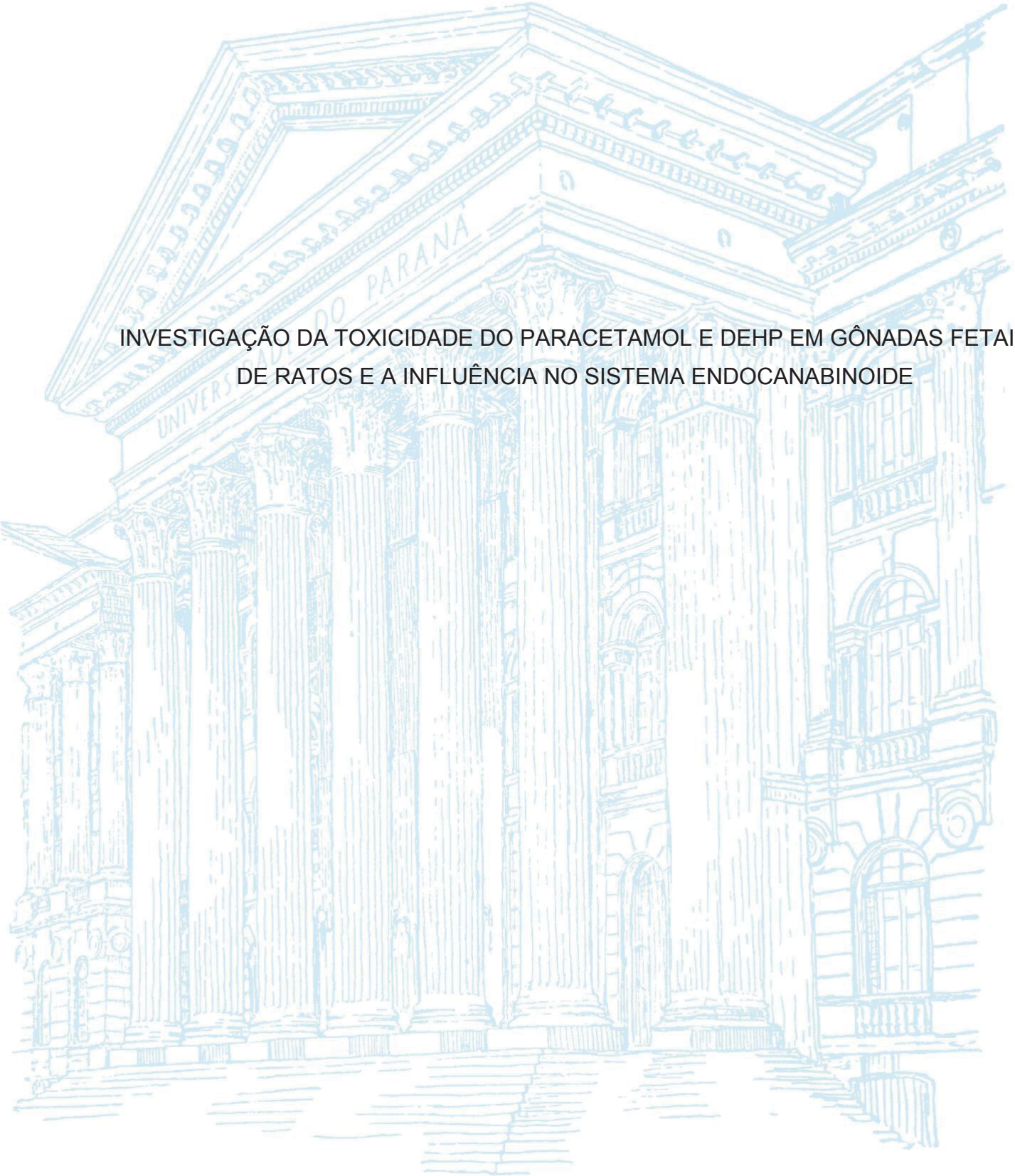
UNIVERSIDADE FEDERAL DO PARANÁ

ANDERSON TADEU DE ARAÚJO RAMOS



INVESTIGAÇÃO DA TOXICIDADE DO PARACETAMOL E DEHP EM GÔNADAS FETAIS DE RATOS E A INFLUÊNCIA NO SISTEMA ENDOCANABINOIDE

CURITIBA

2024

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INVESTIGAÇÃO DA TOXICIDADE DO PARACETAMOL E DEHP EM GÔNADAS FETAIS DE RATOS E A INFLUÊNCIA NO SISTEMA ENDOCANABINOIDE

Tese apresentada ao curso de Pós-Graduação em Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Fisiologia.

Orientador: Prof. Dr. Anderson Joel Martino Andrade

Coorientadora: Profa. Dra. Renata Marino Romano

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ATA DE SESSÃO PÚBLICA DE DEFESA DE DOUTORADO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM FISIOLOGIA

No dia vinte e seis de setembro de dois mil e vinte e quatro às 14:00 horas, na sala 103, Auditório do Departamento de Fisiologia (Defesa Híbrida) , foram instaladas as atividades pertinentes ao rito de defesa de tese do doutorando **ANDERSON TADEU DE ARAÚJO RAMOS**, intitulada**: "Investigação da toxicidade do paracetamol e DEHP em gônadas fetais de ratos e a influência do sistema endocanabinoide."**, sob orientação do Prof. Dr. ANDERSON JOEL MARTINO ANDRADE. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação FISIOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: ANDERSON JOEL MARTINO ANDRADE (UNIVERSIDADE FEDERAL DO PARANÁ), REJANE MAIRA GÓES (UNIVERSIDADE EST.PAULISTA JÚLIO DE MESQUITA FILHO), CRISTINA APARECIDA JARK STERN (UNIVERSIDADE FEDERAL DO PARANÁ), FABRICIA DE SOUZA PREDES (FACULDADE EST. DE FILOSOFIA, CIÊNCIAS E LETRAS DE PARANAGUÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutor está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, ANDERSON JOEL MARTINO ANDRADE, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 26 de Setembro de 2024.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FISIOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ANDERSON TADEU DE ARAÚJO RAMOS** intitulada: **"Investigação da toxicidade do paracetamol e DEHP em gônadas fetais de ratos e a influência do sistema endocanabinoide."**, sob orientação do Prof. Dr. ANDERSON JOEL MARTINO ANDRADE, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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NOTA EXPLICATIVA

Esta tese é apresentada no formato de artigos para publicações de acordo com as normas do Programa de Pós-Graduação em Fisiologia da Universidade Federal do Paraná. Neste formato constam: uma introdução e revisão de literatura, objetivos do trabalho, artigos científicos, discussão e a conclusão geral da tese. O primeiro artigo é uma revisão que discorre a respeito do papel do sistema endocanabinoide no desenvolvimento do testículo fetal e possíveis interferências mediadas por desreguladores endócrinos e, um artigo original que destaca os efeitos do paracetamol e do ftalato DEHP no testículo e ovário fetais de ratos Wistar. Os artigos foram formatados conforme as normas propostas pelos periódicos aos quais serão submetidos.

AGRADECIMENTOS

Escrever a seção de agradecimentos é sempre uma tarefa difícil. Aqui, destaco quem foi importante para o meu doutorado.

Primeiramente, agradeço à minha companheira Vitória Ribeiro Vila, pelo seu carinho, acolhimento e amor proporcionado ao longo desses últimos anos. Você com certeza tornou a minha vida especial.

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Por fim, agradeço a todos os pesquisadores que vieram antes de mim, que criaram uma base sólida no campo dos desreguladores endócrinos e gonadogênese, ao tornarem possível eu construir a minha tese de doutorado. Parafraseando o Google Scholar, “sobre os ombros de gigantes”.

Mire na lua. Mesmo que você erre, cairá entre as estrelas (Norman Vincent Peale, 1988).

RESUMO

As gônadas são órgãos fundamentais do sistema reprodutor, cuja principal função envolve a produção de hormônios e gametas. Para que essas funções ocorram adequadamente, o processo de gonadogênese — o desenvolvimento das gônadas durante o período embrio-fetal — precisa ser rigorosamente regulado. Alterações nesse processo podem levar a desordens do desenvolvimento sexual. Um dos mecanismos críticos nesse contexto é o sistema endocanabinoide, que, nos últimos anos, tem sido reconhecido como uma importante via de sinalização, envolvida na proliferação e diferenciação celular, produção hormonal, e regulação epigenética, entre outros processos. Contudo, há evidências de que desreguladores endócrinos, como o paracetamol e o DEHP (di-2-etilhexil ftalato), conhecidos por seus efeitos antiandrogênicos e potenciais danos ao desenvolvimento gonadal, podem interferir no sistema endocanabinoide das gônadas fetais. Este estudo teve como objetivo avaliar a toxicidade gonadal fetal induzida pela exposição ao paracetamol e ao DEHP, além de investigar a possível interação desses agentes com componentes do sistema endocanabinoide. Foram desenvolvidos dois artigos: o primeiro, uma revisão que compila a literatura existente sobre o papel do sistema endocanabinoide na gonadogênese, destacando a escassez de estudos nesse campo; o segundo, um estudo experimental original em ratos Wistar, no qual foram conduzidos modelos de exposição *in vivo* (entre os dias gestacionais 15 e 18) ao paracetamol (50 e 250 mg/kg/dia) e ao DEHP (750 mg/kg/dia), e também exposição *in vitro* utilizando paracetamol, AM404 (um metabólito do paracetamol que interage com o sistema endocanabinoide), rimonabanto (antagonista do receptor CB1) e MEHP (metabólito ativo do DEHP). Os resultados mostraram que o DEHP reduziu a distância anogenital e a produção de testosterona, além de aumentar a expressão dos genes *Cnr2* no testículo e ovário e *Napepld* no testículo no modelo *in utero*. No modelo *in vitro*, o MEHP elevou a liberação de testosterona. Por outro lado, não foram observados efeitos significativos associados à exposição ao paracetamol. Este trabalho inédito fornece evidências de que o sistema endocanabinoide é um alvo molecular do DEHP nas gônadas fetais de ratos, lançando novas luzes sobre as vias de toxicidade gonadal e a potencial interferência de desreguladores endócrinos no desenvolvimento reprodutivo. Esta tese contribui significativamente para o entendimento da toxicidade induzida por agentes como o DEHP, ressaltando o papel crucial do sistema endocanabinoide na modulação desses efeitos durante a gonadogênese.

Palavras-chave: Gonadogênese. Sistema Endocanabinoide. Acetaminofeno. Desreguladores endócrinos. Toxicologia reprodutiva.

ABSTRACT

Gonads are essential organs of the reproductive system, primarily involved in hormone and gamete production. For these functions to occur properly, the process of gonadogenesis—the development of gonads during the embryonic-fetal period—must be strictly regulated. Disruptions in this process can lead to sexual development disorders. One of the critical mechanisms in this context is the endocannabinoid system, which in recent years has been recognized as an important signaling pathway involved in cellular proliferation and differentiation, hormone production, and epigenetic regulation, among other processes. However, there is evidence that endocrine disruptors such as paracetamol and DEHP (di-2-ethylhexyl phthalate), known for their anti-androgenic effects and potential harm to gonadal development, may interfere with the endocannabinoid system in fetal gonads. This study aimed to evaluate fetal gonadal toxicity induced by exposure to paracetamol and DEHP and to investigate the potential interaction of these agents with components of the endocannabinoid system. Two articles were developed: the first is a review compiling existing literature on the role of the endocannabinoid system in gonadogenesis, highlighting the scarcity of studies in this field; the second is an original experimental study in Wistar rats, where *in vivo* exposure models (between gestational days 15 and 18) to paracetamol (50 and 250 mg/kg/day) and DEHP (750 mg/kg/day) were conducted, along with *in vitro* exposures using paracetamol, AM404 (a paracetamol metabolite that interacts with the endocannabinoid system), rimonabant (CB1 receptor antagonist), and MEHP (active metabolite of DEHP). Results showed that DEHP reduced anogenital distance and testosterone production, while increasing the relative gene expression of *Cnr2* in the testis and ovary and *Napepld* in the testis in the *in utero* model. In the *in vitro model*, MEHP increased testosterone release. On the other hand, no significant effects were observed with paracetamol exposure. This novel work provides evidence that the endocannabinoid system is a molecular target of DEHP in fetal rat gonads, shedding new light on gonadal toxicity pathways and the potential interference of endocrine disruptors in reproductive development. This dissertation significantly contributes to the understanding of toxicity induced by agents like DEHP, highlighting the crucial role of the endocannabinoid system in modulating these effects during gonadogenesis.

Keywords: Gonadogenesis. Endocannabinoid system. Acetaminophen. Endocrine-disrupting chemicals. Reproductive toxicology.

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SUMÁRIO

1 INTRODUÇÃO

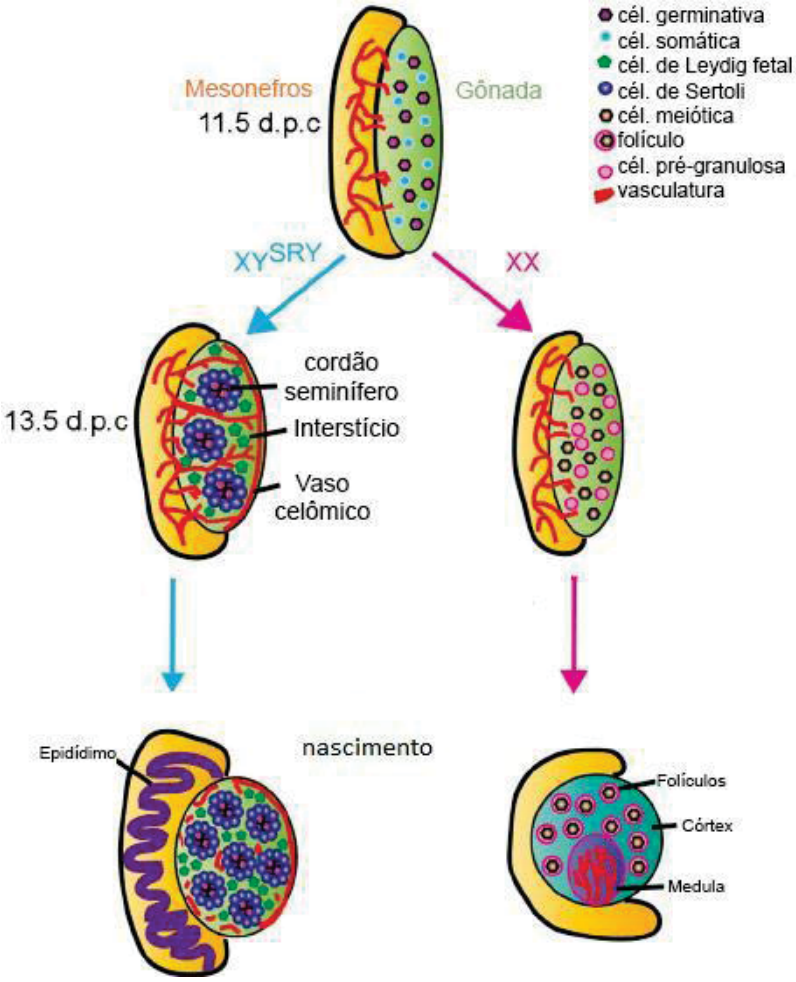
As gônadas são órgãos pertencentes ao sistema reprodutor, cuja função está relacionada principalmente à produção de hormônios e gametas. Os testículos, presentes em machos, são responsáveis pela síntese de hormônios, principalmente androgênios, como a testosterona, além da geração de espermatozoides (Fietz; Bergman, 2017). Por outro lado, os ovários estão presentes nas fêmeas cuja função está relacionada à produção de estrogênios (i.e., 17β-estradiol, estriol e estrona) e progesterona (por exemplo, a progesterona) e a geração de ovócitos, que estão encerrados nos folículos ovarianos (Richard; Pangas, 2010). Contudo, para que estes órgãos desempenhem as suas funções endócrinas e exócrinas de forma apropriada, é necessário que durante o processo de gonadogênese, isto é, a geração e o desenvolvimento gonadal no período embrio-fetal aconteça de forma extremamente regulada. Caso contrário, prejuízos no desenvolvimento gonadal podem resultar em desordens do desenvolvimento sexual (Eggers et al., 2014).

1.1 DESENVOLVIMENTO GONADAL

O primeiro passo envolvido na gonadogênese, chamado de determinação do sexo genético, ocorre ainda na fecundação do ovócito secundário pelo espermatozoide. Devido a segregação dos cromossomos homólogos durante a meiose, os gametas apresentam somente um conjunto de cromossomos (Wilhelm et al., 2007). Relativos aos cromossomos sexuais, o ovócito II, uma vez que não tenham havido problemas durante a meiose, sempre possuirá um cromossomo X. Quanto aos espermatozoides, podem apresentar ou o cromossomo X ou o Y. Na fecundação, os pró-núcleos do espermatozoide e do ovócito II se fundem, restaurando o número de cromossomos da espécie (Wilhelm et al., 2007), onde tal restauração irá determinar se o indivíduo será geneticamente fêmea (XX) ou macho (XY).

