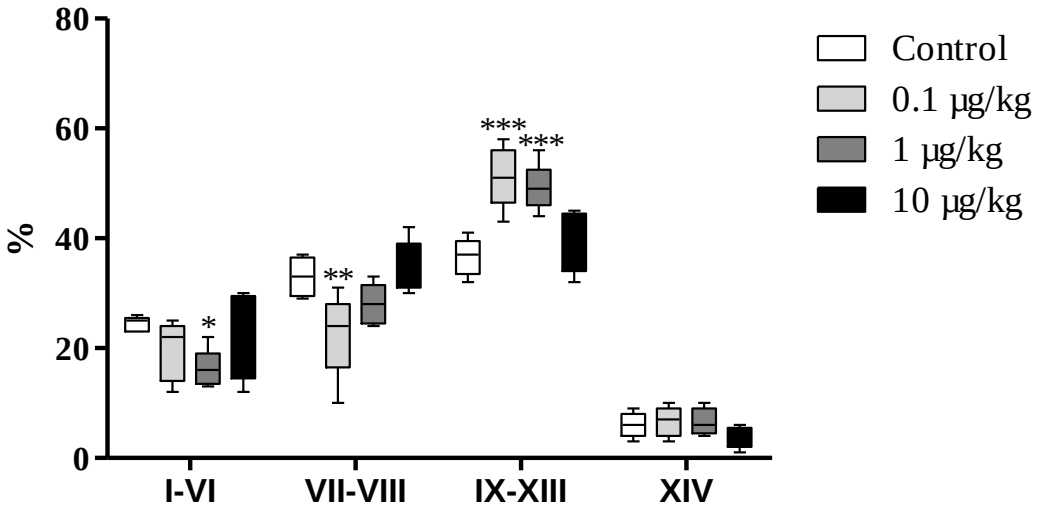

Figure 2 – Stages of the seminiferous tubules of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty. Values expressed in median (Q1-Q3), 9-10 rats/group. Kruskal-Wallis with Dunn's posterior test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the control group.

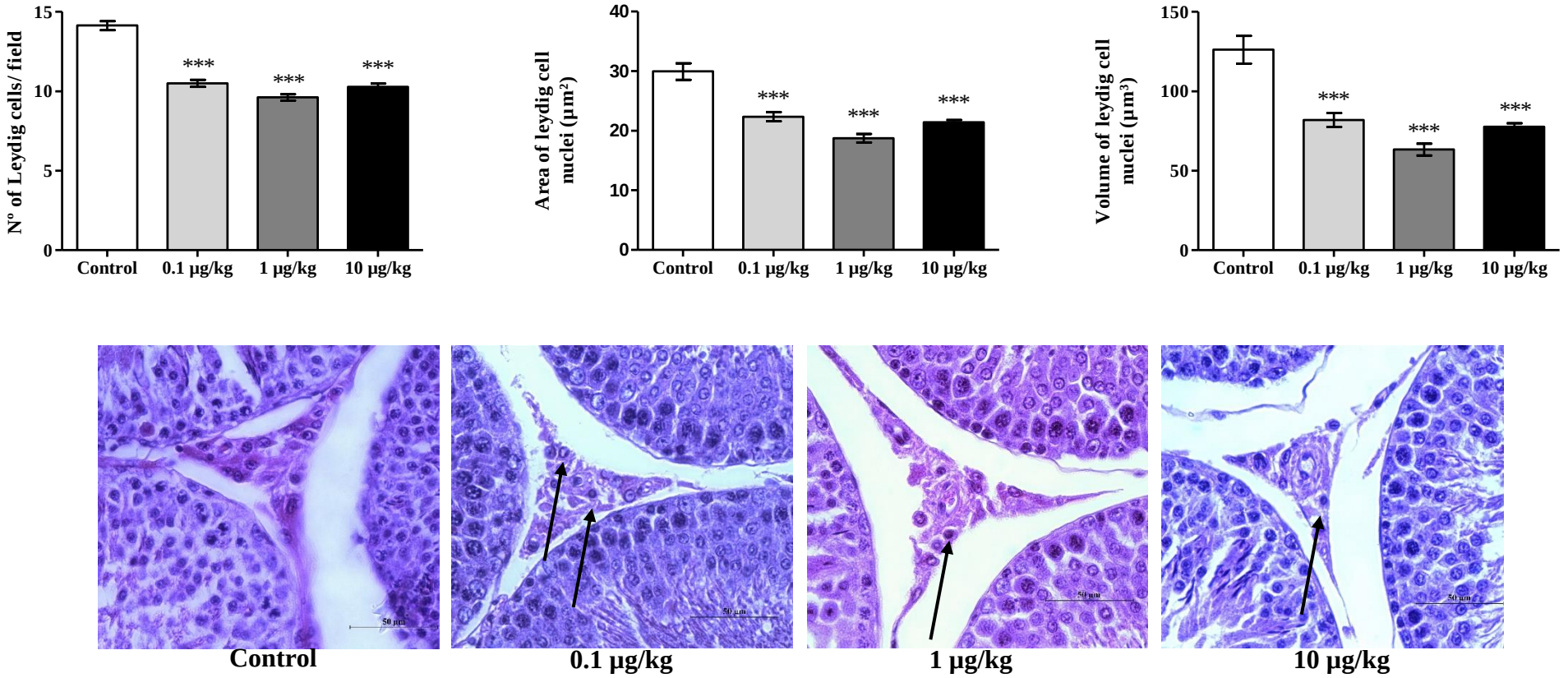

Figure 3 - Leydig cell nuclei count and measurements (area and volume) in the interstitial tissue of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty. Values expressed as mean ± SEM, 5 rats/group. Analysis of variance - ANOVA with Dunett's posterior test.*** p <0.001 compared to the control group. Figures of Leydig cell of all the groups with magnification of 40X. The arrows indicate atrophy.