Em humanos, na quinta) semana gestacional (Garcia-Alonso et al., 2022), em camundongos no dia embrionário 10.5 (E10.5) (Eggers et al., 2014), e em ratos no 12° dia pós-coito (dpc) (Johansson et al., 2017), há o aparecimento da gônada indiferenciada, ou seja, pode diferenciar-se em testículo ou em ovário. A depender de sinais genéticos devido ao conjunto de cromossomos XX ou XY, a gônada

indiferenciada passará por intrincadas redes de sinalização para então diferenciar-se em testículo ou ovário (Eggers et al., 2014). Esta estrutura desenvolve-se a partir das cristas gonadais, adjacentes ao mesonefro (“rim primitivo”) (Wilhelm et al., 2007), ao receber células provenientes do mesotélio, mesênquima subjacente e do saco vitelino (de origem extra-embrionária), onde este último fornece as células germinativas primordiais (CGP), que resultarão na linhagem gamética (Mäkelä et al., 2019). As CGP são ainda especificadas cedo no desenvolvimento, antes mesmo da gastrulação. Em humanos, a sua especificação ocorre provavelmente na segunda semana do gestacional e, em camundongos, no E6 ao E7 (Mäkelä et al., 2019). Eventos-chave na sua especificação envolvem: (1) - repressão do programa somático mesodérmico, (2) - reaquisição de padrão de expressão gênica pluripotente e (3) - reprogramação global do epigenoma (Mäkelä et al., 2019). Os eventos-chave da formação do testículo e ovário fetal estão representados na FIGURA 1 e, os detalhes moleculares serão abordados nas seções seguintes.



FONTE: ADAPTADO DE MCCLELLAND et al. (2012).

FIGURA 1 - Formação do testículo e ovário fetal.

1.1.1 Desenvolvimento do Testículo Fetal

A partir de 42 dpc em humanos e E10.5 em camundongos, as células de suporte precursoras (células pré-Sertoli) presentes na gônada bipotencial de indivíduos XY, oriundas do epitélio celômico, passam a expressar o gene SRY (região determinante do sexo no cromossomo Y) (Mäkelä et al., 2019). O SRY é o fator de transcrição que induz a diferenciação da gônada bipotencial em testículo. A atividade do SRY, em sinergia com o gene NR_5A1 (também chamado de fator esteroidogênico 1 [SF1], do inglês "*Steroidogenic factor 1"*), induz a expressão do fator de transcrição SOX9 e de outros fatores de transcrição que sustentam a alta expressão de SOX9, por exemplo, o fator de crescimento de fibroblastos 9 (FGF9, do inglês "*fibroblast growth factor 9"*), prostaglandina D sintase (PTGDS, do inglês "*prostaglandin D synthase"*) e fator de transcrição 1 relacionado ao duplosexo e mab-3 (DMRT1, do inglês "*doublesex and mab-3-related transcription factor 1"*) (Eggers et al., 2014), o que leva a diferenciação das células pré-Sertoli em células de Sertoli imaturas, devido a transição do fenótipo mesenquimal ao fenótipo de célula epitelial polarizada (Eggers et al., 2014).

Curiosamente, não é o nível de expressão do SRY que é determinante para a via de desenvolvimento da gônada bipotencial em testículo, mas sim o número de células pré-Sertoli que expressam o SRY (Palmer; Burgoune, 1991). Devido a isso, a especificação das células de Sertoli imaturas é um evento crucial na formação do testículo, visto que estas células guiam a diferenciação de outros tipos celulares encontrados no testículo e promove direta e indiretamente a manutenção das estruturas dos ductos de Wolff, além de inibir o programa genético da formação do ovário e reprimir o desenvolvimento dos ductos de Müller (Mäkelä et al., 2019).

A repressão do desenvolvimento dos ductos de Müller promovida pelas células de Sertoli imaturas é mediada pela produção do hormônio anti-Mülleriano (AMH), pertencente à família do fator de crescimento TGFβ (*transforming growth factor* β) (Shrikhande et al., 2020). A expressão do AMH é regulada pela atividade dos fatores de transcrição SOX9, NR_5A1, WT1 (*Wilms tumor protein 1*) e GATA4 (Mäkelä et al., 2019). Os ductos de Müller (ou ductos paramesonéfricos), assim como os ductos de Wolff (ductos mesonéfricos), estão associados ao sistema reprodutor em desenvolvimento. Os ductos de Müller dão origem ao útero, as tubas uterinas e a porção superior da vagina (Mullen; Behringer, 2014), enquanto que os

ductos de Wolff formam o epidídimo, a vesícula seminal e o ducto deferente (Shaw; Renfree, 2014). Desta forma, a produção do AMH impede a formação dos órgãos sexuais acessórios femininos, enquanto que a produção de andrógenos (que será discutida posteriormente) induz o desenvolvimento dos órgãos sexuais acessórios masculinos.

Uma vez presentes no testículo, as células de Sertoli imaturas, assim como outras linhagens de células somáticas (como as precursoras das células mioides peritubulares) e as CGP começam a se agregar para formar os cordões seminíferos, sob a ação dos genes Sox8, Sox9 e Sox10, entre outros (Eggers et al., 2014). Quando encerradas nos cordões seminíferos, as CGP passam a se chamar gonócitos em virtude de alterações significativas em seu transcriptoma (Hill et al., 2018). No testículo fetal, em contraponto ao ovário fetal, os gonócitos não entram na meiose no período fetal, pois as células de Sertoli imaturas expressam a enzima CYP26B1, que degrada o ácido retinoico. Quando presente, o ácido retinoico induz a expressão do gene Stra8 (*Stimulated By Retinoic Acid 8*) (Koubova et al., 2014), que por sua vez, promove a entrada na meiose (De Domenico et al., 2017).

Outra linhagem celular que se desenvolve e desempenha um papel significativo na diferenciação sexual masculina é a célula de Leydig fetal, que em ratos, surge no período E14.5 (Sharpe, 2020). As células de Sertoli imaturas guiam a diferenciação de células progenitoras das células de Leydig fetais, ao liberar sinais parácrinos como a *Desert Hedgehog* (DHH), que por sua vez, liga-se ao receptor Ptch1 (*protein patched homologue 1*) (Jiang; Jorgensen, 2024). Estas células progenitoras são oriundas dos mesonefros e do epitélio celômico e expressam marcadores como o Wnt5, Arx (*X-linked aristaless-related homeobox gene*), NR_5A1 (que induzirá a expressão das enzimas esteroidogênicas STAR, CYP11A1, CYP17A1 e HSDB1). Assim que as células de Leydig fetais se estabelecem, elas passam a produzir o hormônio androgênio androstenediona, devido a expressão de enzimas da via esteroidogênica. A androstenediona, por sua vez, se difunde até a célula de Sertoli imatura, onde é convertida em testosterona, via atividade da enzima HSD17B (*hydroxysteroid dehydrogenase 17B*) (Ivell et a., 2017). A produção de testosterona se faz importante numa janela específica do desenvolvimento, chamada de janela de programação masculina (JPM), onde os altos níveis de testosterona induzem a sexualização do feto, incluindo a formação do pênis, desenvolvimento das estruturas dos ductos de Wolff, aumento da distância

anogenital e, indiretamente, a diferenciação sexual cerebral (McCarthy, 2017; Sharpe, 2020). A JPM ocorre entre o E15.5 e E18.5 em ratos, E13 e E16 em camundongos e entre a sétima e décima quarta semana gestacional em humanos (Sharpe, 2020).

Outro hormônio importante produzido pelas células de Leydig fetais é o peptídeo 3 semelhante à insulina (INSL3, do inglês *insulin-like 3*). Durante a gonadogênese, o testículo está posicionado na região abdominal. Contudo, para que ocorra a descida testicular, é necessário que o testículo fetal produza tanto a testosterona quanto o INSL3 na fase transabdominal, e apenas a testosterona na fase inguinoescrotal, para que haja o desenvolvimento do gubernáculo, um ligamento que traciona o testículo em direção ao escroto (Jiang; Jorgensen, 2024).

1.1.2 Desenvolvimento do Ovário Fetal

Por muito tempo, a via da formação do ovário fetal foi tida como a “via padrão”, sugerindo que fosse um processo passivo em espécies de mamíferos que seguem o padrão “XX e XY” quanto à determinação do sexo genético, ao passo que a presença do gene SRY presente em indivíduos XY “desviaria” a via para o sentido da formação testicular. No entanto, esta visão tem sido contestada conforme mais estudos têm sido desenvolvidos quanto ao desenvolvimento dos ovários, onde uma complexa rede de genes e de sinalização estão envolvidos (Eggers et al., 2014).

Embora um gene com função análoga ao SRY não tenha sido identificado em indivíduos XX, um gene com função importante, o FoxL2 (*Forkhead box L2*) começa a ser expresso no início do desenvolvimento ovário. Demais genes com funções importantes incluem o Rspo1 (*R-spondin 1*) e o Wnt4, que são ativados pela ação transcricional da β-catenina (proteína Ctnnb1) (Eggers et al., 2014). Esses genes agem através de uma inibição recíproca entre os genes pró-ovário e pró-testículo. Por exemplo, o gene Foxl2 (pró-ovário) inibe a atividade do Sox9 e Dmrt1 (pró-testículo), sendo o Foxl2 também inibido pelo Dmrt1; Rspo1 e Wnt4 (pró-ovário) também inibem o Sox9, que por sua vez, também são inibidos pela Sox9; Wnt4 e Fgf9 se inibem reciprocamente (Eggers et al., 2014).

Após a chegada das CGP na gônada bipotencial em indivíduos XX, estas células passam por uma intensa atividade mitótica com citocinese incompleta, formando aglomerados (*clusters)* ou cistos de ovogônias, delimiatados pelas células

originadas do epitélio celômico (Grive; Freiman, 2015). Em seguida, no E13.5 em camundongos e entre a décima primeira e décima segunda semana gestacional em humanos, estas células entram na meiose sob a ação do ácido retinoico (Eggers et al., 2014) e, portanto, recebem a denominação de ovócito primário. A meiose é interrompida na fase de diplóteno da prófase I (MacLennan et al., 2015). Próximo ao período de nascimento em camundongos e na décima sexta semana gestacional em humanos, os cistos de ovócitos se separam (Grive; Freiman, 2015). Neste momento, os ovócitos que não sofrem apoptose, são envoltos por uma camada simples de células da pré-granulosa, com fenótipo pavimentoso, formando um *pool* de folículos primordiais (Grive; Freiman, 2015). Estas células da pré-granulosa são derivadas de três populações de células que expressam os marcadores FOXL2, LGR5 (*Leucine-rich repeat-containing G-protein coupled receptor 5*) e NR2F2 (também conhecido como COUP-TFII) (Rastetter et al., 2014) e a montagem dos folículos, isto é, a associação entre o ovócito primário e as células pré-granulosa é mediada pelas vias de sinalização como Notch, Kit, FIGLA, NOBOX e TAF4b) (Li et al., 2021).

Conforme descrito acima, o processo de gonadogênese consiste em uma intrincada rede de sinalização mediada entre genes e hormônios, que devem seguir passos sequenciais em janelas específicas. Contudo, influências ambientais podem perturbar estes processos e resultar em desordens do desenvolvimento sexual, que serão discutidos na seção a seguir.

1.2 SÍNDROME DA DISGENESIA TESTICULAR E DESREGULADORES ENDÓCRINOS

Desde a segunda metade do século XX, estudos têm mostrado que a saúde reprodutiva de homens tem se deteriorado, com o aumento da incidência de distúrbios do trato reprodutor, como hipospádia, criptorquidismo, baixa contagem espermática e câncer testicular de células germinativas (Kilcoyne; Mitchell, 2017). Em conjunto, esses distúrbios constituem a síndrome da disgenesia testicular (SDT) (FIGURA 2), que apresenta como uma característica central a baixa produção e/ou ação de andrógenos durante o período fetal, crítico para a diferenciação sexual fenotípica (Wohlfahrt-Veje et al., 2009).

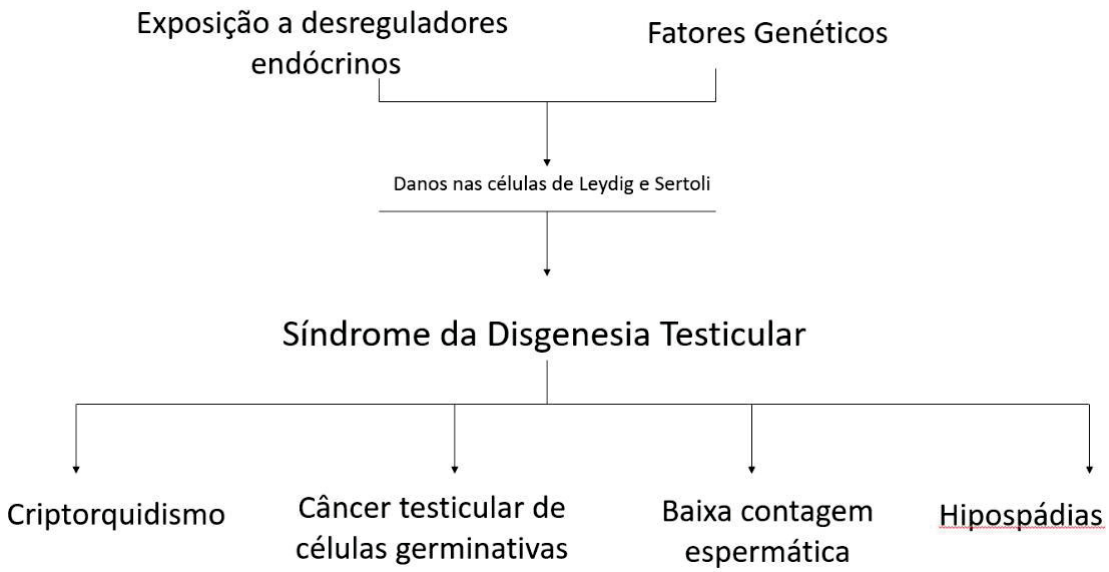


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FIGURA 2 - Hipótese da Síndrome da Disgenesia Testicular e seus sintomas, possivelmente causada pela exposição pré-natal aos desreguladores endócrinos e por fatores genéticos.

Em tese, a hipótese da SDT propõe que estas condições patológicas sejam resultantes de causas comuns, que levam à problemas do desenvolvimento do testículo por meio da disfunção de células de Sertoli e de Leydig durante a janela de diferenciação sexual (Sharpe; Skakkebaek, 2008). A SDT possui o status de hipótese devido à dificuldade de sua avaliação em humanos, uma vez que o desenvolvimento do testículo ocorre entre a sexta e a décima segunda semana gestacional, que seria o período onde a SDT supostamente acontece, o que torna o testículo fetal inacessível (Sharpe; Skakkebaek, 2008). Além do mais, a determinação da concentração de andrógenos durante o período fetal é um fator limitante. Alternativamente, estudos experimentais e epidemiológicos têm utilizado a mensuração da distância anogenital (DAG) como um marcador anatômico sexualmente dimórfico e positivamente correlacionado com níveis de andrógenos no período pré-natal (Eisenberg; Lipshultz, 2015; Kristensen et al., 2011; Swan et al., 2005). Sabe-se também que meninos com criptorquidismo e/ou hipospádia ao nascerem apresentam uma DAG reduzida (Kilcoyne; Mitchell, 2017). Outro desafio para a corroboração desta hipótese é que embora roedores de laboratórios forneçam informações significativas quanto à exposição de substâncias anti-androgênicas ou estrogênicas e a incidência de distúrbios reprodutivos masculinos,

até o momento não foi observado o desenvolvimento de câncer testicular de células germinativas nestes animais (Wohlfahrt-Veje et al., 2009). Apesar disto, acredita-se que a presença de gonócitos multinucleados em roedores possa ser correlacionado com o câncer testicular de células germinativas em humanos (Wilson, 2021). Adicionalmente, faltam estudos a longo prazo que buscam estabelecer as relações entre todas as condições urogenitais citadas acima com a DAG e exposição pré-natal a desreguladores endócrinos (Kilcoyne; Mitchell, 2017).

Fatores genéticos e ambientais podem contribuir para o desenvolvimento da SDT. Um exemplo de fator genético ligado à SDT envolve a síndrome da insensibilidade androgênica, que é resultado de uma mutação do receptor androgênico em indivíduos XY e culmina em sub-masculinização da genitália externa e demais sintomas ligados à SDT (Kilcoyne; Mitchell, 2017). Quanto à fatores ambientais, um agente etiológico que se destaca é a exposição aos desreguladores endócrinos durante a janela de diferenciação sexual (Kristensen et al., 2011; Sharpe; Skakkebaek, 2008; Wohlfahrt-Veje et al., 2009). De acordo com a Agência de Proteção Ambiental dos Estados Unidos (US EPA, 1997) um desregulador endócrino é definido como “agente exógeno que interfere com a produção, liberação, transporte, metabolismo, ligação, ação ou eliminação de hormônios naturais no corpo, que são responsáveis pela manutenção da homeostase, reprodução, desenvolvimento e/ou comportamento”. Os mecanismos de desregulação são muito variados, mas incluem por exemplo o agonismo e antagonismo de receptores hormonais, alteração na expressão das enzimas que sintetizam e degradam hormônios e que interferem ativamente na concentração final do hormônio, alteração no transporte e metabolização dos hormônios, interação com co-fatores transcricionais e etc (Yang et al., 2015). Muitos dos desreguladores endócrinos conhecidos possuem atividade antiandrogênica (isto é, inibem de alguma forma a sinalização do receptor androgênico) ou pró-estrogênica (isto é, estimulam a atividade do receptor estrogênico). Como discutido nas seções anteriores relacionadas ao desenvolvimento gonadal, é necessário que os hormônios sexuais sejam produzidos em quantidades adequadas e em períodos específicos na janela de sexualização, para que não haja o aparecimento de distúrbios urogenitais e reprodutivos.

Diversas classes de substâncias incluem compostos com atividade endócrina, como metais tóxicos, medicamentos, cosméticos, retardantes de chamas, solventes, agrotóxicos e plastificantes (Diamanti-Kandarakis et al., 2010; Lymperi; Giwercman, 2018). Dentre elas, o fármaco paracetamol e o plastificante da família dos ftalatos di(2-etilhexil)ftalato (DEHP) são conhecidos por promoverem efeitos negativos na formação do testículo e ovário fetais, que serão discutidos em detalhes mais adiante.

1.3 PARACETAMOL

Paracetamol, também conhecido como acetaminofeno, ou ainda como acetil-p-aminofenol (APAP), foi sintetizado pela primeira vez em 1878 por Morse, a partir do precursor fenacetina e foi introduzido para uso médico em 1883 (Sharma; Mehta, 2014). Esta molécula consiste em um anel de benzeno substituído por um grupo hidroxila (OH^-)e um grupo acetamida ($NHCOCH3^-$) em posições opostas no padrão *para* (1,4) (Sharma; Mehta, 2014), conforme demonstrado na FIGURA 3.

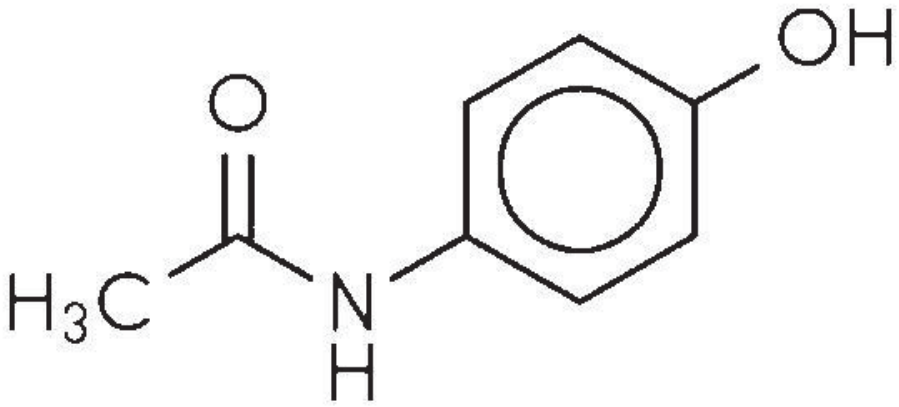

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FIGURA 3 - Estrutura molecular do paracetamol (acetaminofeno).

Devido às suas atividades antipiréticas e analgésicas, o paracetamol se destaca como provavelmente o fármaco mais utilizado no mundo, utilizado no tratamento de dor e febre (Roberts et al., 2016). No entanto, os seus mecanismos de ação ainda não são completamente conhecidos. É amplamente aceito que o paracetamol diminui a concentração tecidual de prostaglandinas e mediadores pró-inflamatórios. Porém, o paracetamol desempenha baixa atividade anti-inflamatória (Sharma; Mehta, 2014). Além disso, o paracetamol é capaz de inibir a atividade da enzima ciclooxigenase-2 (COX-2), uma isoforma presente constitutivamente no

sistema nervoso central. As enzimas COX atuam metabolizando o ácido araquidônico, presente na membrana plasmática das células em prostaglandinas G_2 (PGG_2), e PGG_2 em PGH_2, o que aumentariam as respostas inflamatórias e o limiar de dor (Moriarty; Carroll, 2016). Outros mecanismos do paracetamol envolvem a alteração da sinalização colinérgica, noradrenérgica, opioide e serotoninérgica a nível central, espinhal e supraespinhal (Bertolini et al., 2006; Sharma; Mehta, 2014). Uma das formas pelas quais o paracetamol consegue interagir com sinalização destes neurotransmissores é por meio da conversão do paracetamol em N-araquidonoil aminofenol (AM404), pela enzima FAAH (*fatty acid amide hydrolase*), sendo o AM404 uma molécula capaz de interagir com o sistema endocanabinoide e modular as respostas de dor ao inibir a recaptação de anandamida, que é um agonista do receptor canabinoide 1 (CB1) (McCrae et al., 2018). O AM404 também é capaz de se ligar ao receptor de potencial transitório vaniloide tipo 1 (TRPV1), que desempenha um papel fundamental na nocicepção (Slattery; Klegeris, 2018).

Outras vias de conversão do paracetamol envolvem a formação de conjugados com glicuronídeo e sulfatos, que são excretados na urina. Também, ocorre a metabolização pelas enzimas hepáticas da família citocromo P450, convertendo o paracetamol em n-acetil-p-benzoquinonaimina (NAPQI), que posteriormente é conjugado com glutationa e excretado como conjugados de cisteína e ácido mercaptúrico (McCrae et al., 2018). O NAPQI, por ser um metabólito reativo, é responsável pelas principais respostas de toxicidade do paracetamol, como por exemplo, a hepatotoxicidade (Ferner et al., 2011). O NAPQI liga-se covalentemente a grupos sulfidrila da glutationa. No entanto, quando os estoques intracelulares de glutationa estão depletados, o NAPQI liga-se a proteínas celulares, o que por sua vez, libera uma cascata de radicais livres (Ferner et al., 2011). Desta forma, o antídoto mais indicado para intoxicação mediada pelo paracetamol é a acetilcisteína, que age como doador de grupos sulfidrila (Bateman, 2023).

Outros alvos de toxicidade mediados pelo paracetamol envolvem o trato gastrointestinal, sistema cardiovascular, sistema renal, sistema nervoso e sistema endócrino e reprodutor[1] (McCrae et al., 2018). De fato, há diversas evidências epidemiológicas e experimentais que apontam o paracetamol como desregulador endócrino, interferindo por exemplo na síntese de testosterona e do peptídeo 3 semelhante à insulina (INSL3) (Mazaud-Guittot et al., 2013; Kristensen et al., 2011; Holm et al., 2015; Holm et al., 2016; Kilcoyne; Mitchell, 2017; Bauer et al., 2021).

O que torna o uso do paracetamol preocupante é justamente o fato deste fármaco ser amplamente utilizado por gestantes, tornando-o um fator de risco durante o período de diferenciação sexual. Por exemplo, 47% das gestantes dinamarquesas e mais de 50% de gestantes estadunidenses reportaram o uso do paracetamol (Kilcoyne; Mitchell, 2017). No Brasil, foi relatado que 43% das gestantes afirmaram terem utilizado o paracetamol (Melo, 2008), no entanto, por se tratar de um estudo antigo, estes dados podem estar desatualizados.

Visto que a inibição da testosterona no testículo fetal não parece ocorrer por meio da inibição da síntese de prostaglandina (Kristensen et al., 2011) e, dada a capacidade do paracetamol em interagir com o sistema endocanabinoide, como mencionado anteriormente, surge a necessidade de testar a hipótese de que o paracetamol ao ser convertido em AM404 pela atividade da FAAH, seja um dos possíveis mecanismos de desregulação endócrina promovido pelo paracetamol.

1.4 FTALATOS E DEHP (DI-2-ETILHEXIL FTALATO)

Ftalatos consistem em uma classe de substâncias, as quais são ésteres do ácido 1,2-benzenodicarboxílico (também conhecido como ácido ftálico) com cadeias laterais de carbono com tamanho variado (Wang; Qian, 2021). Os ftalatos são amplamente utilizados como plastificantes, tornando os plásticos mais flexíveis e maleáveis. Além disso, são empregados em cosméticos, produtos de higiene e de saúde e etc (Wang; Qian, 2021). Dentre os ftalatos conhecidos como desreguladores endócrinos, destacam-se o DiPEP (diisopentil ftalato), DIBP (diisobutil ftalato), DEP (dietil ftalato), DMP (dimetil ftalato), DInP (diisononil ftalato) e o DEHP (di-2-etilhexil ftalato) (Wang; Qian, 2021), representados na FIGURA 4.

Diversas evidências apontam os ftalatos como potentes anti-androgênicos, isto é, capazes de inibir a síntese de androgênios como a testosterona (Martino-Andrade et al., 2009; Bertoncello-Souza et al., 2018; Gray et al., 2021; Wang; Qian, 2021; Sree et al., 2023). Inclusive, a administração de ftalatos durante a JPM culmina em uma série de desfechos reprodutivos caracterizados pela má formação da genitália externa, criptorquidismo, distância anogenital reduzida, retenção de mamilos em machos, alterações histopatológicas no testículo e epidídimo e baixa contagem de espermatozoides. Todas estas alterações em conjunto são conhecidas

como “síndrome dos ftalatos” (Willson, 2021), o que corresponde muito bem à síndrome da disgenesia testicular.

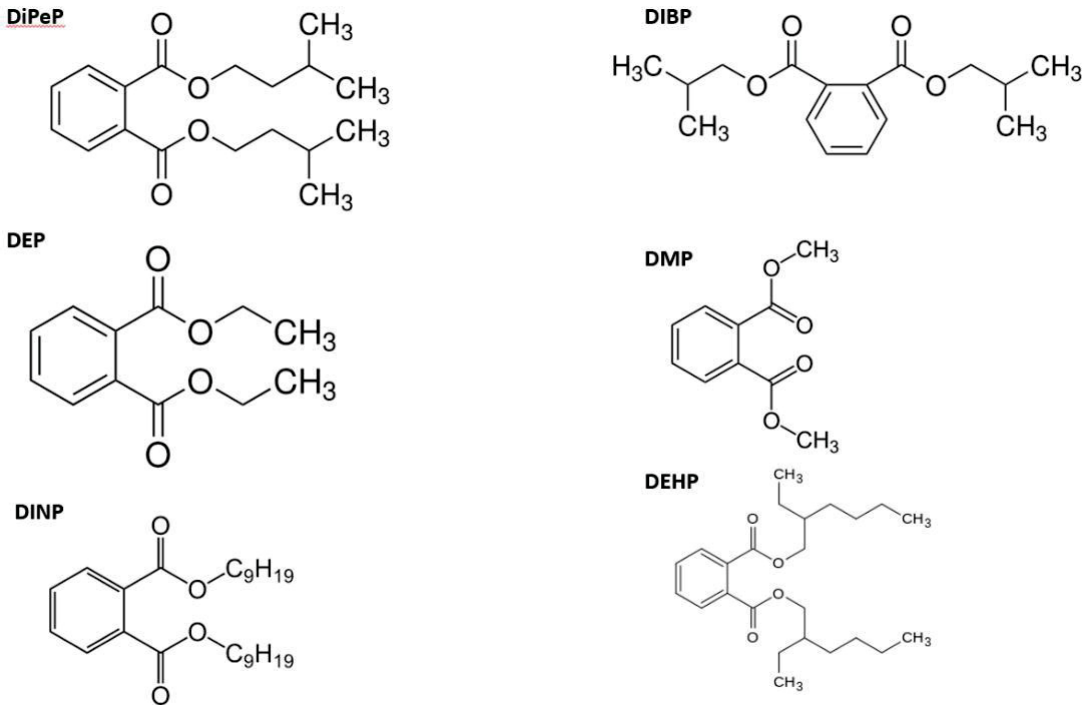

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FIGURA 4 - Estrutura molecular dos ftalatos DiPEP, DIBP, DEP, DMP, DINP e DEHP.

Assim como o paracetamol, os mecanismos de toxicidade dos ftalatos não estão completamente elucidados e, parece haver também alguma interação com o sistema endocanabinoide. Forner-Piquer et al. (2018) demonstraram em zebrafish que a exposição ao DINP pode alterar a expressão gênica do sistema endocanabinoide no testículo e no ovário. No entanto, não há estudos demonstrando os impactos no DEHP no sistema endocanabinoide gonadal.

Portanto, destaca-se a importância de avaliar se existe alguma conexão entre o DEHP e o sistema endocanabinoide para uma melhor compreensão de seus mecanismos toxicológicos.

1.5 SISTEMA ENDOCANABINOIDE E DESENVOLVIMENTO GONADAL

O sistema endocanabinoide, ilustrado na FIGURA 5, consistem em uma rede de sinalização e interações envolvendo os endocanabinoides, os receptores ao

quais se ligam (CB1, CB2 e TRPV1), o transportador de membrana (EMT) e também as enzimas envolvidas na sua produção de degradação (Saito et al., 2010). Os seus principais ligantes endógenos são a N-araquidonoil etanolamina (ou anandamida, AEA) e o glicerol 2-araquidonoil (2-AG). A síntese da AEA é catalisada pela fosfolipase D N-acil fosfatidil etanolamina (NAPE-PLD), enquanto que a produção de 2-AG ocorre por meio da hidrólise de fosfolipídeos de inositol via fosfolipase C (PLC), gerando diacilglicerol, que posteriormente é convertido em 2-AG pela enzima diacilglicerol lipase (DAGL). A degradação dos endocanabinoides ocorre principalmente pela ação da hidrolase de amida de ácidos graxos (FAAH), que possui maior afinidade pela AEA, e pela ação da enzima monoacilglicerol lipase (MAGL), que degrada seletivamente o 2-AG (Barchi et al., 2020). A AEA além de ativar os receptores CB1 e CB2, pode ligar-se em uma região do domínio intracelular do TRPV1, modificando a condutância dos íons Na^{+} e Ca^{2+}. A AEA também desempenha alguns efeitos genômicos, ao promover a heterodimerização dos receptores PPAR (receptores ativados por proliferador de peroxissoma) com o RXR (receptor retinoico X) e promover a transcrição de genes associados aos seus elementos responsivos (Sullivan; Kendall, 2010). Por outro lado, o 2-AG possui maior afinidade pelo CB2, atuando como agonista (Meccariello et al., 2014).

É bem documentado que o sistema endocanabinoide atua nos sistemas nervoso, imunológico e reprodutor. No sistema nervoso, o sistema endocanabinoide age principalmente em vias de sinalização retrógrada, isto é, o neurônio pós-sináptico secreta endocanabinoides que inibem a exocitose de mais neurotransmissores (Mackie, 2008). No sistema imunológico, o sistema canabinoide exerce ambas funções pró-inflamatória e anti-inflamatória, sendo a função predominante determinada pela célula-alvo e molécula sinalizadora envolvida (Chiurchiu et al., 2015). No sistema reprodutor masculino, o sistema endocanabinoide desempenha diversos papéis, como a regulação da mitose de células germinativas, a formação do acrossomo dos espermatozoides e a sua capacitação, ao retardar o ganho de motilidade, via CB1 (Cacciola et al., 2013). Além disso, o sistema endocanabinoide age centralmente, modulando o funcionamento de neurônios GnRH, via neurônios kisspeptina e a ativação de sistemas GABAérgicos, dopaminérgicos, opioidérgicos, que são capazes de inibir a

secreção de GnRH, além de reprimir os sistemas glutamatérgicos e noradrenérgicos, envolvidos na promoção da secreção de GnRH (Pierantoni et al., 2009).

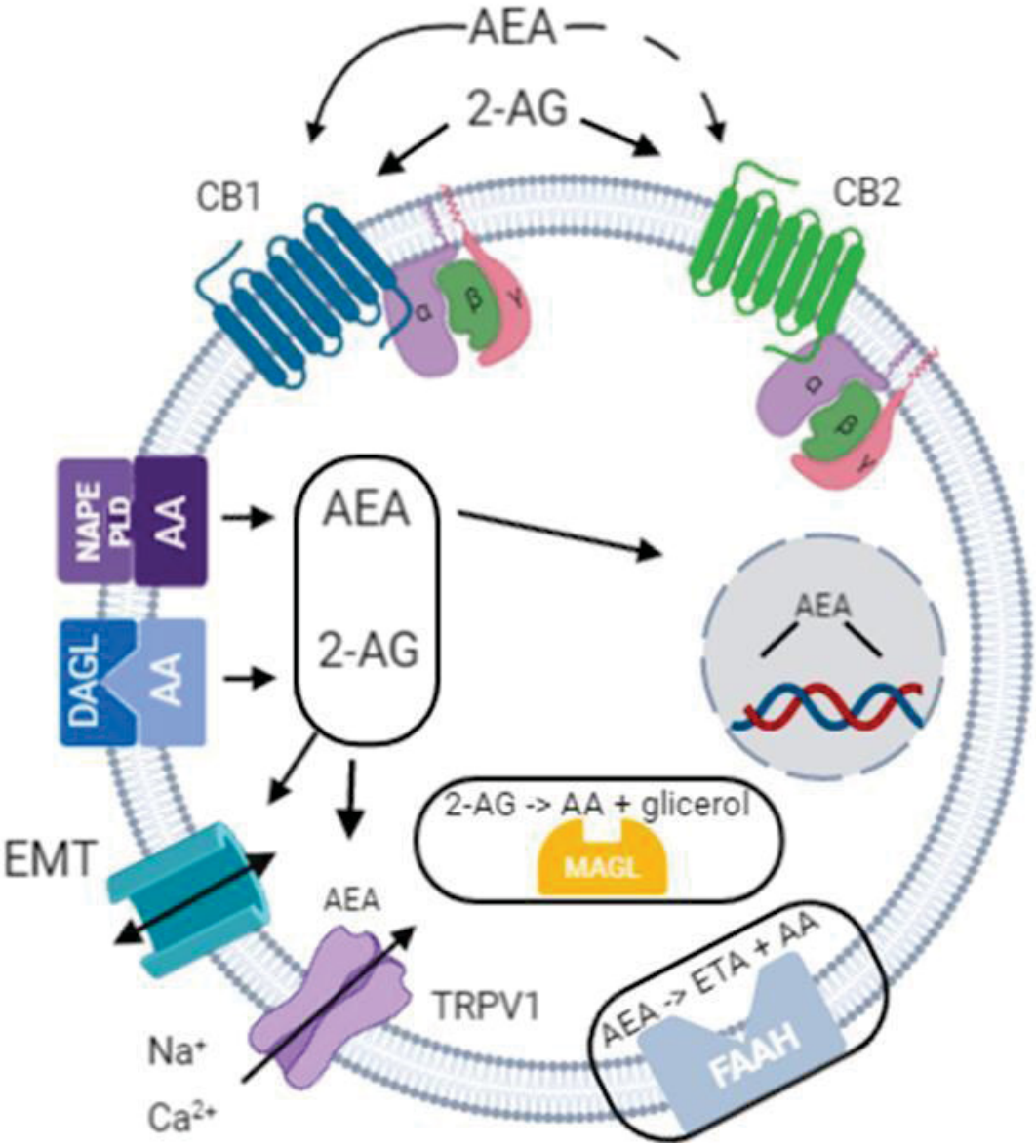

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FIGURA 5 - Representação de uma célula hipotética contendo todos os componentes do sistema endocanabinoide. Na região superior, a seta cheia indica a alta afinidade pelo receptor, ao passo que a seta tracejada indica a baixa afinidade da anandamida pelo CB2. AEA - anandamida; 2-AG - 2-araquidonoil glicerol; AA - ácido araquidônico; ETA - etanolamina; EMT - *Endocannabinoid membrane transporter*; FAAH - *Fatty acid amide hydrolase*; MAGL - *Monoacylglycerol lipase*; NAPE-PLD - *N-acylphosphatidylethanolamine specific phospholipase D*; *DAGL - Diacylglycerol lipase*; CB1 - *Cannabinoid receptor 1*; CB2 - *Cannabinoid receptor 2*; TRPV1 - *Transient potential type 1 vanilloid receptor*.