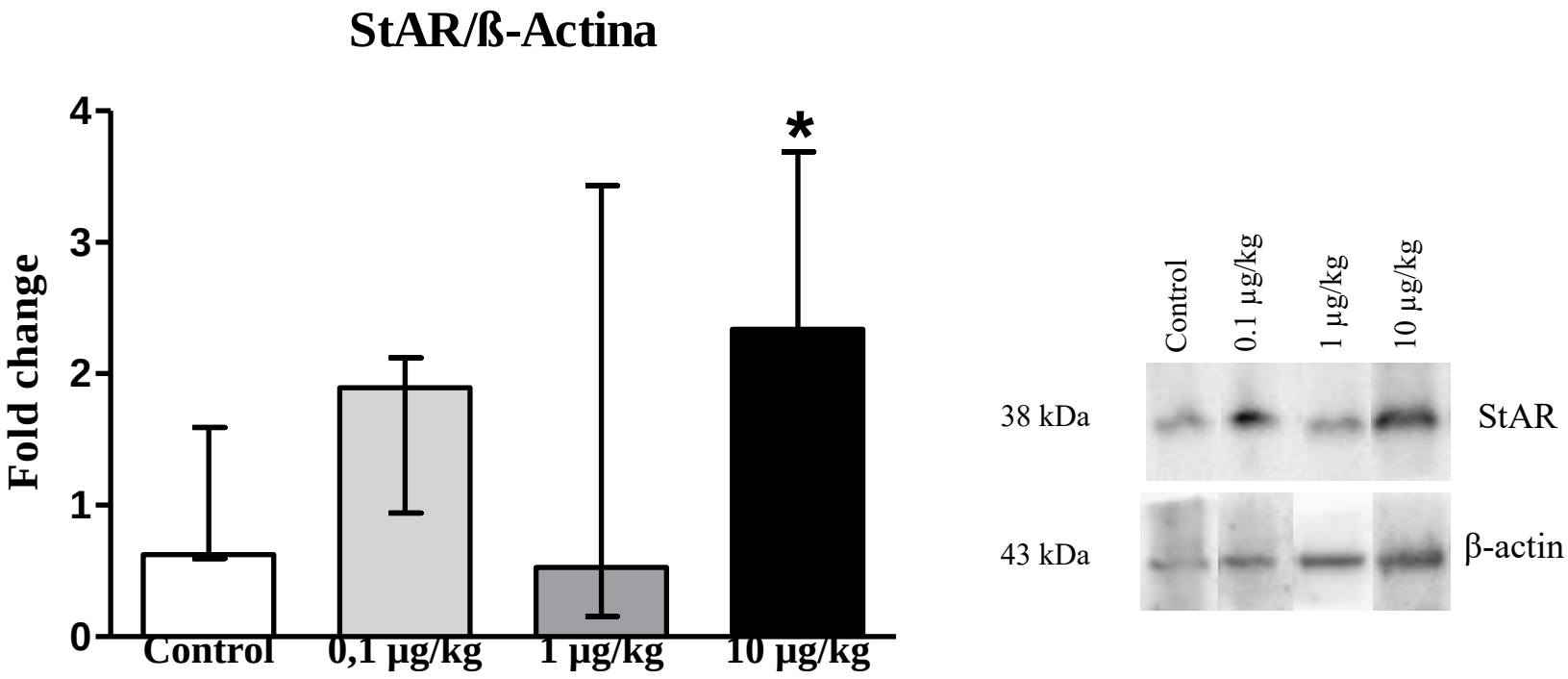

Figure 4 – Image and plot for expression of StAR protein in testis (PND 90) by western blot of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty. Median with interquartile range. Kruskal-Wallis.

Supplementary material

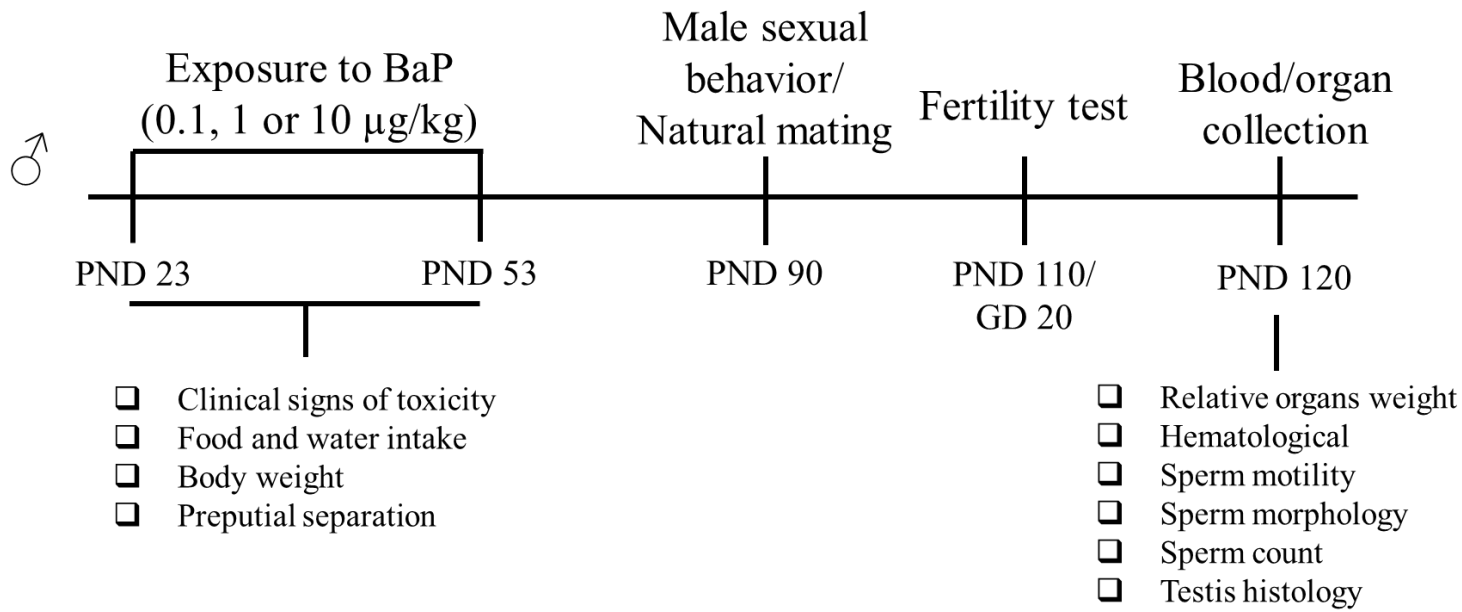

Figure 1 – Experimental design

Table 1 – Final body weight and water and food consumption of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty.

Parameters	Experimental groups			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Initial body weight (g)	69.17 ± 1.67	67.71 ± 1.81	69.72 ± 1.69	68.68 ± 1.46
Final body weight (g)	263.16 ± 7.36	250.73 ± 5.55	264.97 ± 9.45	259.03 ± 8.00
Food intake (g/day)	20.40 ± 0.39	19.76 ± 0.15	21.22 ± 0.35	20.19 ± 0.35
Water intake (ml/day)	31.78 ± 0.40	31.67 ± 0.50	32.54 ± 0.65	31.04 ± 0.50

Values expressed as mean ± SEM, 10 rats/group. Analysis of variance - ANOVA with Dunett's posterior test.

Table 2 – Body weight regarding age to preputial separation of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty.

Groups	Preputial separation (days)	Body weight (g)
Control	44.81 ± 0.44	207.45 ± 5.96
0.1 µg/kg	45.64 ± 0.49	219.09 ± 6.74
1 µg/kg	44.45 ± 0.37	209.65 ± 3.45
10 µg/kg	45.36 ± 0.34	214.44 ± 5.70

Values expressed as mean ± SEM, 10 rats/group. Analysis of variance - ANOVA with Dunett's posterior test.

Table 3 - Hematological parameters of male rats exposed to 0.1, 1 or 10 µg/kg of BaP from juvenile period to peripuberty.