Há evidências de que o sistema endocanabinoide é responsável pela proliferação das células de Leydig imaturas, além de influenciar a sua diferenciação para célula de Leydig adulta, durante o período pós-natal em roedores (Cacciola et al., 2008). Além disso, estudos indicam que o sistema endocanabinoide está envolvido de alguma forma na síntese de andrógenos, pois é bem registrado que o consumo de canabinoides exógenos, pelo uso recreativo de *Cannabis sativa*, causa alterações na produção de testosterona pelas células de Leydig, embora resultados opostos sejam observados (Carvalho et al., 2019; du Plessis et al., 2015). Em adultos, a AEA tem um efeito protetor nas células de Sertoli contra a apoptose, via CB2 (Pierantoni et al., 2009).

Há alguns poucos estudos que avaliaram o papel do sistema endocanabinoide na formação das gônadas fetais. Este tema será melhor abordado mais adiante nesta tese, no artigo 1. Contudo, vale mencionar que ambos o paracetamol e DEHP, capazes de desregular a síntese de androgênios durante o período de sexualização dos fetos, parecem de algum modo interagir com o sistema endocanabinoide, o que poderia explicar, ao menos em parte, o mecanismo de desregulação endócrina.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Esta tese teve como objetivo avaliar a toxicidade gonadal fetal induzida pelo paracetamol e pelo DEHP em ratos, bem como investigar os efeitos e a possível interação desses agentes com componentes do sistema endocanabinoide.

2.2 OBJETIVOS ESPECÍFICOS

- Discutir e demonstrar a relevância do sistema endocanabinoide na formação das gônadas fetais, com ênfase no testículo;
- Investigar experimentalmente em ratos os efeitos da exposição materna ao paracetamol e ao DEHP, na janela gestacional GD15 ao GD18, crítica para a produção de testosterona no testículo fetal;
- Avaliar se a exposição ao paracetamol e ao DEHP alteram a expressão de genes importantes para o desenvolvimento gonadal (*Insl3*, *Lgr5*, e *Stra8*), do sistema endocanabinoide (*Cnr2*, *Napepld*, *Mgll*) e de enzimas esteroidogênicas (*Star* e *Cyp11a1*);
- Em um modelo *in vitro* de cultivo de testículos fetais de curta duração, examinar o impacto do paracetamol e de seu principal metabólito (AM404), e do MEHP (metabólito do DEHP) individualmente ou em combinação com o antagonista do CB1 rimonabanto, sobre a produção de testosterona e a expressão de genes críticos para a esteroidogênese e a sinalização canabinoide.

3 ARTIGOS CIENTÍFICOS

3.1 ARTIGO 1 – ROLE OF THE ENDOCANNABINOID SYSTEM IN GONADAL DEVELOPMENT: IMPLICATIONS FOR ENDOCRINE DISRUPTION AND REPRODUCTIVE TOXICITY

Este trabalho consiste em uma revisão narrativa, onde são levantados os papéis já conhecidos do sistema endocanabinoide na formação do testículo fetal, especulações de outras possíveis funções ainda desconhecidas e sugestão de futuras pesquisas para esclarecer tais lacunas. O presente artigo foi submetido para o periódico “Reproductive Toxicology” (ISSN: 0980-6238) e formatado conforme as normas da revista.

Role of the Endocannabinoid System in Gonadal Development: Implications for Endocrine Disruption and Reproductive Toxicity

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Abstract: The endocannabinoid system (ECS) plays a pivotal role in reproductive physiology, including gonadal development, though its influence on testis and ovary development has only recently gained attention. The ECS comprises lipid-derived ligands such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), along with cannabinoid receptors CB1 and CB2, which are expressed in various gonadal cells. Emerging research indicates that ECS signaling is critical for testosterone synthesis and gonadal cell proliferation and differentiation. This review explores the expression and function of ECS components in developing gonads, highlighting the differential roles of CB1 and CB2 receptors in species-specific contexts. Furthermore, the ECS has been implicated in the adverse effects of endocrine-disrupting chemicals (EDCs) on reproductive development. EDCs, such as phthalates, may interfere with ECS signaling, potentially leading to reproductive abnormalities that resemble the human Testicular Dysgenesis Syndrome (TDS). Understanding the molecular interactions between EDCs and the ECS could reveal novel mechanisms underlying reproductive toxicities. Future research should focus on the detailed localization and temporal expression of ECS components in fetal gonads, the mechanisms of cannabinoid-mediated testosterone inhibition, and the potential direct interaction of EDCs with the ECS. This knowledge could be crucial for developing strategies to mitigate reproductive health risks associated with EDC exposure.

Keywords: endocrine-disrupting chemicals; sexual development, fetal testosterone; cannabinoid.

Overview

The gonadal development relies on a complex network of genetic and environmental factors [1], and one possible emerging mechanism related to these processes is the

endocannabinoid system (ECS). The components of this signaling pathway comprehend several synthesizing and degrading enzymes, endogenous lipid-derivative molecules, cannabinoid and non-cannabinoid receptors, and membrane transporters. The endogenous ligands include Narachidonoylethanolamine, also known as anandamide (AEA), and 2- arachidonoylglycerol (2-AG), both derived from arachidonic acid (AA), as well as endocannabinoid-like molecules N-palmitoylethanolamine (PEA) and Noleoylethanolamine (OEA). These molecules can bind to a variety of receptors, including the canonical G-coupled type 1 and type 2 cannabinoid receptors (CB1 and CB2, respectively), the non-CB1/CB2 cannabinoid G protein-coupled receptors GPR55 and GPR118, and PPAR (peroxisome proliferator-activated receptor) family and ion-channel TRPV1 (transient receptor potential cation channel subfamily V member 1) receptors [2]. Over the past two decades, several studies have revealed the relevance of the ECS to reproductive physiology. In fact, both the testis and ovary express the complete endocannabinoid machinery, suggesting the ECS's relevance in testicular and ovarian functioning [3,4]. Some examples of such relevance were demonstrated in studies where marijuana users have been associated with sperm impairments and altered testosterone production [2,5,6], suggesting a negative control of androgen synthesis. Also, cannabinoid receptors could induce acrosome aberrations and epigenetic changes in sperm [7], revealing the participation of epigenetic mechanisms and chromatin remodeling. In the frog Rana esculenta, cannabinoid signaling can influence the seasonal reproductive cycle [8]. Endogenous cannabinoids can also modify the GnRH secretion on the hypothalamus through kisspeptin modulation [9,10] and LH and FSH production in gonadotrophs in the pituitary [4]. However, only recently have a few studies begun to explore the role of the endocannabinoid system (ECS) in the development of the testes and ovaries. In this narrative review, we discuss the potential roles of the ECS in gonadal development, with a focus on the testis, as fewer studies have examined ovarian development. Additionally, we propose the ECS as a novel pathway through which endocrinedisrupting chemicals (EDCs) may interfere with reproductive development.

Expression and function of cannabinoid receptors in the developing gonads

Both oocytes and (pre)granulosa cells from primordial and primary follicles

express CB1 and CB2 receptors, with CB2 being expressed at higher levels than CB1 [11]. Additionally, studies on human granulosa-like KGN cells have shown the expression of CB1, CB2, DAGL, FAAH, GPR55, MAGL, NAPE-PLD, and TRP1, as well as the production of anandamide, an endogenous cannabinoid [12].

Totorikaguena et al. [13] demonstrated that ovaries from Cnr1-/- and Cnr1-/-Cnr2-/- mice were smaller and had a reduced follicle pool compared to controls. In these knockout models, the number of secondary follicles increased while primordial and primary follicles decreased, indicating an accelerated folliculogenesis. Interestingly, CB2 knockout alone did not affect follicle pool size or development, and no additive effects were observed in the double-knockout (CB1-CB2), suggesting that CB1 plays a dominant role in follicular regulation. Supporting the role of CB1, Castel and colleagues [14] showed that fetal exposure to the CB1 agonist WIN55212 reduced follicle numbers in adult rats, while exposure to the CB1 inverse agonist SR141716 increased the follicle pool. Interestingly, co-exposure to WIN55212 and SR141716 mitigated these effects, emphasizing the importance of CB1 activity in determining follicle numbers and regulating follicular maturation.

Cannabinoid receptors also influence oocyte maturation, as demonstrated by Cecconi et al. [15]. During oocyte maturation, the mRNA expression of Cnr1, Cnr2, and Gpr55 (encoding CB1, CB2, and GPR55, respectively) was highest in the germinal vesicle (GV) stage, reduced in metaphase I (MI), and lowest in metaphase II (MII). At the protein level, CB2 and GPR55 expression sharply increased during the MI phase, and GPR55 expression further increased in MII. Additionally, CB1 was found compartmentalized in lipid rafts during the GV-MI transition. Using CB1 (SR1) and CB2 (SR2) antagonists, they showed that the GV-MI transition was inhibited, accompanied by cAMP accumulation, underscoring the role of cannabinoid receptors in oocyte maturation [15]. Weizman et al. [16] further highlighted the effects of cannabinoids on granulosa cells, reporting that in vitro treatment with THC (Δ9-tetrahydrocannabinol) increased CB2 receptor expression but did not affect CB1 levels. Despite these findings on granulosa cells, the precise in vivo impact of exogenous cannabinoids on the developing ovary remains unclear. However, Castel et al. [14] provided evidence that prenatal exposure to WIN55212 (CB1 agonist) disrupts normal ovarian development, suggesting that modulation of CB1 activity could have potential implications for reproductive health.

Related to testis development, Dochez-Arnault and colleagues [17] have recently published an important paper addressing the expression patterns of cannabinoid receptors and enzymes in human fetal testis, in which Leydig cells (LC), Sertoli cells (SC), and germ cells (GC) all express both CB1 and CB2 classical receptors. Contrary to the mice model [18], human testis expresses Cnr1 at higher levels than the Cnr2 gene. In Sprague-Dawley rats, CB1 protein expression starts at 19.5 days post-coitum (dpc) and is specifically localized on gonocytes, as demonstrated by Migliaccio et al. [19].

By exposing the human fetal testis to Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), two main marijuana components, Dochez-Arnault and colleagues [17] verified several impacts on the testis physiology, such as the decrease of testosterone and AMH secretion, and impaired proliferation profile of LC, SC, and GC. Similarly, Cacciola et al. [20] found that the ECS could be implicated in LC differentiation and proliferation in the postnatal rodent testis. The CB1 receptor was detected in immature non-mitotic LC, whereas in the mitotic counterpart, CB1 could not be detected by immunolocalization assays. CB1 knockout leads to an increased number of LC population in mice, suggesting that CB1 signaling may block the proliferation of these cells. However, as mentioned before, the human fetal testis expresses CB1 early during development, similar to mice, where CB1 mRNA can be detected as early as 11.5 dpc [18,21]. In contrast, Sprague-Dawley rats express CB1 only in gonocytes starting at 19.5 dpc [19], highlighting an inter-species difference. On the other hand, overactivation of the CB2 receptor can lead GCs to premature entry into meiosis, as demonstrated by De Domenico et al. [18]. In female mice, JWH133 (a CB2 agonist) exposure induced 13.5 and 15.5 dpc gonocytes to start meiosis, and this effect was blocked by AM630 (a CB2 antagonist). This effect was probably mediated by the increased Stra8 and the reduced Nanos2 expression, genes related to meiotic entry and meiotic arrest, respectively. However, this premature meiotic entry was not enough to sustain meiosis, since it was observed an increased level of GC apoptosis and reduction in the ovarian follicle pool. This experiment suggests a putative role of the CB2 receptor in the initializing events of meiosis entry. According to Migliaccio et al. [19], CB1 is expressed in gonocytes at the mitotic arresting stage, since it is expressed only at 19.5 dpc, when the gonocytes are mitotically arrested. During puberty, CB1 is likely involved in meiosis resumption. Thus, CB1 and CB2 may be complementary, since CB2 induces meiosis

entry and CB1 culminates into meiosis resumption. Thus, early overactivation of both receptors could imply a risk in gonocytes during development, similar to the findings previously described by Dochez-Arnault et al. [17].

Cannabinoid receptors are also related to epigenetic mechanisms in gonocytes. Murine 13.5 dpc gonocytes initiate DNA methylation reactions that are completed before birth (in mitotically arrested gonocytes) and maintained postnatally [7]. Some evidence suggests that cannabinoid exposure interferes with methylation patterns in humans [22,23], rats [24,25], and mice [26]. It seems that CB1 activation in sperm is involved in chromatin remodeling and spermiogenesis, a process during which epigenetic changes occur [7]. Making a parallel to the gonocytes, in which CB1 is also expressed (as mentioned before), it is plausible to suggest that CB1 activation could lead to epigenetic events in these cells as well. Also, CB2 activation in gonocytes can increase histone modifications through H3K4me3, inducing the expression of promeiotic genes c-Kit and Stra8 [18].

Regarding other components of endocannabinoid machinery, Dochez-Arnault et al. [17] demonstrated that the fetal testis expresses all the cannabinoid enzymes. However, no study aimed to immunolocalize the expression of these endocannabinoid components in the fetal gonads has been published yet. Studies are required to uncover the expression patterns and immunolocalization of the cannabinoid marker in testicular fetal cells. Despite the interspecies (human vs rodents) and time point (adult vs fetal) differences, at least in adults human testis, it was already demonstrated that (1) NAPEPLD (anandamide synthesis) was found in SC, peritubular myoid cells, LC, blood vessels, spermatocytes, round spermatids; (2) DAGL (2-AG synthesis) was found in SC, peritubular myoid cells, LC, blood vessels, spermatogonia, and spermatocyte; (3) FAAH (anandamide metabolism) was found in spermatocytes, SC (in mice), [27], and LC; (4) MAGL (2-AG metabolism) was found in spermatid, SC, LC, and blood vessels [28]. Further studies should characterize the immunolocalization of cannabinoid enzymes at protein level in fetal testis. However, which cell in the fetal testis can be the source of the endogenous cannabinoids? Also demonstrated by Dochez-Arnault et al. [17], imaging analysis showed that 2-AG presence was correlated to LC marker CYP11A1 localization, implying that LC can be the main producing cells, and probably express 2-AG synthesizing MAGL enzyme. Anandamide levels were below the detection limits of the MALDI technique. Tracing a parallel to the adult mouse testis, anandamide is probably produced in LC

and SC, but in trace levels. 2-AG is likely produced mainly by LC, and to a lesser extent in seminiferous cord cells, primarily in SC and spermatogonia, as shown in Figure 1B.

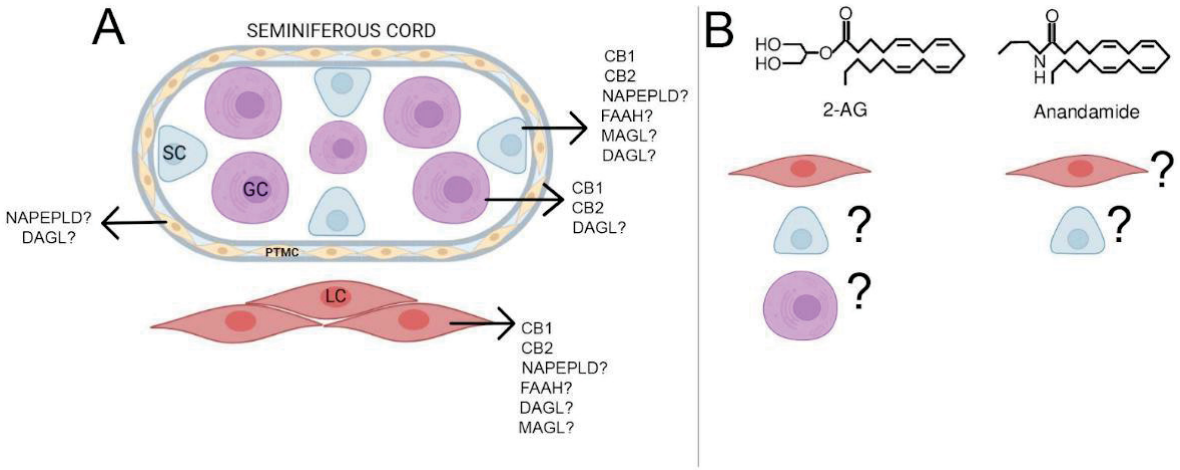

Figure 1 - A) A fetal testis scheme, showing the spatial expression of CB1 and CB2 receptors, and likely cannabinoid-enzymes expression based on adult testis. B) Likely source of production of the cannabinoid ligands 2-AG and AEA. LC - Leydig cell; SC - Sertoli cells; GC - gonocyte; PTMC - peritubular myoid cell; NAPEPLD - N-acyl phosphatidylethanolamine-specific phospholipase D; FAAH - Fatty-acid amide hydrolase 1; MAGL - Monoacylglycerol lipase; DAGL - Diacylglycerol lipase. ? - means the possibility of enzyme expressions and ligands production.