Parameters	Experimental groups			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Hematocrit (%)	48.70 ± 0.95	49.55 ± 0.94	48.7 ± 0.67	46.30 ± 1.76
Leukocytes (10^3/mm^3)	9.70 ± 0.87	9.06 ± 0.79	11.15 ± 0.66	11.19 ± 0.47
Lymphocyte (%)	63.40 ± 1.32	63.11 ± 3.03	59.50 ± 2.74	55.11 ± 2.16*
Neutrophil (%)	24.90 ± 1.46	22.67 ± 2.56	24.10 ± 2.57	24.90 ± 2.20
Monocyte (%)	9.40 ± 1.17	10.40 ± 1.90	11.60 ± 0.98	13.40 ± 1.77
Eosinophil (%)	1.80 ± 0.42	1.90 ± 0.41	3.00 ± 0.65	2.80 ± 0.53
Basophile (%)	0.50 ± 0.22	1.60 ± 0.43	1.80 ± 0.42	1.70 ± 0.47
Blood cells (10^6/mm^3)	8.24 ± 0.16	7.90 ± 0.28	8.24 ± 0.21	8.31 ± 0.16
Platelets (10^3/mm^3)	18.67 ± 2.17	12.00 ± 7.14*	13.78 ± 1.94	10.80 ± 9.75**
Hemoglobin (g/Dl)	15.02 ± 0.31	14.35 ± 0.48	14.79 ± 0.27	13.25 ± 0.72*
MCV	57.99 ± 1.34	61.71 ± 3.01	58.01 ± 1.38	55.74 ± 2.01
MCH	17.96 ± 0.62	18.26 ± 0.60	18.03 ± 0.57	16.36 ± 0.82
MCHC (%)	30.30 ± 0.74	29.82 ± 0.62	30.36 ± 0.58	29.37 ± 1.13

Values expressed as mean ± SEM, 9-10 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. * $p < 0.05$ and ** $p < 0.01$ compared to the control group.

Capítulo II - O manuscrito intitulado "**Reproductive impairment in the offspring mediated by paternal exposure to benzo(a)pyrene during juvenile period to peripuberty in rats**" será submetido para publicação no periódico "Journal of Toxicology and Environmental Health, Part A". Fator de impacto: 2.706.

REPRODUCTIVE IMPAIRMENT IN THE OFFSPRING MEDIATED BY PATERNAL EXPOSURE TO BENZO(A)PYRENE FROM JUVENILE PERIOD TO PERIPUBERTY IN RATS

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ABSTRACT

Developmental programming is defined how any injury that can alter normal trajectory of organism during a critical developmental window. Endocrine-disrupting chemicals (EDCs) can change reproductive parameters and produce negative impacts not only in the exposed individual but also in the offspring and subsequent generations. The potential EDC evaluated in this study is the benzo(a)pyrene (BaP), a substance present in cigarette smoke and burning of organic compounds. Our objective was to evaluate the reproductive impacts of the offspring (males and females) mediated by paternal exposure to BaP from juvenile period to peripuberty. Male rats (23 days) were treated with BaP (0, 0.1, 1, or 10 µg/kg) for 31 consecutive days (gavage). In adulthood, these males were mated with non-treated females to obtain the offspring. The initial sexual development and the onset of puberty were evaluated in both male and female pups. Estrous cyclicity, fertility test and ovary histology were evaluated in female offspring and the sperm parameters in the males. The body weight was decreased in the female offspring of the low and mid doses and relative anogenital distance (AGD) was altered in males exposed to the lowest dose. Vaginal opening and first estrus were anticipated in the lowest dose group while the testicular descent and preputial separation were delayed (0.1 µg/kg) when compared to control group. In adulthood, the number of estrous and fertility parameters were altered (0.1 µg/kg), as well as follicle and corpora lutea counts of the treated-groups. In conclusion, paternal exposure to low-doses to BaP causes negative impacts on the reproductive parameters in the offspring, suggesting alterations in sperm provoked by this substance.

Keywords: endocrine disruptor, benzo(a)pyrene, paternal programming, fertility, offspring.

1. INTRODUCTION

Any injury that alters the normal trajectory of development during the critical developmental window (Robinson, 2006; Patisaul, 2009) and may result in persistent effects on offspring phenotype can be defined as developmental programming, a response to a specific challenge to the mammalian organism (Rabadán-Diehl and Nathanielsz 2013; Zambrano et al. 2014). The consequences of molecular and cellular alterations that occur during this exposure can remain in latency and to manifest only in adulthood (Zambrano et al. 2014), and/or in their offspring (Patisaul 2009; Sookoian et al. 2013).

Several studies with maternal exposure to certain substances have proved they can cause disorders in the adult life of offspring due to disturbances in the intrauterine phase, via placenta (Kiss et al., 2012; Santos et al., 2016; Cloarec et al., 2018), but the paternal contribution to developmental origins of health and disease (DOHaD) is poorly understood and explored. Endocrine-disrupting chemicals (EDCs) play a role in DOHaD and can provoke epigenetic alterations in the paternal germ cells (Gore et al. 2016), leading to damage in offspring. The EDCs are an exogenous compounds class that interfere with any hormonal aspect, such as inhibition, stimulation or alteration of hormonal metabolism,, affecting the functions regulated by it. (Diamanti-Kandarakis et al. 2009; Gore et al. 2016).

Benzo(a)pyrene (BaP) is a substance formed by incomplete combustion of organic compounds and belongs to the polycycle aromatic hydrocarbons (PAH) class (Domingo and Nadal 2015; Marzooghi and Di Toro 2017). It is present in cigarette smoke, motor vehicle exhaust, grilled food, contaminated water and soil and industrial processes (US-EPA 2017). BaP is a substance ubiquitous in the environment and exposure to BaP occurs via dermal, inhalation and digestive (Abdel-Shafy and Mansour

2016; US-EPA 2017; Ribière et al. 2016). Due to its ability to accumulate in various organs and its genotoxic nature, BaP has become a compound for many toxicological investigations (for revision, see Verma et al. 2012), and epidemiological and animal models studies confirmed the toxic effects of this substance in high doses (Sadeu and Foster 2011; Yu et al. 2011; Xia et al. 2011).

Due to its potential for endocrine disruptor, this study aimed to investigate if exposure to low-doses of BaP from juvenile period to peripuberty in male rats can provoke paternal programming and produce reproductive impairment in male and female offspring.

2. MATERIALS AND METHODS

2.1. Animals

12 female Wistar (90 days old) and 12 Wistar males (90 days old) were obtained from the UNESP Central Animal Bioterium for natural mating. Male offspring (23 days old) was randomly divided into four experimental groups The animals were maintained under controlled temperature (22 ± 2ºC) and lighting conditions (12 h light:dark cycle) with free access to food and water and were not exposed to identifiable endocrine disruptors. All procedures are according with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee for Animal Experimentation at the Institute of Biosciences of Botucatu/UNESP (958/2017)**.**

2.2. Experimental Design

Male juvenile rats Wistar on 23 post-natal days (PND) were randomly distributed into four experimental groups (n= 7 animals/group), one control (received corn oil + DMSO (0,5%)) and three groups treated with different doses of

benzo(a)pyrene (0.1, 1 or 10 µg/kg) during 31 consecutive days (gavage), on PND 23 to 53, encompassing from juvenile period to peripuberty of male rats (a critical window of development) (Picut, Remick, et al. 2015). The choice of doses was based on human exposure to BaP, where the median BaP found in European food is 0.2 µg/kg (EFSA, 2008) and an active smoker is exposed to approximately 0.7 µg/kg/day of BaP (ATSDR, 2009). In adulthood (PND 90), there males were naturally mated with non-treated females and their offspring were evaluated in the present study (n= 7 litters/group).