ECS and Fetal Steroidogenesis

The effects of cannabinoid signaling on testosterone secretion are well-known for decades. The pioneering studies conducted by Dalterio demonstrated that the marijuana components, THC and CBD, can decrease testosterone production in fetal [29,30], perinatal and adult mice testis [31]. Also, epidemiological studies in humans show that marijuana users have lower circulating testosterone levels and sperm abnormalities [2,5], even though not all studies confirm this effect [32]. Currently, it is clear that the human fetal LC expresses CB1 and CB2 receptors [17]. In rodents, the pattern of cannabinoid receptor expression in LCs varies during different stages of prenatal development [19]. During the male programming window (MPW) in rats (will be discussed in more depth later), which extends from 14.5 to 18.5 dpc and coincides with the peak of testosterone production and major prenatal masculinizing effects [33], LCs express only CB2, while CB1 is detected only at 19.5 dpc. Activation

of CB2 during the MPW is likely responsible for the reduction of testosterone secretion observed by administering THC and CBD in both human and rodent testes. One possible mechanism that may explain this, at least in part, is the fact that both CB1 and CB2 are inhibitory G-coupled receptors [34]. Steroidogenesis requires cholesterol mobilization from the cytoplasm to the inner mitochondrial membrane, a process promoted by STAR (steroidogenic acute regulatory protein). For cholesterol mobilization to occur, STAR must be phosphorylated [35]. This phosphorylation results from LHCG receptor activation, which elicits the adenylate cyclase pathway, leading to increased cAMP and PKA activation. It is worth mentioning that ACTH can bind to the LHCG receptor and induce steroidogenesis in the rodent fetal testis [36]. Activation of CB2 would decrease cAMP levels and PKA activity, leading to reduced phosphorylation of STAR protein. As a result, less cholesterol would be mobilized to the mitochondria, thereby inhibiting the initiation of steroidogenesis. However, additional experimental data are needed to confirm this hypothesis.

AEA, the main ligand of the cannabinoid receptors, can block testosterone synthesis, as demonstrated by Wenger et al. [37], even though this blockage could be mediated through central inhibition. No study has specifically addressed the involvement of 2-AG on testosterone synthesis, however, it is plausible to suggest that, similar to anandamide, 2-AG could have an inhibitory effect on steroidogenesis. Proper endocannabinoid production during development could play an important role in testosterone production by balancing its production and preventing excessive androgenization. However, overactivation of this system could lead to inhibition of testosterone synthesis. Additionally, a study in the Pelophylax esculentus frog [9] found that anandamide can enhance the aromatase (CYP19) expression, an enzyme involved in testosterone conversion into estradiol. The rise of estradiol levels and reduced testosterone levels result in the disruption of proper sexualization [38]. Figure 2 summarizes the possible role of endocannabinoids in steroidogenesis.

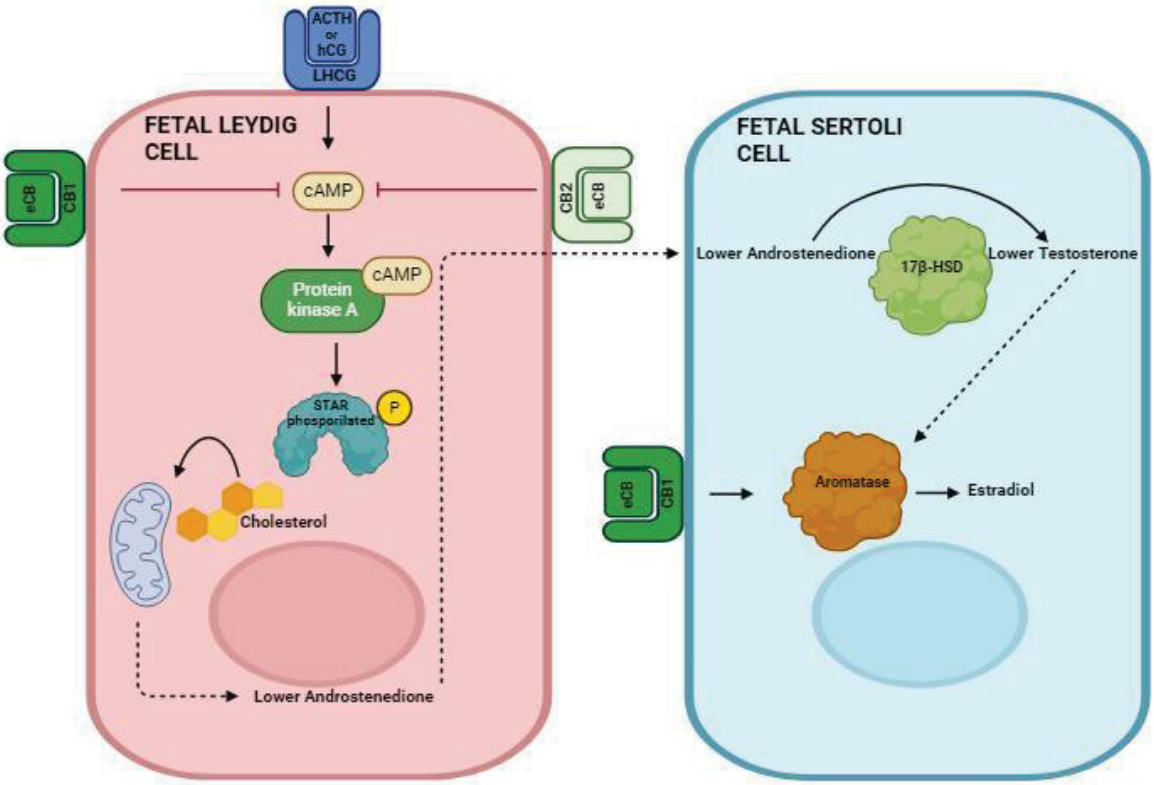

Figure 2 - Hypothetical mechanism of hormone steroid disruption due to endocannabinoid signaling in fetal Leydig and Sertoli cells. ACTH - Adrenocorticotropic hormone; cAMP - cyclical adenosine monophosphate; eCB - endocannabinoid ligand; LHCHR - luteinizing hormone/choriogonadotropin receptor; P - phosphate; STAR - Steroidogenic acute regulatory protein.

Several substances with anti-androgenic (reducing testosterone signaling) and pro-estrogenic (enhancing estradiol signaling) effects can lead to various impairments in the male reproductive system, including cryptorchidism, hypospadias, testicular germ cell cancer, and low sperm counts in adulthood [39]. These issues are particularly associated with disruptions occurring during the male programming window (MPW) — a critical fetal developmental period marked by a peak in testosterone production [40]. This testosterone surge is essential for inducing male sexual differentiation, which includes the formation of the penis, accessory reproductive organs (such as the prostate, seminal vesicles, and vas deferens), and brain masculinization, establishing a “male phenotype” [41]. The MPW is highly sensitive to hormonal fluctuations and, therefore, vulnerable to interference by endocrine-disrupting chemicals (EDCs) [42]. These interrelated reproductive disorders collectively form what is known as Testicular Dysgenesis Syndrome (TDS),

which may stem from abnormal testicular development in utero influenced by both genetic and environmental factors [43]. A common link among TDS disorders is impaired androgen signaling, which may result from increased exposure to anti-androgenic EDCs [44]. In this context, the testicular endocannabinoid system (ECS) might represent a target for exogenous substances, potentially disrupting testosterone synthesis indirectly, as explored in the sections below.

Interplay among Testicular Endocannabinoid System, Endocrine-Disrupting Chemicals, and Testicular Dysgenesis Syndrome

A large set of substances are capable of interfering with the endocrine system, by acting through several mechanisms: inhibiting or increasing hormone synthesis; interacting with membrane and nuclear receptors; modulating gene expression of key enzymes involved in hormone synthesis; altering hormone transportation, depuration, and elimination. These substances are known as endocrine-disrupting chemicals (EDCs) [45]. Many substances can interact with sexual steroid signaling, for instance, mimicking or blocking the actions of testosterone and estradiol. Since these hormones are crucial for proper sexual development during critical developmental windows, exposure to EDCs can promote long-lasting and devastating effects on the gonads, accessory organs, and sexual dimorphism of the brain, and have been implicated in the origins of the TDS [42,46,47]. As mentioned in the previous section, TDS comprises the following reproductive abnormalities: cryptorchidism, a condition in which one or both of the testes fail to descent from the abdominal cavity into the scrotum; hypospadias, a penile malformation characterized by the ectopic opening of the urethra in the ventral aspect of the penis; testicular germ cell cancer; and low sperm count at adulthood [43]. Certain EDCs are known to promote TDS-like phenotypes in animal models; an example is the class of phthalates. Phthalates are a group of substances used as plasticizers in polyvinyl chloride (PVC) plastics, making them more flexible [48]. Phthalates are esters of 1,2-benzene dicarboxylic acid (phthalic acid), with ester side chains of varying lengths. Examples include DEHP (di-2-ethylhexyl phthalate), DINP (diisononyl phthalate) DMP (dimethyl phthalate), DEP (diethyl phthalate), DBP (dibutyl phthalate), DIBP (diisobutyl phthalate), and DIPeP (diisopentyl phthalate). Certain phthalates can disrupt fetal Leydig and Sertoli cell functions in rodents and promote downstream hormonal changes, including deficient

production of insulin-like factor 3 (Insl3), involved in the process of testis descent, and testosterone [48]. As a result, male offspring rats exposed in utero to these compounds exhibit various male reproductive abnormalities, known as the rat phthalate syndrome, that share remarkable similarities with the human TDS and include cryptorchidism, hypospadias, testicular dysgenesis, presence of multinucleated gonocytes, and low sperm counts [48]. Despite advances in understanding the hormonal and cellular factors disrupted by in utero phthalate exposure, the upstream molecular initiating events remain unknown. Recently, some studies have shown that certain phthalates may interact with components of the ECS. DINP exposure promotes infertility in adult zebrafish, reduced gonadosomatic index (GSI), and impaired ECS gene expression profile in both male and female gonads [49]. In our laboratory, we found that DEHP exposure between 15 and 18 dpc can increase the Cnr2 expression in the testis and ovary of Wistar rats (unpublished data). In humans, germ cell tumors, a component of testicular dysgenesis syndrome (TDS), are believed to originate from gonocytes that fail to undergo proper maturation (germ cell neoplasia in situ - GCNIS). The molecular mechanisms of the initial malignant transformation from a precursory cell to a GCNIS cell are still unclear. Barchi and colleagues [7] raised the hypothesis that signaling disruption during the early development of primordial germ cells or gonocytes could culminate into GCNIS, and cannabinoid signaling could play a crucial role in this process. Interestingly, in utero phthalate exposure may lead to a transient increase in the number of multinucleated gonocytes in the fetal and neonatal rat testis [50]. This phenomenon is considered a pathological hallmark of the rat phthalate syndrome and may be associated with defective Germ-Sertoli cell interactions. The induction of multinucleated gonocytes occurs independently of phthalate-induced testosterone deficiency [50], but the types of phthalates that induce these effects, as well as their rank order of potency, are consistent, suggesting a common, yet unidentified, upstream molecular initiating event [50,51]. In this regard, the ECS may constitute a potential phthalate target to be assessed in future research. Other EDCs could pose a risk to the ECS and, consequently, the reproductive system. Bisphenol-A (BPA), a component of polycarbonate plastics and epoxy resins, was also implicated in cannabinoid signaling and impacted the gonad function in zebrafish [52,53]. Molecular docking studies revealed that BPA can bind to the CB1 [53]. Paracetamol is another EDC [54,55] and there is some evidence it can interact with the ECS.

Paracetamol can be metabolized into AM404 by the FAAH enzyme [56], a molecule that can weakly bind to CB1 and EMT, which could inhibit the AEA reuptake [57]. However, until now there is no evidence that endocrine disruption induced by paracetamol could be mediated through cannabinoid interaction. Negative effects on the female reproductive system cannot be ruled out, especially considering that the molecular mechanisms involving the cannabinoid system in fetal ovaries are less understood compared to those in the fetal testis. As discussed in previous sections, cannabinoid modulation can influence follicle numbers and oocyte maturation progression via CB1-dependent mechanisms [13,14]. Additionally, as mentioned before, CB2 activity has been shown to induce early meiotic commitment [18], potentially leading to a reduced reproductive lifespan and premature ovarian failure. The reduction of the follicle pool is a particularly critical issue, as it is associated with an increased risk of health conditions such as cardiovascular disease and osteoporosis in women, due to the sharp decline in estradiol production [58]. This highlights the importance of further investigating the impact of cannabinoid signaling on ovarian development and its long-term implications for female reproductive health.

Conclusion

The ECS is a complex network involved in several processes in reproductive physiology, including the development of the testis and ovary. Particularly, its role in gonadal development has been neglected for a long time, and only recently some studies aimed to uncover its role during this process. There is enough evidence supporting the involvement of ECS in testosterone production, gonadal cell proliferation and differentiation. Recently, toxicologists have focused on unraveling the molecular initiating events involved in the adverse outcome pathways of EDCs and other reproductive toxicants [59]. It is reasonable to think that exposure to some EDCs, such as phthalates, could (1) directly bind to CB1 and/ or CB2 receptors, or (2) alter the levels of AEA and 2-AG, which could lead to aberrations in hormone production, and in cell proliferation and differentiation. This could explain some mechanisms of toxicity leading to reproductive system malformations and reduced fertility capacity. Future studies should investigate the immunolocalization of other components of the endocannabinoid system (enzymes and transporters) in fetal gonads and their temporal expression patterns. It's crucial to determine whether CB1

receptors, CB2 receptors, or both, are involved in testosterone reduction by using specific agonists and antagonists. Additionally, the precise mechanism of testosterone inhibition by cannabinoid receptors should be explored, focusing on the decrease in cAMP levels and STAR phosphorylation. Researchers should also test whether 2-AG is capable of reducing testosterone. Finally, molecular modeling studies, such as molecular docking, could evaluate the interaction between endocrine-disrupting chemicals (like phthalates) and cannabinoid receptors and enzymes.

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3.2 ARTIGO 2 – DEHP BUT NOT PARACETAMOL DISRUPTS THE GONADAL DEVELOPMENT IN BOTH MALE AND FEMALE WISTAR RATS: A FOCUS ON THE ENDOCANNABINOID SYSTEM

Este é um artigo original onde foi testado se a exposição pré-natal ao paracetamol e ao DEHP é capaz de induzir uma má formação no testículo e no ovário fetais, e ainda correlacionar se tais desfechos podem ser associados com a interação com o sistema endocanabinoide. Este artigo será submetido no periódico “Toxicological Sciences” (ISSN: 1096-0929) e foi formatado conforme as normas da revista.

DEHP but not Paracetamol Disrupts the Gonadal Development in both Male and Female Wistar Rats: A Focus on the Endocannabinoid System

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ABSTRACT: Paracetamol and di-2-ethylhexyl phthalate (DEHP) are two endocrine-disrupting chemicals (EDCs) known to disturb prenatal gonadal development and inhibit testosterone synthesis in some experimental models. However, their precise mechanism of toxicity remains to be elucidated. We have hypothesized that both substances can interact with the endocannabinoid system, which may represent a potential mechanism for the induction of abnormal testis and ovary development. By using *in vivo* (exposing pregnant rats during GD15 and GD18 to 50 and 250 mg.paracetamol.day^{-1} and DEHP 750 mg.kg.day^{-1}) and short *ex vivo* exposures to 10 µM paracetamol, 10 µM AM404, 10 µM rimonabant, and 100 µM MEHP, we have demonstrated that DEHP decreased the anogenital distance and testosterone production in males, and increased the relative expression of *Cnr2* (that encodes CB2 receptor) in both testis and ovary and increased the relative expression of *Napepld* in fetal testis. MEHP increased the testosterone secretion in the *ex vivo* model. We did not observe any changes after paracetamol exposures. This study provides some evidence for the first time that the ECS in rodent developing gonads is a molecular target of DEHP in rodent gonads.

Keywords: Acetaminophen; endocrine disruptor; male programming window; testicular dysgenesis syndrome; phthalate.

INTRODUCTION

The Developmental Origins of Health and Diseases (DOHaD) concept proposes that the effects of environmental factors during critical windows of development can damage the developing organism and cause several diseases in the adult organism (Heindel et a. 2015). Examples of DOHaD related to infertility include testicular dysgenesis syndrome (TDS) and ovarian dysgenesis syndrome (ODS), even though the latter is still controversial (Wohlfahrt-Veje et al. 2009; Johansson et al. 2017). One important etiologic factor that could be attributed to these conditions is the exposure to endocrine-disrupting chemicals (EDC) during critical windows of sexual development. Two important ECDs that are known to disrupt *in utero* gonadal development are the plasticizer di-2-ethylhexyl phthalate (DEHP) (Sree et al. 2023) and the drug paracetamol (or acetaminophen) (Kilcoyne and Mitchell 2017).

DEHP belongs to the class of phthalates, which are substances used to make polyvinyl chloride plastics (PVC) more flexible and durable, in cosmetics, healthcare products, medical devices, and other industrial applications (Wang and Qian 2021). DEHP is a potent inhibitor of androgen synthesis (Parks et al. 2000; Culty et al. 2008; Ha et al. 2016; Sree et al. 2023). Despite various proposed mechanisms of disruption, such as by affecting PPAR family receptors, oxidative stress, interacting with histones and promoting epigenetic disturbances, alterations in cell proliferation, and stimulation of the IGF-1R–mediated PI3k/AKT/mTOR signaling pathway (Sree et al. 2023), the precise mechanism by which steroidogenesis is impaired remains unclear. The impacts of DEHP in the ovary include abnormal follicle assembly, decreased PTEN levels, apoptosis induction in granulosa cells, altered estrous cyclicity, and disruption in hormone synthesis (Hannon and Flaws, 2015; Mu et al. 2015; Tripathi et al. 2019).

Paracetamol is an antipyretic and analgesic drug. Its mechanism of action is still unknown. However, some mechanisms were proposed: serotoninergic interactions, selective inhibition of COX-3 in the brain, and interactions with the endocannabinoid system (Moriarty and Carroll 2016). Even though paracetamol is considered a safe drug, several studies have reported potential adverse effects, including impairments in the development of both the male and female reproductive systems. Regarding sexual development, paracetamol has been shown to disrupt

testosterone production in various, but not all, experimental models (Kristensen et al. 2011; Axelstad et al. 2014; van den Driesche et al. 2015; Kilcoyne and Mitchell 2017; Gray et al. 2021), alter sexual behavior (Coria-Avila et al. 2024), and impact the number of oocytes and gonocytes, with long-lasting effects (Holm et al. 2016). Females seem more sensitive to paracetamol, as a reduction in the primordial follicle pool can have devastating effects on the reproductive lifespan, accelerating the depletion of the ovarian reserves (Holm et al. 2016).

The mechanisms of reproductive toxicity associated with developmental exposure to paracetamol and DEHP remain unclear and warrant further investigation. We have hypothesized that one of the possible toxicological mechanisms could be the interaction between paracetamol or DEHP with the endocannabinoid system (ECS). The ECS comprises a set of signaling lipid compounds (mainly anandamide and 2-arachidonoyl glycerol), receptors (mainly CB_1 and CB_2), transporters (endocannabinoid-membrane transporter, EMT), and biosynthetic and degrading enzymes (FAAH, NAPEPLD, DAGL, and MAGL) (Stasiulewicz et al. 2020). This complex system is known to be involved in several physiological processes, such as retrograde synaptic signal (Mackie 2008), inflammation and immune responses (Chiurchiu et al. 2015), cell proliferation and differentiation (Almeida et al. 2022), and regulation of the reproductive system. This includes acrosome formation, retardations of motility acquisition, DNA remodeling, regulation of Kisspeptin and GnRH secretion (Pierantoni et al. 2009; Maccarrone et al. 2021), steroidal hormone production and secretion (du Plessis et al. 2015; Carvalho et al. 2019), Leydig cell proliferation and differentiation (Cacciola et al. 2008), among others.

Some studies have shown that phthalates can interact with the ECS. Bisset et al. (2011) showed that DEHP inhibits the CB1 signaling *in vitro* and Forner-Piquer et al. (2018) demonstrated that DiNP (di-isononyl phthalate, a DEHP substitute) can induce alterations in ECS gene expressions and FAAH activity in the gonads of zebrafish.

Paracetamol is a target of FAAH activity, being converted to AM404 (Rehfeld et al. 2022). AM404 acts as a weak agonist of CB_1 and inhibits the reuptake of anandamide by blocking EMT (Slattery and Klegeris 2018). Increased CB1 activation or enhanced anandamide bioavailability could represent a potential mechanism of altered reproductive development. However, the physiological role of ECS on

gonadal development is still unknown. A study showed that hyperactivation of CB_2 could accelerate meiosis entry in the fetal ovary, impacting the time-regulated development (De Domenico et al. 2017). Also, it was demonstrated that ECS is important for the postnatal development of the testis, by regulating the Leydig cell maturation and proliferation.

This study aimed to examine the *in utero* effects of paracetamol and DEHP on fetal rat gonads by assessing testosterone production in the fetal testis and the mRNA expression of key genes involved in steroidogenesis, testis descent, and ovarian development, as well as genes related to endocannabinoid synthesis, degradation, and signaling. Additionally, we investigated the short-term effects of co-administering paracetamol or its metabolite AM404 with the CB1 antagonist rimonabant in fetal rat testes cultured in vitro.

MATERIAL AND METHODS

Animals

Male and female adult Wistar rats (aged 60 days) were obtained from the animal facilities of the Universidade Federal do Paraná. The protocol was approved by the local Ethics Committee on Animal Use (CEUA, registration number: 1419). Animals underwent acclimatization for one month until the beginning of the experiments when they were approximately 90 days old. During all experimental period, animals were kept under a 12 h light／dark cycle (light on at 10:00 and off at 22:00), controlled temperature (22 ± 2 °C), and housed in groups of 3–4 animals in polypropylene cages (414 mm × 344 mm × 168 mm). Standard pellet food (NUVILAB-CR1; QUIMTIA, Colombo, Brazil) and tap water were provided *ad libitum*.

Chemicals

Paracetamol used in the *in vivo* experiment was purchased at Nissei® Drugstore and commercial Canola oil was from a local retail market. Rimonabant (SML0800, CAS #158681-13-1), AM404 or N-(4-hydroxyphenyl)-arachidonylamide (H1911, CAS #183718-77-6), mono-ethylhexyl phthalate or MEHP (796832, CAS #4376-20-9), DEHP (D201154, CAS #117-81-7), M199 modified medium without

phenol red (M3769), paracetamol (A7085, CAS #103-90-2) used in the *in vitro* experiment, recombinant human chorionic gonadotropin (hCG, C1063, CAS #9002-61-3), and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt [ABTS], A2319) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO, 41641, CAS #67-68-5) was purchased from Fluka. Polyclonal anti-testosterone antibody (R 156) and respective horseradish peroxidase-testosterone conjugated were obtained from Coralie Munro at the University of California (Davis, USA). Trizol® (5596018, CAS #136426-54-5), PowerUp® SYBR® Green Master Mix (A25742), Glycogen (10814-010), RNase Away® Reagent (15410564), MicroAmp® Optical Adhesive Film (4311971), and MicroAmp® Fast 96-Well reaction Plate (4346907) were purchased from ThermoFischer Scientific, while GoScriptTM Reverse Transcription System kit was from Promega.

Study design

Female rats were mated with males at the proportion 2 or 3:1 during the last 3 hours of the dark period (from 07:00 to 10:00). Mating was identified by the presence of sperm in vaginal smears and considered as gestational day 0 (GD0). The study was divided into two different experiments: A short-term 4-day *in utero* assay and a short-term 3-hour *in vitro* exposure protocol of fetal testes, as illustrated in Figure 1.

Experiment I: *In utero* exposure to paracetamol and DEHP

Paracetamol was administered in canola oil (vehicle) at doses of 50 and 250 $mg.kg.day^{-1}$. The first dose was selected by its pharmacological relevance, considering the maximum daily dose of 4000 mg of paracetamol for humans, which corresponds to approximately 57 mg/kg/day for a 70 kg adult, while the highest dose represents a dose within the therapeutic range, considering allometric extrapolations to rats (Reagan-Shaw et al. 2008). Additionally, the highest dose was selected to characterize the inhibitory effect of paracetamol on testosterone production without overt maternal toxicity, as observed in a study by Kristensen et al. (2011). DEHP was also administered in canola oil at the dose of 750 $mg.kg.day^{-1}$, which has been shown to induce a significant reduction in testosterone production by the fetal rat testis (Passoni et al. 2018; Krebs et al. 2020). Pregnant rats were treated daily with canola

oil, Paracetamol (50 and 250 mg.kg.day^{-1}), and DEHP (750 mg.kg.day^{-1}) from GD15-18, by oral gavage.

The selected exposure window corresponds to the critical period of masculinization in rats and includes the peak of testosterone production, which occurs approximately on GD18 (Livera et al. 2006). One set of dams and fetuses (N = 5-8 dams/group) was euthanized on the last day of vehicle, paracetamol or DEHP administration (GD18) and the other set and their pups on postnatal day 7 (PND7) (N = 4-7 litters/group). The day of birth was considered as PND1. Dams or pups (PND 7) received sevoflurane in a closed chamber and were rapidly decapitated with a guillotine or a scissor, while fetuses (GD18) were decapitated without anesthetics. Dam organs (ovary, uterus, adrenals, liver, kidney, and spleen) were recorded on GD18 or PND7 to obtain the absolute and relative organ weights.

In the assessment of GD18 fetal gonads, the fetuses were removed from the gravidic uterus, kept alive in saline solution on a heated plate, weighed, and subsequently decapitated. Sex was determined by visual inspection of the gonads, which were removed for gene expression analysis, and in the cases of fetal testes, also for assessment of testosterone production.

For dams that were allowed to deliver, pups were sexed on PND1, and weighed on PND1, PND4, and PND7. Anogenital distance (AGD) from each pup was recorded using a digital caliper twice or three times (if the coefficient of variance was above 10%) by a blind researcher on PND4. The anogenital distance index (AGDi) was obtained by dividing AGD by the cube root of body weight.

Fetal Testosterone Production

Fetal testis testosterone production was assessed in fetuses collected on GD18. Both testes from two fetuses per litter were individually incubated at 37°C in humidified ambient air in a 24-well plate containing 500 µL of M199 media for 3 hours. For each fetus, one testis was incubated with human chorionic gonadotropin (hCG, 200 IU) and the other without, to explore the testis response to hCG stimulation. After incubation, testes were snap-frozen in nitrogen liquid and stored at -80°C until analysis. Testosterone was quantified by ELISA using a polyclonal anti-testosterone antibody (R 156/71:7500 dilution) and respective horseradish

peroxidase-testosterone conjugate obtained from Coralie Munro at the University of California (Davis, CA, USA). 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt [ABTS]) was used as a substrate buffer containing hydrogen peroxide. Absorbance measurements were performed at 405 nm in a microplate reader (BioTek, Winooski). Results were expressed as pg/testis/3 hours and the assay sensitivity was 2.3 pg/well. All samples were evaluated in duplicate. The testosterone analysis was performed in a single run and the intra-assay coefficients of variation were less than 10 %.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

After removal, fetal or PND7 testis and ovaries from 3-6 fetuses or pups from each litter (N = 3-7 litters/group) at each collection time point) were snap-frozen in liquid nitrogen and then stored at -80°C. The selected genes for fetal testis were: *Tbp* (the keephouse gene), *Insl3*, *Star*, *Cyp11a1*, *Cnr2*, *Napepld*, and *Mgll*; for postnatal testis were: *Tbp*, *Star*, *Cyp11a1*, *Cnr2*, *Napepld*, and *Mgll*; for fetal ovary were: *Stra8*, *Lgr5*, *Cnr2*, *Mgll*, and *Napepld*; and for postnatal ovary were: *Lgr5*, *Cnr2*, *Mgll*, and *Napepld*. *Insl3* codifies the peptide hormone called insulin-like 3, responsible for the transabdominal phase of testis descending (Tremblay 2008), Stra8 is a transcription factor responsible for the meiosis entry in germ cells (Anderson et al., 2008), *Star* and *Cyp11a1* are enzymes linked to the first steps of steroidogenesis (Zirkin and Papadoulos, 2018), *Lgr5* is a marker of granulosa cells (Rastetter et al. 2014), and *Cnr2* codifies the CB2 protein, *Napepld* and *Mgll* codifies the cannabinoid enzymes Napepld and MAGL. It is worth mentioning that we failed to standardize the cannabinoid genes *Cnr1*, *Faah*, and *Dagl* by RT-qPCR due to their very low expression levels in fetal gonads. The *Rpl19* gene was used as an internal control.

Each pair of frozen gonads was powdered and 50 ng was transferred to microtubes. Total RNA isolation was performed by the guanidine-phenol-chloroform extraction (Chomczynski and Sacchi 2006) using 500 µL Trizol®, according to fabricant instructions. One microliter of glycogen was added at each fetal sample during the precipitation phase. After extraction, 2.5 µg RNA was used to synthesize cDNA by reverse transcription reaction using oligoDT with the resources of GoScript™ Reverse Transcription System kit (Promega, Madison, USA). Samples plus oligoDT were incubated in a thermal cycler (Axygen Maxygene, term-1000)

under 70°C for 5 min. Next, samples were immediately cold to 4°C and kept at this temperature for 5 min. Then, to each sample we added a solution containing 2 µL enzyme buffer (GoScript 5X), 0.75 µL RNAse free ultrapure water, 0.5 µL of ddNTPs, 0.25 µL RNAsin, 1 µL MgCl2, and 0.5 µL GoScript Reverse Transcriptase enzyme. The reaction with a total volume of 10 µL was incubated in a thermal cycler initially for 5 min under 25°C, followed by 1 h under 42°C. In each reaction, 10 µL of cDNA was obtained with a final estimated concentration of 250 ng. μL^{-1}. The forward and reverse primers of the selected genes and reference sequences are shown in Table S1. The qPCR was performed using PowerUp® SYBR® Green Master Mix (Applied Biosystems, ThermoFisher Scientific, USA). Amplification was done in StepOnePlus™ Real-Time PCR System (Applied Biosystems, Singapore), according to the following cycle configurations: 50°C for 2 min, 95°C for 2 min, and 45 cycles of 95°C for 15 s and 60°C for 60 s. Mean values of threshold cycle (Ct) were determined by StepOne™ Software v2.3 (Applied Biosystems) and quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Experiment II: *In vitro* exposure of fetal testes to Paracetamol, MEHP, AM404 and Rimonabant

Another set of mating was conducted in the same conditions described in the Experiment I section. At GD18, *naïve* dams were euthanized and fetal testis were cultured in the same conditions described in the *Ex Vivo* Testosterone Production Section. To investigate a possible interplay between paracetamol toxicity and endocannabinoid signaling in fetal testes, the following treatments were tested: vehicle (DMSO), 10 µM Rimonabant (CB1 antagonist), 10 µM Paracetamol, 10 µM AM404, 100 µM MEHP (an active metabolite of DEHP),10 µM Rimonabant + 10 µM Paracetamol and 10 µM Rimonabant + 10 µM AM404. After incubations, testes were collected for gene expression analysis (RT-qPCR) and the culture media were used for assessment of testosterone testosterone production (ELISA), as described above.

RT-qPCR

After incubation for testosterone measurements, the pair of testes was snap-frozen in liquid nitrogen and subjected to gene expression analysis, following the same procedures described in Experiment I. Here, the genes analyzed were: *Star*, *Cyp11a1*, *Mgll*, *Napepld*, and *Cnr2.*

Statistical Analyses

The statistical analysis was performed using the Graph-Pad Prism software version 6.0 (Graph-Pad, San Diego, CA, USA). Normality, heterogeneity of variances, skewness, and kurtosis were evaluated for every data set before the test choice. In the *in utero* experiment, testosterone production was evaluated by mixed-ANOVA, with treatment as the between-subjects factor and hCG as the within-subjects factor. Post-hoc analyses were conducted with Dunnett's test for comparison of exposed versus control groups at each level of hCG treatment, and Sidak's test to assess the effect of hCG within each treatment group. Maternal data, AGD and AGDi, testosterone production (in vitro assay), and gene expression were analyzed by One-Way ANOVA followed by Dunnett's post-hoc test. Data are expressed as the mean ± standard error of the mean (SEM). The litters were used as the statistical units. Results were considered significant when $p \leq 0.05$.

RESULTS

Experiment I: Maternal Data

Maternal body weight, organ weights and gestational data of dams examined on GD18 or PND7 are presented in the supplementary Tables S2 and S3, respectively. No major effects on body weight or absolute and relative organ weights were detected, besides a reduction in the spleen of DEHP dams examined on GD18 (DEHP = 0.64 g and 0.21% vs control = 0.81 g and 0.24%, respectively). No differences were seen in the litter size, weight and number of male and female fetuses and pups, or early reabsorptions from both sets of dams.

Experiment I: Anogenital Distance and Testosterone Production

AGD and AGDi were measured on PND4 in both male and female pups, as shown in Table 1. Only DEHP treatment in males resulted in a reduction in AGD (4.02 mm ± 0.19 vs 5.01 mm ± 0.14, p = 0.002) and AGDi (1.94 ± 0.06 vs 2.40 ± 0.06, p = 0.0006). No effects were seen after paracetamol treatments.

In the assessment of fetal testosterone production, we observed significant main effects of both treatment (p < 0.0001) and hCG stimulation (p < 0.0001), as well as a significant interaction between the two factors (p = 0.0016). hCG significantly increased testosterone production compared to the basal condition in all experimental groups, except for DEHP. *In utero* DEHP exposure significantly reduced fetal testicular testosterone production under both basal and hCG-stimulated conditions, compared to the control group. However, no significant effects were observed in basal or hCG-stimulated testosterone production with either dose of paracetamol (Figure 2).

Experiment I: Relative Gene Expression

- Fetal Testis: we analyzed the mRNA relative expression of the cannabinoid genes *Cnr2*, *Mgll*, and *Napepld*, the steroidogenic genes *Star* and *Cyp11a1*, and the peptidic hormone *Insl3*, as shown in Figure 3. An increase in the expression of *Cnr2* (3.63 ± 1.04 vs 0.86 ± 0.07, p = 0.0008) and *Napepld* (3.15 ± 0.96 vs 1.09 ± 0.22, p = 0.006) was observed in the DEHP-exposed group compared to the control group. *Star* expression was reduced in the DEHP group (0.24 ± 0.04 vs 1.04 ± 0.13,p = 0.01) compared to controls. Although DEHP nearly abolished expression levels of *Insl*3 (0.04 ± 0.02 vs. 1.16 ± 0.28, p = 0.16), *Mgll* (0.31 ± 0.08 vs. 1.20 ± 0.34, p = 0.20), and *Cyp11a1* (0.12 ± 0.04 vs. 1.30 ± 0.49, p = 0.23), no statistically significant differences were detected. The high variability observed in the control group for these genes may have contributed to the lack of significant findings. On the other hand, no significant differences were observed for any of the analyzed genes in GD18 testes at either dose of paracetamol.