2.3. Initial evaluation of sexual development

At birth (PND 1), the offspring were reduced to 4 female and 4 male pups per litter. On PND 1, 13 and 22, the anogenital distance (AGD) was measured manually with digital caliper from the genital tubercle to the fetuses' anus, as well as body weight. Relative AGD was obtained by divided anogenital distance (mm) by cube root of body weight (g). From PND 15, the date of the testicular descent was evaluated by palpation of the scrotum of male offspring.

In the female offspring, the external signs of puberty onset were evaluated (since PND 30) by detection of the vaginal canal opening and this day, the body weight of the females were noted. Vaginal lavage was performed to identify the first estrous, with the predominance of cornified cells in light microscopy. In the male offspring, the date of preputial separation was detection through manual retraction of the foreskin and the body weight of the males were noted.

After the onset of puberty of the female pups (approximately on PND 42), two females per litter were killed for collection of organs and histological analysis of the ovary. On PND 90, one female of each litter was also killed for collection of organs and

histological analysis, and another female for to fertility test. In adult life (PND 90), the male offspring were killed for collection of organs and sperm parameters analysis.

2.4. Female offspring evaluation

2.4.1. Estrous cycle

During fifteen consecutive days (8:00 to 10:00 a.m.) vaginal smears was collected (n= 14 females/group) on PND 70 to 85, to determinate the phases of the estrous cycle: estrous, metaestrous, diestrous or proestrous. The collection was performed with 20 µl of saline solution (0.9% NaCl) deposited on the edge of the vagina with a pipette and quickly pulled and deposited the material in histological slide. Immediately, the material was analyzed with microscopic light in the magnification 200 x and the stage of the estrous cycle was determined by the characteristic cellular proportions. Estrous: cornified cells; metaestrous: leukocytes, cornified and nucleated cells; diestrous: predominance of leukocytes and some cornified and nucleated cells; proestrous: nucleated cells. Thus, the number of days in each phase, the duration of the cycles and the number of complete cycles were determined (Marcondes et al., 2002).

2.4.2. Fertility tests

Vaginal material was collected for detection of estrous phase for natural mating with non-treated male in overnight. If sperm was found in vaginal cytology we considered it gestational day zero (GD 0). On GD 20, the pregnant rats were killed by decapitation and their uterine horns exposed by exploratory laparotomy. All the fetuses and placenta were weighted, and other parameters were measured: number of reabsorptions, number of fetus live, number of corpora lutea, fertility potential, sex ratio, post-implantation and pre-implantation loss index (Barros et al., 2016).

During the fertility test, all fetuses were classified according to body weight for gestational age in fertility test (n=6/7 litters/group). The mean body weight of control group ± SD (multiplicated for 1.7) is used to determine the range of body weight classification in large, appropriate or small for pregnancy age. If the fetus was smaller than mean – SD (control group) is considerable smaller for gestational age and if the fetus was bigger than mean + SD (control group) is considerable large for gestational age and between this range is appropriate for gestational day (Calderon, 1988). The placental efficiency also was determinate. For this, the body weight of fetuses was divided by weight of placenta for determination of efficiency nutrition of dams to pups (Fowed et al, 2009).

2.4.3. Collection of organs

Approximately on PND 42, two females per litter were killed for collection of organs (uterus, ovaries, kidney, adrenal, thyroid, spleen, liver, lung and heart). On PND 90, one female of each litter was killed by inhalation of CO_2 and cardiac puncture for collection of the blood and organs (to measured relative organs weight).

2.4.4. Histological analyses

The right ovary was fixed in Bouin's solution for one day and subsequent changed dehydrated in ethanol. It was paraplast embedded, sectioned at 5 µm (3 sections per animal) and stained with hematoxylin and eosin (H&E). On PND 42 and 90, ovarian follicles were count and classified as: primordial/primary, preantral, antral, corpora lutea or atretic

2.5. Male offspring evaluation

2.5.1. Collection of organs

On PND 90, the males were killed by inhalation of CO_2 and cardiac puncture for collection of the blood and organs (testis, epididymis, vesicle gland, ventral prostate, kidney, adrenal, thyroid, spleen, liver, lung and heart) to measured relative organs weight. The left testis and epididymis were stored in the freezer until the analysis described below was performed.

2.5.2. Sperm count (testis and epididymis)

Spermatids resistant to homogenization (stage 19 of spermiogenesis) and spermatozoa on the caput/ corpus and cauda of the epididymis were counted as previously described by Robb et al. (1978), with adaptations adopted by Fernandes et al. (2007). Briefly, each right testis, decapsulated and weighted soon after collection, was homogenized in 5mL of 0.9% NaCl containing 0.5% TritonX100. After dilution of 10 times, the sample was transferred to Neubauer chambers (4 fields per animal), counting mature spermatids. For the calculation of the daily sperm production (DSP) the number of spermatids in stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium. Likewise, portions of the caput/corpus and epididymal cauda were cut into small fragments and homogenized, and sperm count as described as for the testis. The transit time of sperm through the epididymis was determined by dividing the number of sperm in each portion by the DSP.

2.5.3. Sperm morphology

The left vas deferens of the animals were sectioned at the proximal and distal extremities, and washed with a needle and syringe, with 1 ml of 10% formalin solution. 20 µl of this duct lavage was deposited on histological slides for the qualitative evaluation of spermatozoa. Thus, 200 sperm per animal were evaluated under light

144 microscopy (400X magnification). Morphological abnormalities found in spermatozoa
145 were classified as head and tail abnormalities (Filler, 1993).

146 **2.6. Statistical analysis**

147 All results obtained in the present study were submitted for normality test and if
148 considerable normal distribution was done ANOVA with post-test Dunnett. If
149 considered no normal distribution, was done Kruskal-Wallis with post-test Dunn. In
150 classification according to body weight was performed by fisher test. The results were
151 considered significant when $p < 0.05$.

152 **3. RESULTS**

153 In the female offspring from males treated with the lower and mid doses of BaP,
154 there was a reduction in the body weight in all evaluated ages, while the relative AGD
155 was unchanged (Table 1). In the same way, the male offspring showed a decrease in the
156 body weight on PND 1 (all treated-groups), 13 (1 µg/kg) and 22 (0.1 and 1 µg/kg), as
157 well as, a reduction in the relative AGD (0.1 and 1 µg/kg) (Table 1).

158 Concerning puberty installation, both the vaginal opening and first estrous were
159 antecipated (Figure 1A) and the body weight also decreased on vaginal opening day
160 (Figure 1B) in the group that the parents received the lowest dose of the BaP. The
161 testicular descent was delayed in relation to the control group in all treated groups and
162 the preputial separation was delayed in the lowest group (Figure 1C). The body weight
163 on preputial separation day was decreased in the lowest and mid group (Figure 1D).

164 In the adulthood, the frequency of estrous days was decreased of the groups 0.1
165 and 1 µg/kg (Table 2). In fertility test, the group exposed to the lowest dose showed
166 several alterations, with reduction in the following parameters: final maternal weight,
167 uterine + fetus weight, number of live fetuses, implantations and corpora lutea. The

placenta weight showed increased when compared to control group (Table 3). Placental efficiency was also altered in the group of lowest and mid dose (Figure 2A). The distribution of body weight according to pregnancy age was shifted to right when compared to the normal distribution of the control group, increased the large fetuses for pregnancy age and diminished of appropriate fetuses for pregnancy age (Figure 2B).

The body weight on PND 45 and 90 were reduced when compared to control group and the relative organs weight remained unchanged (Table 4). The ovarian structure was changed, and follicles count was altered in both PND 45 and 90. In ovarian histology (PND 45), there was decreased of antral follicles and number of corpora lutea in all treated-groups. There was an increase of primordial/primary and atretic follicles when compared to control group (Figure 3A). On PND 90, the ovarian structure remained altered, with increased in the number of primordial/primary follicles (0.1 µg/kg), preantral and atretic follicles, as well as a decrease in the corpora lutea (Figure 3B).

On PND 90, the male offspring of the mid dose present a reduction in the absolute number of spermatids and daily sperm production in the testis (Table 5). The other sperm parameters were not changed in relation to control group.

4. DISCUSSION

Studies have provided evidence supporting the impact of exposure to endocrine disruptors on developmental programming (Grandjean et al., 2015) during the critical periods of fetal and child development. The results obtained in this study reinforce this hypothesis, demonstrating that the paternal exposure to low-doses of BaP from juvenile period to peripuberty causes negative reproductive effects in male and female offspring.

In mammals, the anogenital distance (AGD) is a sexually dimorphic, hormone-dependent parameter and an external marker of masculinization and feminization, with males having a AGD measure twice as large as females (Clark et al. 1990; Rhess et al. 1997). It is known that exogenous substances that have altered the hormonal action during prenatal development can alter the process of masculinization (Thankamony et al., 2016). Thus, the reduction in AGD of the male offspring found in the present study may indicate a possible feminization of these animals, with the interference of an antiandrogenic substance

Low birth weight is a predictive parameter for the development of diseases in adulthood (Collman 2011; Zambrano et al. 2014), such as obesity and metabolic diseases (Wadhwa et al. 2010). Alharthy and collaborators (2017) showed a relation between the exposure to PAHs (polycyclic aromatic hydrocarbons) and low birth weight of the offspring, in epidemiological and experimental studies. The groups of 0.1 and 1 μg/kg presented greater reduction in body weight (males and females) in all the measurements performed and thus can be related to the repercussions in several altered reproductive parameters in adult life, such as precocious (female) and delay (male) puberty.

The complex transition process of childhood to adult life is highly hormone-dependent, and any alteration in this trajectory may cause impaired fertility in adulthood (Mantovani and Fucic. 2014). Some substances that act as EDCs are inductors of precocious puberty in females and delayed puberty in males (Rojas et al., 2015), data also found in our study. In the female offspring, changes caused by BaP in the paternal sperm produced estrogenic response patterns, such as precocious puberty and decreased fertility. Male offspring responded differently, presenting a decreased AGD and delayed testicular descent and preputial separation, produced anti-androgen response patterns. It

217 is known that onset of the puberty is related to the nutritional status of the individual,
218 for example: girls of the same age who present greater body weight had precocious
219 puberty when compared to girls with weight within normal (Rojas et al. 2015).
220 However, the data obtained in this study may seem to be conflicting since the females
221 showed precocious puberty and a decrease in body weight in the lower dose group.

222 The term “estrous” in rodents refers to the period that females are sexually
223 receptive to males. It is start with increase of estradiol circulant and is displayed by
224 cornified cells in vaginal cytology, starting after proestrous until early to metaestrous
225 (Jerome M Goldman, Murr, and Cooper 2007). The reduction in the frequency of
226 estrous days can cause a decreased in fertility, since the females were less receptive
227 days when compared to control group. This evaluation is important because any
228 changed in the estrous cycle may reflect the interference of ECDs in the hypothalamus-
229 pituitary-ovary axis (Gore et al. 2016).

230 Besides the decrease in the number of days in estrous, the females of the lower
231 dose group showed a reduction in several fertility parameters and alteration in ovarian
232 structure. A study *in vitro* indicated that BaP compromises preimplantation embryo
233 development and embryo quality inducing oxidative stress, DNA damage and apoptosis
234 (Zhan et al. 2015), reinforcing that BaP is a toxic agent for the ovary. (Neal et al. 2007)
235 and corroboration with our findings.

236 The efficiency placental is defined as the grams of fetus by grams of placenta
237 and the capacity to placenta nutrients and hormones to fetus and dam. Any
238 morphological alterations in the placenta can means that adaptations of efficiency
239 placental due to adverse uterine conditions (Fowden et al. 2009; Sandovici et al. 2012)
240 and these adaptations can occur to maximize fetal development and growth.
241 Corroborating with data, it is well documented that substances that cause displacement

242 in the normal size distribution of newborns to pregnancy age (small or large) increase
243 the risk for disease in adult life (Fowden et al. 2009), as observed in the present study.

244 Studies that showed the BaP as ovarian toxicity are relevant, involving in vitro
245 and in vivo (rodent and human) (Sadeu and Foster 2011; Neal et al. 2007; Zhan et al.
246 2015). Its known that for follicular growth its necessary many factors and
247 communication between follicular cells and oocyte, and alterations in this process can
248 cause reproductive damage. *In vitro*, exposure to BaP causes a delay in follicular
249 development and progression to the antral follicle (Sadeu and Foster 2011), reinforced
250 by the present study, since that results showed a reduce antral follicles and increased of
251 preantral and atretic. Other study with exposure to 100 µg/kg de BPA also increased the
252 number of atretic follicles pathways then expression to BCL2-associated X protein
253 (Bax) to the factor B cell lymphoma 2 (Bcl2), leading to follicle atresia (Peretz, Craig,
254 and Flaws 2012) and in vivo with chronic exposure to BPA lead to for induction to
255 caspase-3-associated apoptosis (Patel et al. 2015; Lee et al. 2013).