- PND7 Testis: we analyzed the RNAm expression of the cannabinoid genes *Cnr2*, *Mgll*, and *Napepld*, and the steroidogenic genes *Star* and *Cyp11a1*, as shown in Figure 4. No statistically significant differences in comparison to the control group were detected in any analyzed gene.

- Fetal Ovary: we have analyzed the RNAm expression of the cannabinoid genes *Cnr2*, *Mgll*, and *Napepld*, the granulosa cell marker *Lgr5*, and the meiosis-entry-related gene *Stra8*, as shown in Figure 5. No statistical difference were detected.

- PND7 Ovary: we analyzed the RNAm expression of the cannabinoid genes *Cnr2*, *Mgll*, and *Napepld*, and the granulosa cell marker *Lgr5*, as shown in Figure 6. *Cnr2* expression was increased in the DEHP group compared to the control (1.99 ± 0.31 vs 1.06 ± 0.13, p = 0.004). DEHP also increased *Napepld* expression, but due to high data variability, the difference was not statistically significant (2.11 ± 0.87 vs 1.03 ± 0.09, p = 0.17). In the *Lgr5* analysis, it was not possible to conclude if DEHP could decrease its expression (0.61 ± 0.13 vs 1.12 ± 0.19) due to the limited sample size in the DEHP group (n = 2).

Experiment II: Testosterone Production

We evaluated testosterone production in fetal testes incubated *in vitro* for 3 hours with paracetamol, its metabolite AM404, and the CB1 antagonist rimonabant, either individually or in combination. Additionally, one experimental group included fetal testes exposed to MEHP, the primary metabolite of DEHP. No significant differences were observed between experimental groups and the vehicle control (DMSO), except for an increase in testosterone production in testes exposed to MEHP (2.38 ng/testis/3 hours ± 0.12 vs 5.25 ng/testis/3 hours ± 1.23, p = 0.02).

Experiment II: Gene expression

We analyzed the RNAm expression of the cannabinoid genes *Cnr2*, *Napepld*, *Mgll*, and the steroidogenic genes *Star* and *Cyp11a1*, as shown in Figure 8. No significant effects were observed for any gene except Cnr2 Its expression was significantly increased by AM404 + rimonabant (2.22 ± 0.70) compared to control (1.03 ± 0.10, p = 0.04), rimonabant-only (0.69 ± 0.12, p = 0.004), paracetamol (0.59 ± 0.08, p = 0.002), AM404-only (0.77 ± 0.08, p = 0.015), MEHP (0.41 ± 0.07, p = 0.001), and paracetamol+rimonabant (0.85 ± 0.08, p = 0.001).

DISCUSSION

This study evaluated for the first time the likely interaction of paracetamol and DEHP with ECS in the developing gonads in rats. We showed that *Cnr2* expression from both testis and ovary is sensitive to prenatal exposure to DEHP. Also, *Napepld* is also a target for DEHP toxicity in fetal testis. Paracetamol exposure was not enough to alter the testosterone synthesis in Wistar rats, whereas both DEHP and its metabolite MEHP, as expected, induced endocrine disruption.

GD15-18 window is a critical period in which testosterone is produced, and consequently, masculinizes the fetus (MacLeod et al. 2010). No differences were seen in AGD and AGI from paracetamol exposures in males and females. On the other hand, DEHP treatment reduced AGD and AGDI only in males.

Although we did not find any change in testosterone secretion in both 50 and 250 $mg.kg^{-1}$ paracetamol-administered groups, many studies reported testosterone reduction after paracetamol administration. For example, Kristensen et al. (2011; 2012) showed in both pregnancy exposure and organotypic culture of Sprague-Dawley fetal testis the capacity of paracetamol to reduce testosterone synthesis. Van den Driesche (2015) also reported testosterone reduction induced by paracetamol exposure, but in human fetal testis in a xenograft model in castrated mice. However, not all studies showed testosterone reduction after paracetamol administration. Gray et al. (2021) did not find any testosterone reduction after 400 $mg.kg.day^{-1}$ paracetamol administration in fetal Sprague-Dawley fetal testis but in the GD17-21 window. Taken all together, one possible explanation for our lack of testosterone reduction in paracetamol-treated groups relies on the fact that Wistar rats could be resistant to paracetamol toxicity, as demonstrated in hepatotoxicity studies (Sinoriya 2019). No work showed testosterone endocrine disruption caused by paracetamol in the Wistar rat model, but only in other models, such as Sprague-Dawley and humans.

In our short exposure *in vitro* model, only MEHP, the active metabolite of DEHP, showed an endocrine-disrupting property. DEHP metabolism involves the rapid hydrolysis to mono(2-ethylhexyl) phthalate (MEHP) to increase the solubility, an active metabolite known for the antiandrogenic effects (Hanioka et al. 2019). We expected a reduction in testosterone synthesis, but we observed an increase in its

release. Other studies also showed testosterone increased levels after MEHP administration (Lehraiki et al. 2009; Zhao et al. 2012), whereas others reported a reduction (Svechnikov et al. 2008; Njembele and Trembley 2021). But, we cannot exclude a possible endocrine-disrupting property of paracetamol in Wistar rat testis, since we did not test in a long-term exposure model, such as the 3-day organotypic model (Livera et al. 2006). Kristensen et al. (2012) showed a reduction in testosterone synthesis in Sprague-Dawley rat testis in a 3-day organotypic model. More studies are recommended to test if Wistar rat testis is insensitive to the testosterone-reducing effect of paracetamol.

We observed a reduction in *Star* expression in the DEHP-exposed group in our *in utero* experiment. The STAR protein plays a crucial role in mobilizing cholesterol to initiate steroid hormone synthesis (Miller and Auchus 2011). Consistent with findings from other studies (Yamada et al, 2015; Wang et al. 2016;), phthalates, including DEHP, can reduce *Star* expression levels, which partially explains their antiandrogenic properties, corroborating our findings on testosterone production. Although we observed a marked reduction in *Cyp11a1* and *Insl3* expression (Figure 2), these changes were not statistically significant. *Cyp11a1* is a key gene in the testosterone biosynthesis pathway, encoding an enzyme that converts cholesterol into pregnenolone (Miller and Auchus 2011). Similarly, *Insl3* encodes insulin-like 3, a protein essential for testicular descent (Ivell et al. 2022). Several other studies have reported that DEHP exposure can reduce the expression levels of both genes (Borch et al. 2006; Wang et al. 2016;). A potential explanation for the lack of statistical significance in our results is the limited sample size (n = 3–4).

For the first time, it was reported an interaction between DEHP exposure and the ECS in a rodent model, in which we observed an increase in *Cnr2* and *Napepeld* gene expression in fetal testis, and an increase in *Cnr2* gene expression in postnatal ovary, indicating a long-term effect and demonstrating that both testis and ovary endocannabinoid system are sensitive to phthalate exposure. Forner-Piquer (2018) reported endocannabinoid gene expression changes in the zebrafish model after DInP exposure, indicating that other phthalates could interact with the ECS.

It is important because the whole toxic mechanism of toxicity of phthalates is not known (Sree et al. 2023), and the ECS could be one of these mechanisms.

Current toxicological approaches involve the search for molecular initiating events (MIEs) and adverse outcome pathways (AOPs) (Browne et al. 2017). The interaction with the ECS, and more specifically, through CB2 receptor expression may represent an important MIE. A few studies aimed to explore the impact of the ECS in the developing gonads, so, there is a knowledge gap during this period. But De Domenico et al. (2017) showed that CB2 (encoded by the gene *Cnr2*) activity induced meiotic entry in fetal germ cells. Dochez-Arnault et al. (2023) showed that exposure to THC-only, CBD-only, and THC-CBD promoted several modifications in fetal testis, such as different patterns of cell proliferation in Leydig, Sertoli, and germ cell populations. However, this effect could not be attributed only to the CB2 receptor because THC and CBD bind to both CB1 and CB2 receptors (Chayasirisobhon 2021). It is also known that *Cnr2* transcript levels are higher than *Cnr1* in the developing gonads (De Domenico et al. 2017), suggesting that this receptor could reflect important, but still unknown, events in gonadogenesis. Some limitations of our study rely on the fact that we did not quantify the *Cnr1* (that encodes the CB1 receptor), due to its low expression levels, and in our *in vitro* experiment, we tested only a CB1 antagonist and not a CB2 antagonist. Since *Cnr2* levels are higher than *Cnr1* in the developing testis, maybe the inhibition of CB2 activity could reflect in reduced testosterone secretion.

Importantly, the postnatal ovary also showed higher *Cnr2* expression levels. As mentioned before, CB2 activity could induce premature meiotic entry in germ cells, which could affect the pool of follicles and impact the female reproductive lifespan. Also, the rodent ovary is still forming its follicles after birth, when oocyte cysts are broken down and are surrounded by granulosa cells (Johansson et al. 2017). It is still unknown if the CB2 receptor could play a role in follicle assembly, but it raises a concern in this process since DEHP can disrupt follicle assembly (Zhang et al. 2014; Mu et al. 2015).

CONCLUSION

In short, *In utero* exposure to DEHP alters the gonadal expression of ECS components, particularly *Cnr2*, which may serve as a novel genomic biomarker of gestational phthalate exposure. However, further research with other active phthalates is needed to confirm this finding. Future mechanistic studies should also

explore the endocannabinoid system as a potential molecular initiating target for phthalate-induced reproductive toxicity.

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TABLES

Table 1 - Anogenital distance (AGD), anogenital distance index (AGDi) and weight data of PND4 males and females pups after *in utero* exposure to the vehicle, Paracetamol (P), or DEHP (mg/kg/day) from gestation days 15 to 18. Sample size (N) is indicated as the number of litters in each group. Data expressed as litter means ± SEM. One-way ANOVA followed by Dunnett's post-hoc test. *Significantly different from the control group ($p \leq 0.05$).

Parameters	Control (N = 7)(	P50 (N = 6)	P250 (N = 5)	DEHP750 (N =4)
Males AGD	5.01 ± 0.14	4.82 ± 0.19	4.63 ± 0.13	4.02 ± 0.19*
Males AGDi	2.40 ± 0.06	2.26 ± 0.07	2.21 ± 0.07	1.94 ± 0.06*
Females AGD	2.64 ± 0.12	2.61 ± 0.14	2.44 ± 0.09	2.67 ± 0.09
Females AGDi	1.28 ± 0.06	1.23 ± 0.06	1.17 ± 0.04	1.31 ± 0.04
PND4 male weight	9.08 ± 0.34	9.82 ± 0.30	9.30 ± 0.37	8.79 ± 0.38
PND4 female weight	8.79 ± 0.29	9.51 ± 0.28	9.14 ± 0.25	8.50 ± 0.23

FIGURES AND FIGURE CAPTIONS

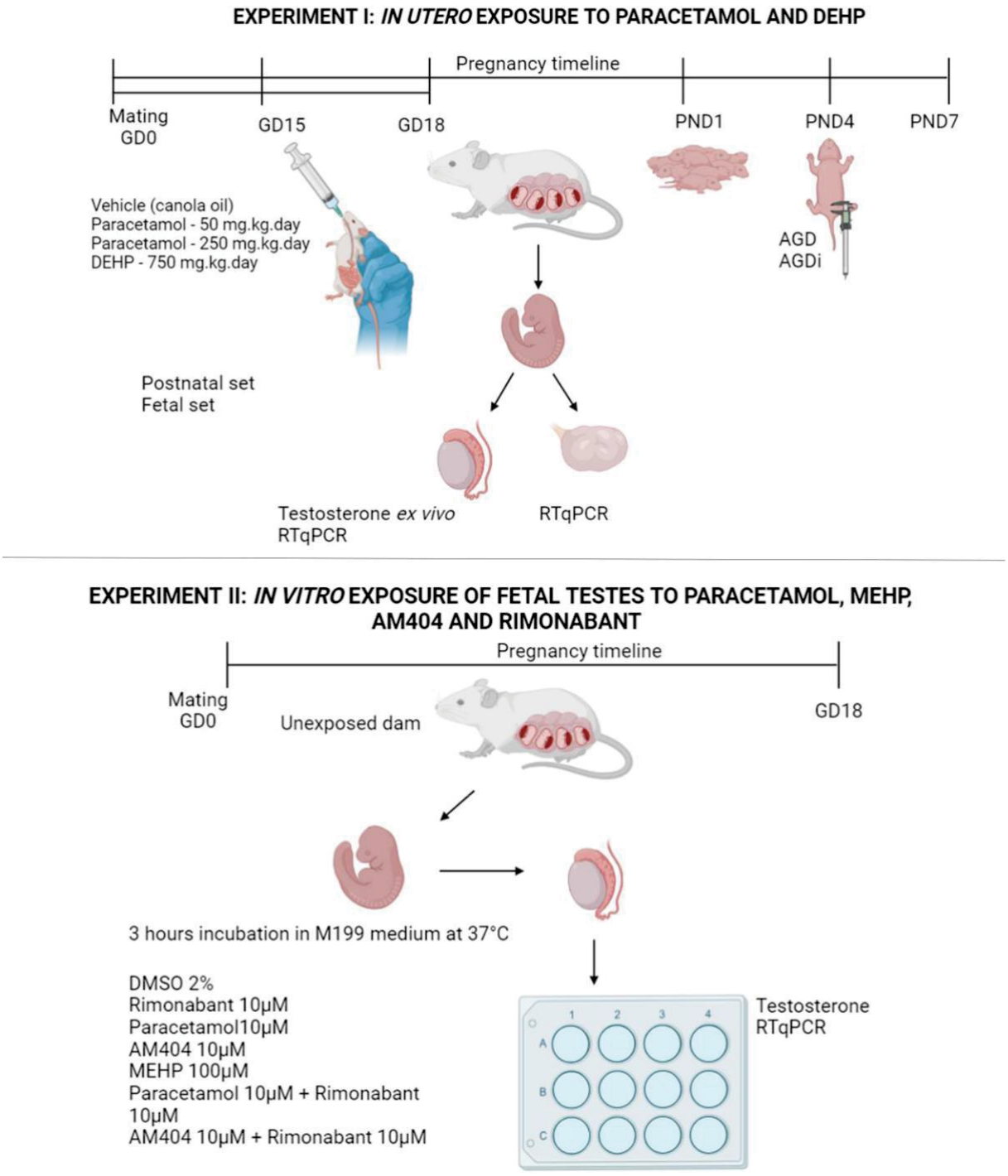

Figure 1 - Study design of Experiment I (*in utero* exposure do paracetamol and DEHP) and Experiment II (*in vitro* exposure to paracetamol, MEHP, AM404 and rimonabant).

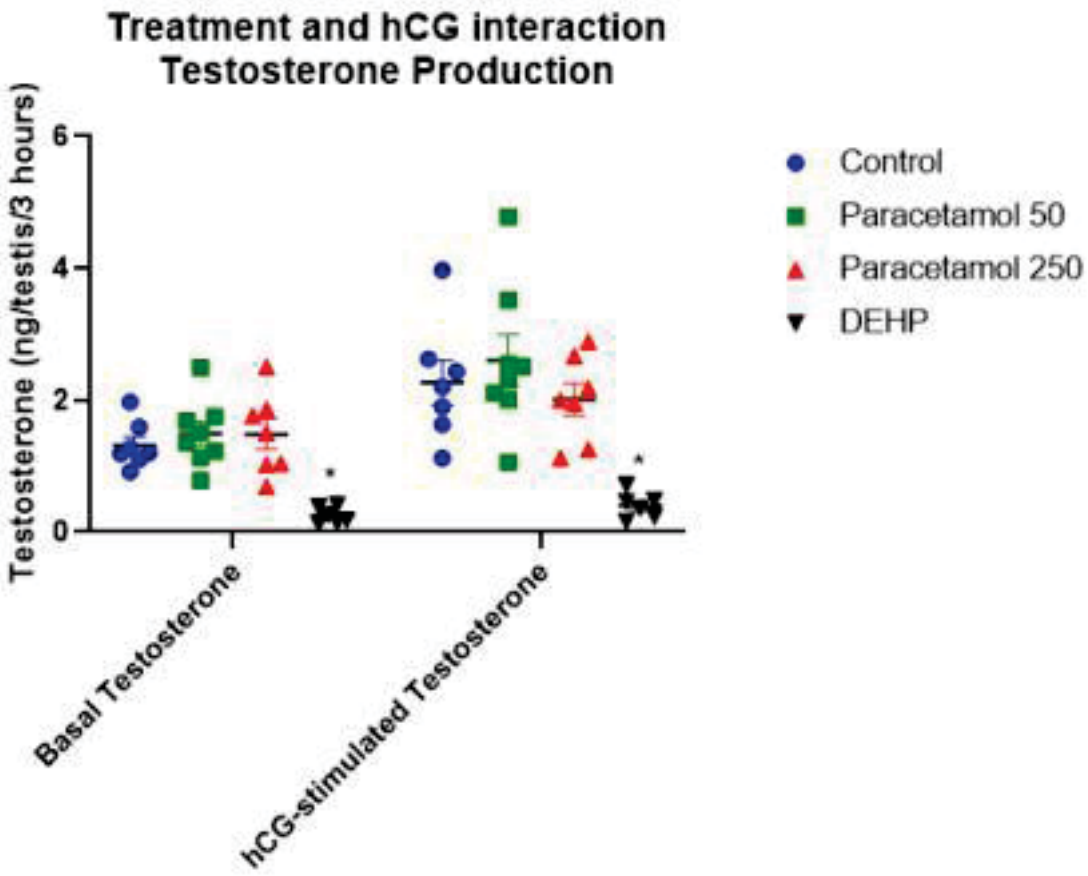

Figure 2 - Testosterone production in GD18 fetal testes, following *in utero* exposure to canola oil (vehicle control), paracetamol or DEHP. The sample size ranged from 6 to 8 litters/group, as indicated in the scatter plots. For each fetus, one testis was incubated with hCG (200 IU) and the other testis was left untreated (basal testosterone). Data expressed as litter means ± SEM. Two-way ANOVA followed by Dunnett's post-hoc test. Significant main effects were observed for both DEHP treatment ($p < 0.0001$) and hCG stimulation ($p < 0.0001$), as well as for their interaction ($p = 0.0016$). *Significantly different from the control group ($p \leq 0.05$).