256 According to our results, the BaP could alter morphological and reproductive
257 aspects that are linked to dependent hormonal action, such as installation of puberty
258 (male and female) and fertility test. The lowest dose group (0.1 µg/kg) reported more
259 changes when compared to the other groups, showing that the BaP did not present the
260 classical dose response curve. This feature is found in substances that acts like to
261 endocrine disruptors chemicals and that, in low-doses, can affect several biological
262 processes, represented by non-monotonic dose-response (NMDR) (Vandenberg et al.
263 2012). The NMDR describes a dose-response relationship characterized by a curve
264 whose slope alters direction within the range of groups with dose test. This non-
265 monotonicity represents a challenge to fundamental concepts in toxicology
266 (Vandenberg et al. 2012; Patisaul 2009; Lagarde et al. 2015).

This study showed that exposure to BaP in critical development window in male rats cause several impacts in reproductive parameters in the offspring, suggesting that substance provoke epigenetic alterations in spermatogonia and affect the next generation. Further studies should be carried out to investigate the ability of BaP to cause changes in the parental germ cells and to generate reproductive impacts for the next generation, and if these impacts can also be observed in F2.

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Table 1 – Body weight (g) and relative anogenital distance (mm/g^3) on PND 1, 13 and 22 of offspring from males exposed to BaP.

Parameters	Experimental groups			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Female				
Body weight (g)				
PND 1	7.20 ± 0.14	6.67 ± 0.13**	6.53 ± 0.09**	7.02 ± 0.11
PND 13	29.80 ± 0.47	28.30 ± 0.35*	26.50 ± 0.50**	29.80 ± 0.38
PND 22	55.81 ± 0.63	52.32 ± 0.71**	50.37 ± 1.05**	55.40 ± 0.72
Relative anogenital distance (mm/g^3)				
PND 1	1.29 ± 0.02	1.28 ± 0.04	1.34 ± 0.02	1.24 ± 0.02
PND 13	2.58 ± 0.03	2.62 ± 0.03	2.56 ± 0.05	2.70 ± 0.42
PND 22	3.43 ± 0.06	3.49 ± 0.05	3.34 ± 0.05	3.33 ± 0.04
Male				
Body weight (g)				
PND 1	7.45 ± 0.12	6.67 ± 0.21**	6.93 ± 0.11*	6.84 ± 0.11*
PND 13	30.43 ± 0.37	29.50 ± 0.36	27.80 ± 0.68*	30.88 ± 0.47
PND 22	55.79 ± 0.80	52.41 ± 0.85*	51.64 ± 1.39*	56.72 ± 0.73
Relative anogenital distance (mm/g^3)				
PND 1	2.40 ± 0.03	2.27 ± 0.03*	2.45 ± 0.04	2.36 ± 0.04
PND 13	3.81 ± 0.06	3.62 ± 0.03*	3.61 ± 0.04*	3.81 ± 0.06
PND 22	5.50 ± 0.09	5.20 ± 0.08*	5.20 ± 0.10*	5.25 ± 0.07

Values expressed as mean ± SEM, 7 litters/group. Analysis of variance - ANOVA with Dunett's posterior test. * $p < 0.05$ and ** $p < 0.01$ compared to control group. PND: post-natal day.

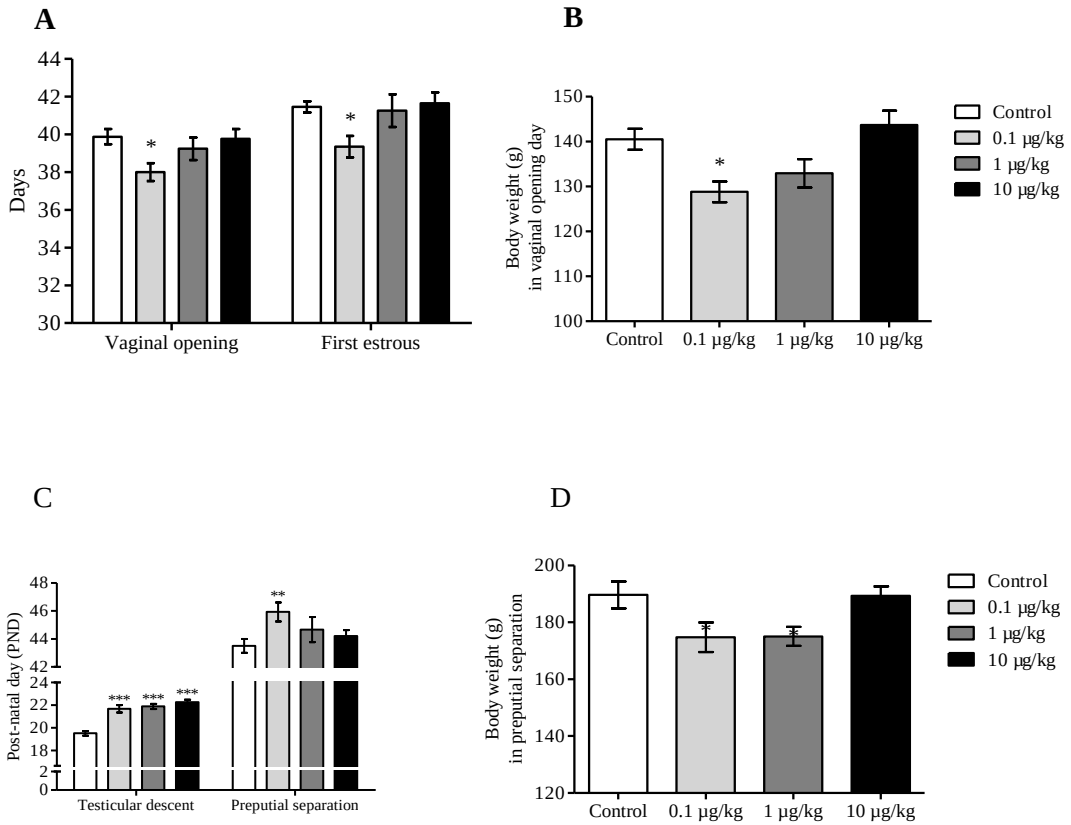

Figure 1 – Vaginal opening, first estrous days (A) and body weight (g) on vaginal opening day (B); testicular descent, preputial separation in days (C) and body weight (g) on preputial separation day (D) of offspring from males exposed to BaP. Values expressed in mean ± SEM (28 animals/group) ANOVA with post-test Dunett. *p < 0.05; ** p < 0.01 and ***p < 0.001 compared to control group.

Table 2 – Estrous cycle during fifteen consecutive days on PND 75 to 90 of female offspring from males exposed to BaP

Parameters	Experimental groups			
	Control	**0,1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Number of estrous cycles	2.27 ± 0.14	2.14 ± 0.18	2.15 ± 0.15	2.29 ± 0.24
Estrous cycle length	4.64 ± 0.20	4.33 ± 0.26	4.44 ± 0.27	4.17 ± 0.24
Estrous (days)	5.58 ± 0.51	4.17 ± 0.40*	4.16 ± 0.32*	4.85 ± 0.34
Metaestrous (days)	3.54 ± 0.37	4.23 ± 0.30	4.14 ± 0.25	4.38 ± 0.29
Diestrous (days)	3.67 ± 0.35	5.22 ± 0.64*	4.71 ± 0.42	4.36 ± 0.31
Proestrous (days)	2.77 ± 0.30	2.46 ± 0.31	2.46 ± 0.31	1.82 ± 0.23

Values expressed as mean ± SEM, 14 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. * $p < 0.05$ compared to control group.

Table 3 – Fertility test by exploratory laparotomy on GD (gestational day) 20 of female offspring from males exposed to BaP

Parameters	**Experimental groups**			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Pregnancy rate (%)	100	85.71	100	85.71
#Fertility potential (%)	100 (86 – 100)	91 (64 – 100)	100 (80 – 100)	96.5 (80 – 100)
Final maternal weight (g)	374.83 ± 14.45	319.43 ± 13.43*	331.27 ± 9.54	376.50 ± 20.49
Weight gain (g)	130.50 ± 9.55	98.53 ± 12.53	109.93 ± 6.26	125.80 ± 10.31
Uterine + fetal weight (g)	69.84 ± 7.11	48.85 ± 3.70*	67.76 ± 4.41	74.56 ± 3.47
Placenta weight (g)	0.56 ± 0.02	0.67 ± 0.02**	0.59 ± 0.02	0.54 ± 0.02
Number of live fetuses	12.17 ± 1.45	7.80 ± 0.86*	11.83 ± 0.60	12.50 ± 0.56
Number of implantations	13.67 ± 0.92	9.80 ± 0.37**	12.50 ± 0.43	13.40 ± 0.25
Number of corpora lutea	14.33 ± 0.67	12.17 ± 0.70*	13.00 ± 0.58	14.00 ± 0.36
#Number of resorptions	1.00 (0.00 - 2.00)	1.00 (0.00 - 5.00)	0.00 (0.00 - 3.00)	0.00 (0.00 – 2.00)
#Pre-implantation loss (%)	0 (0 - 14)	8 (0 – 36)	0 (0 – 20)	3.5 (0 – 20)
#Post-implantation loss (%)	6 (0 – 18)	10 (0 - 50)	0 (0 – 23)	0 (0 – 15)
Sex ratio (male/female)	1.86 ± 0.21	1.05 ± 0.21	1.32 ± 0.38	1.77 ± 0.55

Values expressed as mean ± SEM, 6-7 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. * $p < 0.05$ and ** $p < 0.01$ compared to control group. #Values expressed in median (Q1-Q3), 6-7 rats/group. Kruskal-Wallis with Dunn's posterior test. $p > 0.05$

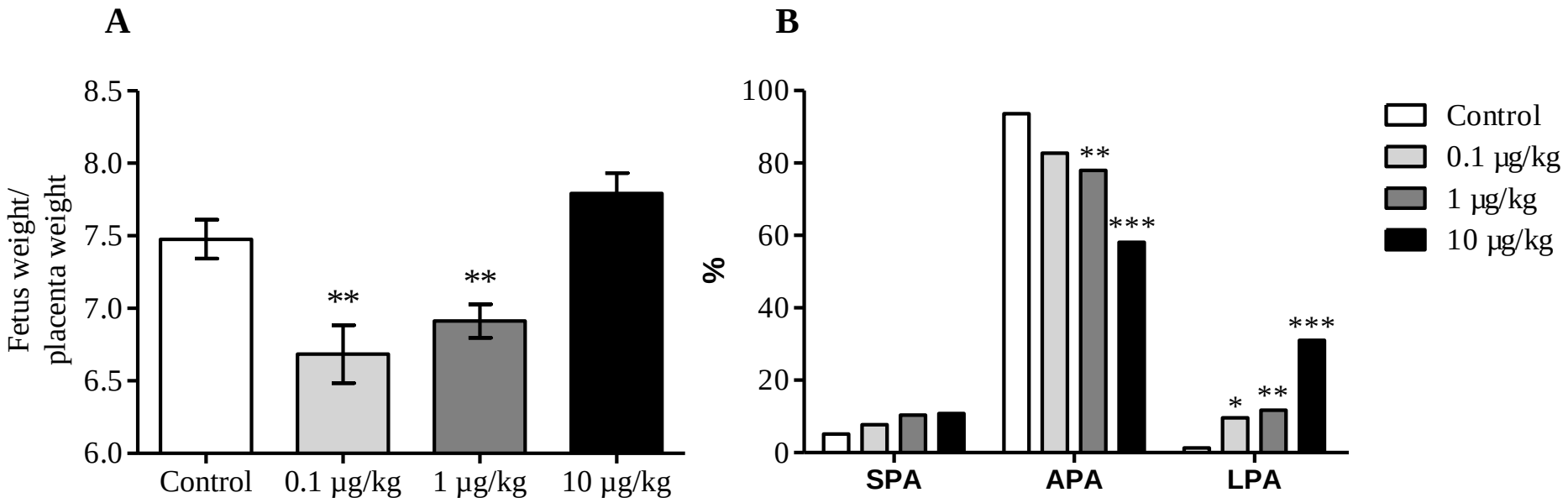

Figure 2 – A. Placental efficiency (fetus weight / placenta weight) and **B** - distribution of normal weight of fetuses in fertility test on GD 20 of female offspring from males exposed to BaP - SPA – small for pregnancy age, APA – appropriate for pregnancy age, LPA – large for pregnancy age. A - Values expressed as mean ± SEM, 6-7 litters/group. Analysis of variance - ANOVA with Dunett's posterior test. **p <0.01 compared to the control group. B – Fisher's test. * p <0.05, ** p <0.01 and *** p <0.001 compared to control group.

Table 4 – Relative organ weight on PND 45 and PND 90 offemale offspring from males exposed to BaP

Parameters	Experimental groups			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
45 days				
Body weight (g)	173.68 ± 3.36	157.26 ± 5.28*	162.68 ± 4.46	166.90 ± 3.90
Ovaries (mg)	43.00 ± 6.44	49.28 ± 4.00	39.17 ± 3.63	31.29 ± 2.44
Uterus + fluid (mg)	150.67 ± 14.78	195.00 ± 14.50	183.67 ± 18.00	139.86 ± 18.91
Liver (g)	4.45 ± 0.33	5.34 ± 0.31	4.85 ± 0.60	4.45 ± 0.38
Kidney (g)	0.45 ± 0.05	0.51 ± 0.02	0.43 ± 0.05	0.43 ± 0.04
Adrenal gland (mg)	16.39 ± 1.85	17.93 ± 0.58	14.95 ± 2.63	13.94 ± 1.76
Spleen (g)	0.24 ± 0.02	0.27 ± 0.01	0.26 ± 0.03	0.25 ± 0.02
90 days				
Body weight (g)	254.24 ± 7.35	220.42±4.54**	231.43 ± 3.00	231.23 ± 9.53
Ovaries (mg)	37.86 ± 3.98	41.83 ± 3.46	46.33 ± 2.82	44.67 ± 1.69
Uterus + fluid (mg)	190.33 ± 10.48	192.29 ± 16.83	229.50 ± 17.62	175.83 ± 5.36
Liver (g)	4.14 ± 0.23	4.16 ± 0.11	3.87 ± 0.11	3.97 ± 0.09
Kidney (g)	0.42 ± 0.01	0.43 ± 0.007	0.43 ± 0.008	0.44 ± 0.007
Adrenal gland (mg)	18.00 ± 0.78	17.84 ± 1.77	18.09 ± 0.78	16.95 ± 0.66
Spleen (g)	0.23 ± 0.00	0.22 ± 0.01	0.23 ± 0.00	0.25 ± 0.01
Heart (g)	0.34 ± 0.00	0.38 ± 0.01	0.40 ± 0.01	0.35 ± 0.01
Lung (g)	0.55 ± 0.02	0.58 ± 0.03	0.56 ± 0.01	0.60 ± 0.02
Thyroid (mg)	2.06 ± 0.32	2.88 ± 0.78	4.02 ± 0.18	3.88 ± 0.79

Values expressed as mean ± SEM, 6-7 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. * $p < 0.05$ and ** $p < 0.01$ compared to control group.

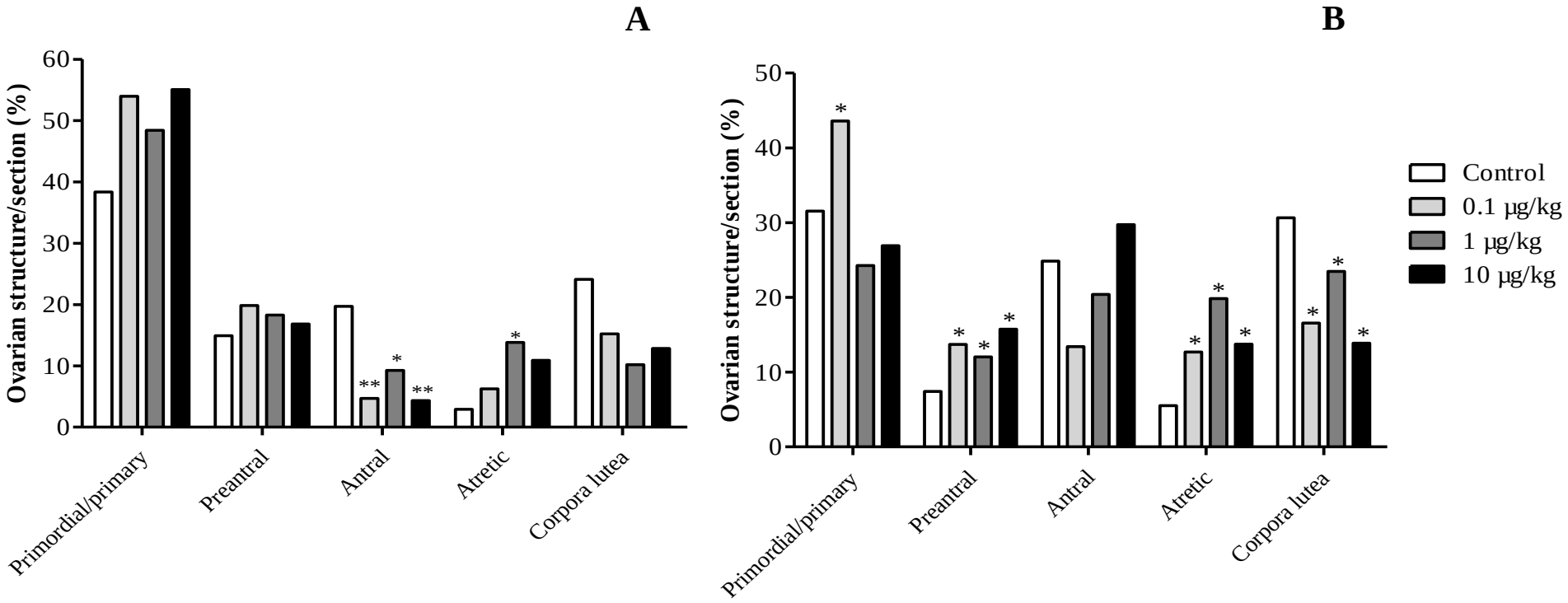

Figure 3 – Follicle count and classification in primordial/primary, preantral, antral, atretic follicle and corpora lutea for section of ovarian structure in PND 45 (A) and PND 90 (B) of female offspring from males exposed to BaP Values expressed as mean ± SEM, 6-7 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. * p <0.05 and ** p <0.01 compared to control group

Table 5 - Sperm count (PND 90) and morphology of male offspring from males exposed to BaP

Parameters	Experimental groups			
	Control	**0.1 µg/kg**	**1 µg/kg**	**10 µg/kg**
Testis				
Number of mature spermatids ($x10^6$)	245,15 ± 7,99	216,23 ± 10,08	189,84 ± 18,12*	230,45 ± 15,07
Relative number of mature spermatids ($x10^6$/g)	188,27 ± 11,80	167,26 ± 10,40	150,92 ± 11,49	177,54 ± 7,97
Daily sperm production	40,19 ± 1,31	35,45 ± 1,65	31,12 ± 2,97*	37,78 ± 2,47
Epididymis – Caput/corpus				
Number of sperm ($x10^6$)	98,93 ± 11,19	91,34 ± 5,77	72,16 ± 10,33	95,05 ± 4,22
Relative number of sperm ($x10^6$/g)	391,04 ± 28,08	406,46 ± 13,51	350,18 ± 22,87	380,83 ± 22,08
Transit (days)	2,33 ± 0,25	2,60 ± 0,07	2,71 ± 0,47	2,74 ± 0,26
Epididymis - Cauda				
Number of sperm ($x10^6$)	149,83 ± 11,36	137,97 ± 15,88	136,64 ± 17,74	162,92 ± 11,91
Relative number of sperm ($x10^6$/g)	870,00 ± 54,23	825,42 ± 55,41	848,50 ± 51,81	836,79 ± 38,82
Transit (days)	3,53 ± 0,22	4,19 ± 0,56	5,37 ± 0,82	4,42 ± 0,44
#Normal sperm	97,50 (97,00–99,50)	97,00 (96,50–97,50)	96,00 (94,00–97,50)	95,50 (94,50–96,50)

Values expressed as mean ± SEM, 7 rats/group. Analysis of variance - ANOVA with Dunett's posterior test. #Kuskall-Wallis. * $p < 0.05$ compared to control group

Considerações finais

Assim, levando em consideração os resultados e a discussão dos dois capítulos apresentados, temos que o BaP age como um desregulador endócrino quando o indivíduo é exposto no período juvenil até a peripuberdade e gera impactos negativos na produção espermática na vida adulta. Além disso, o BaP é capaz de causar uma programação no desenvolvimento dos machos expostos e, via células germinativas, foi capaz de alterar vários parâmetros reprodutivos da sua prole masculina e feminina, deixando claro a importância da programação paterna no desenvolvimento da prole.