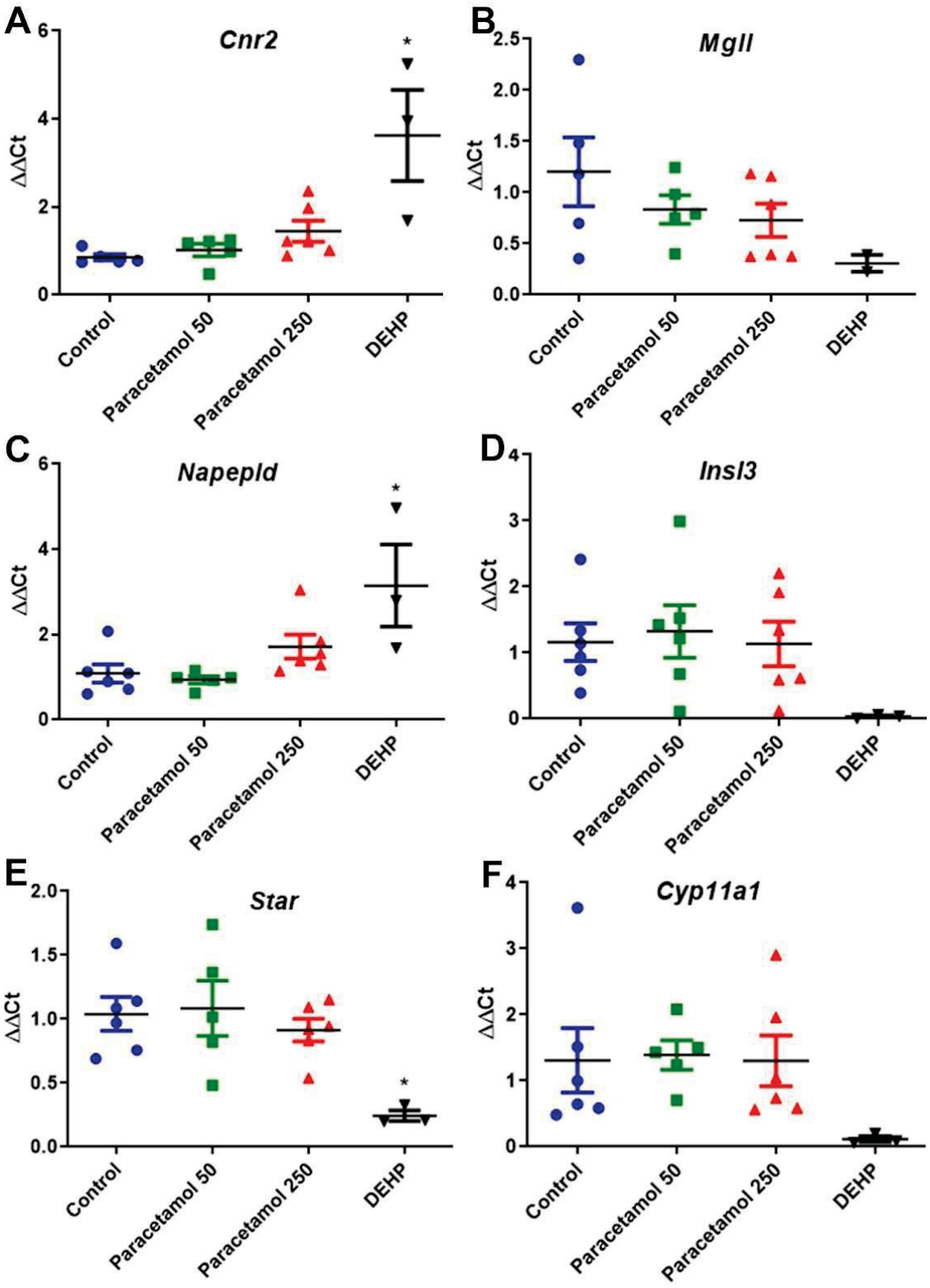

Figure 3 - Relative gene expression in GD18 fetal testes for *Cnr2* (A), *Mgll* (B), *Napepld* (C), *Insl3* (D), *Star* (E) and *Cyp11a1* (F). The sample size ranged from 3 to 6 litters/group, as indicated in the scatter plots. Data expressed as means ± SEM. One-way ANOVA followed by Dunnett's post-test. *Significantly different from the control group ($p \leq 0.05$).

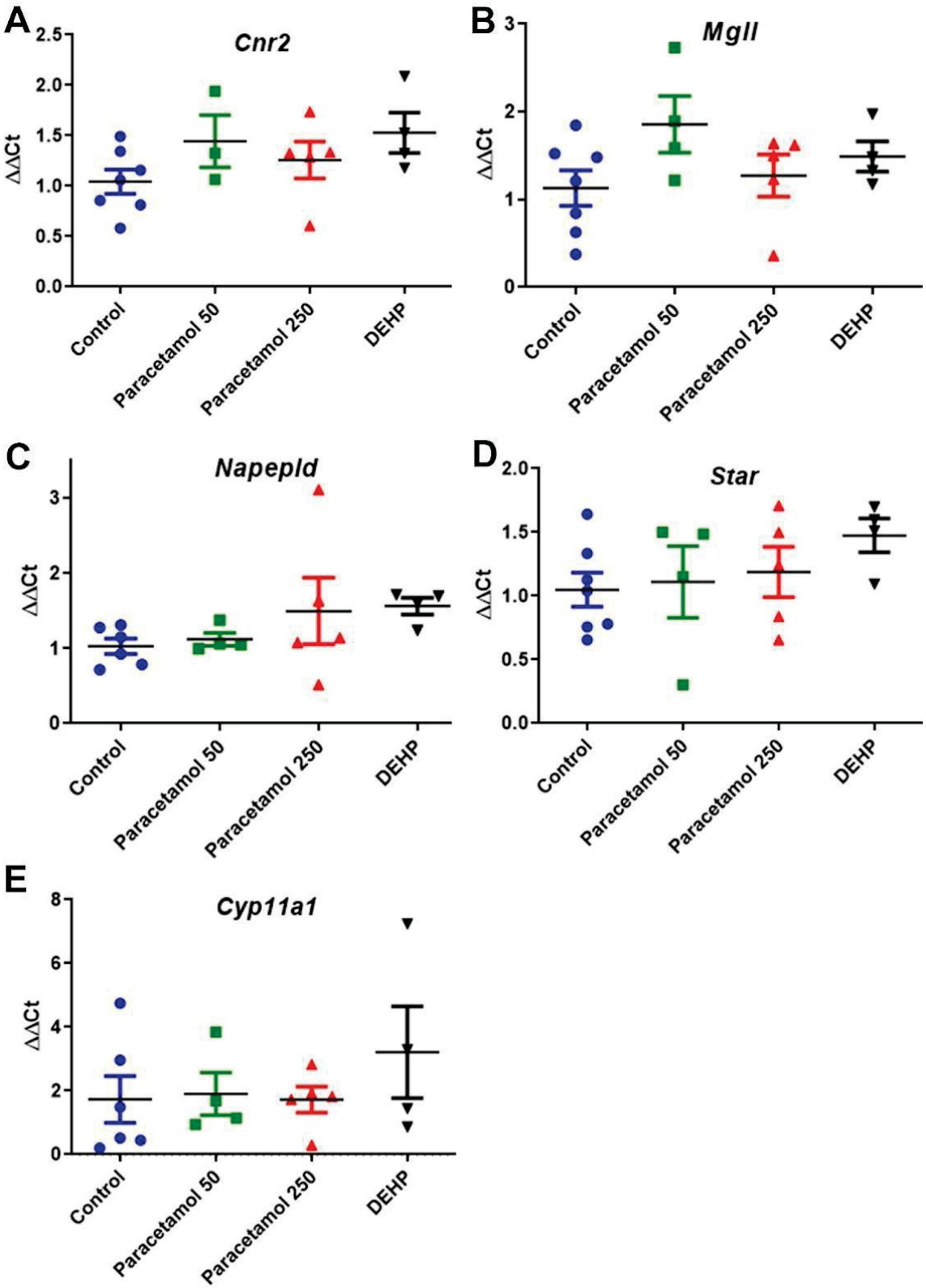

Figure 4 - Relative gene expression in postnatal testes exposed during GD15-18 window for *Cnr2* (A), *Mgll* (B), *Napepld* (C), *Star* (D) and *Cyp11a1* (E). The sample size ranged from 3 to 7 litters/group, as indicated in the scatter plots. Data expressed as means ± SEM. One-way ANOVA followed by Dunnett's post-test. No significant statistical difference were detected.

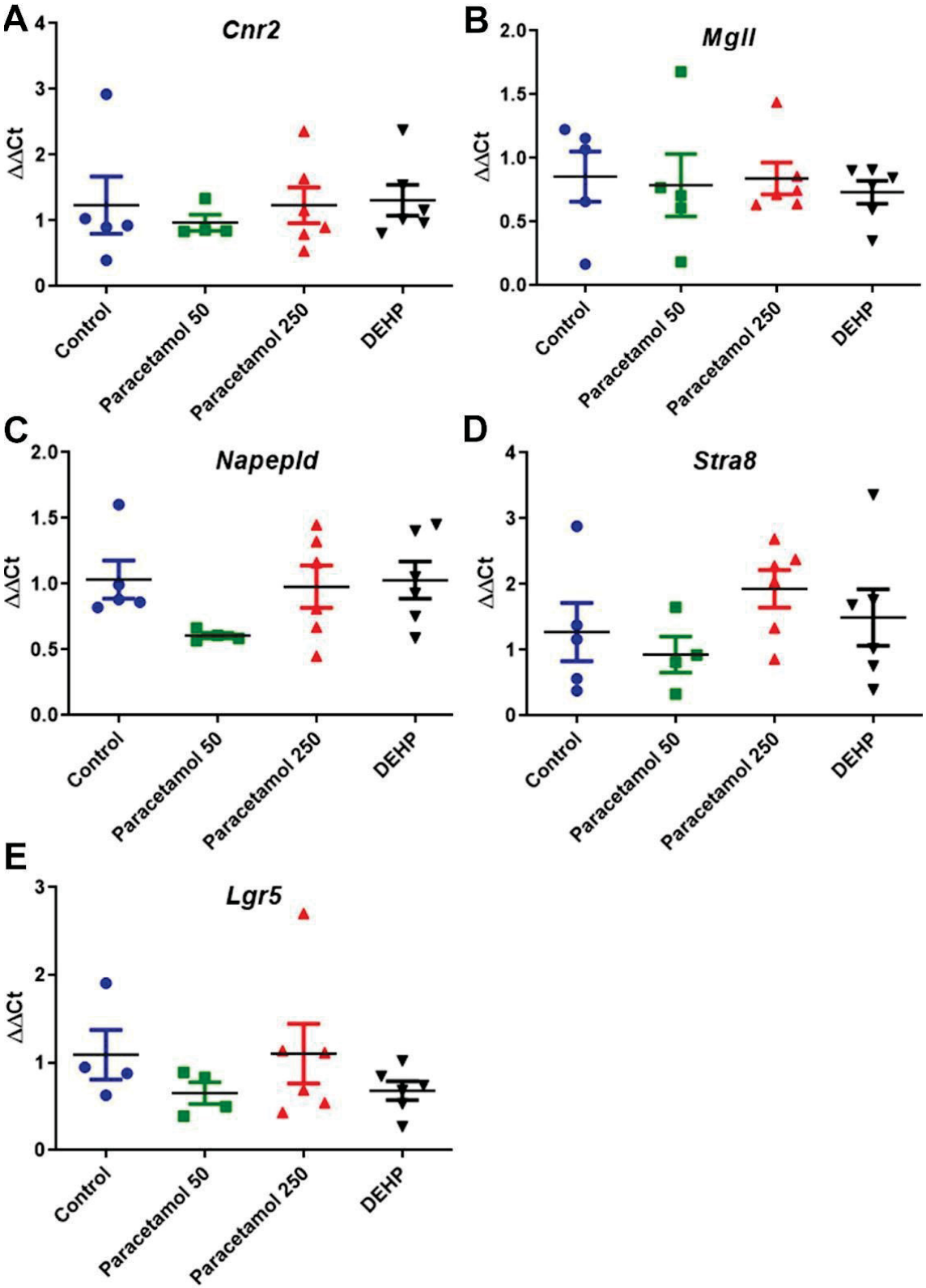

Figure 5 - Relative gene expression in GD18 fetal ovaries for *Cnr2* (A), *Mgll* (B), *Napepld* (C), *Stra8* (D) and *Lgr5* (E). The sample size ranged from 4 to 6 litters/group, as indicated in the scatter plots. Data expressed as means ± SEM. One-way ANOVA followed by Dunnett's post-test. No significant statistical difference were detected.

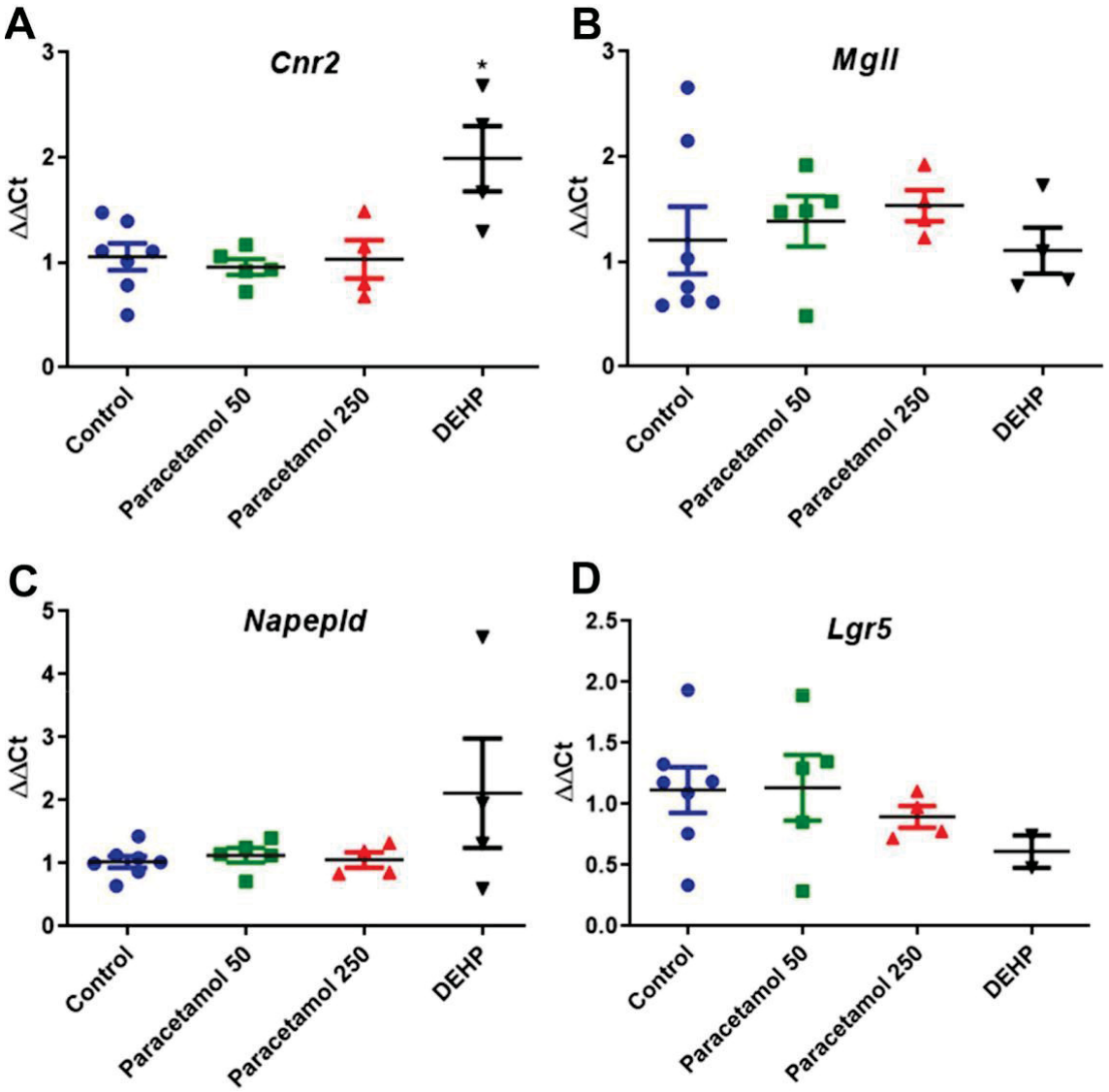

Figure 6 - Relative gene expression in postnatal ovaries exposed during the GD15-18 window for *Cnr2* (A), *Mgll* (B), *Napepld* (C) and *Lgr5* (D). The sample size ranged from 4 to 7 litters/group (except in DEHP in *Lgr5,* N=2), as indicated in the scatter plots. Data expressed as means ± SEM. One-way ANOVA followed by Dunnett's post-test. *Significantly different from the control group ($p \leq 0.05$).

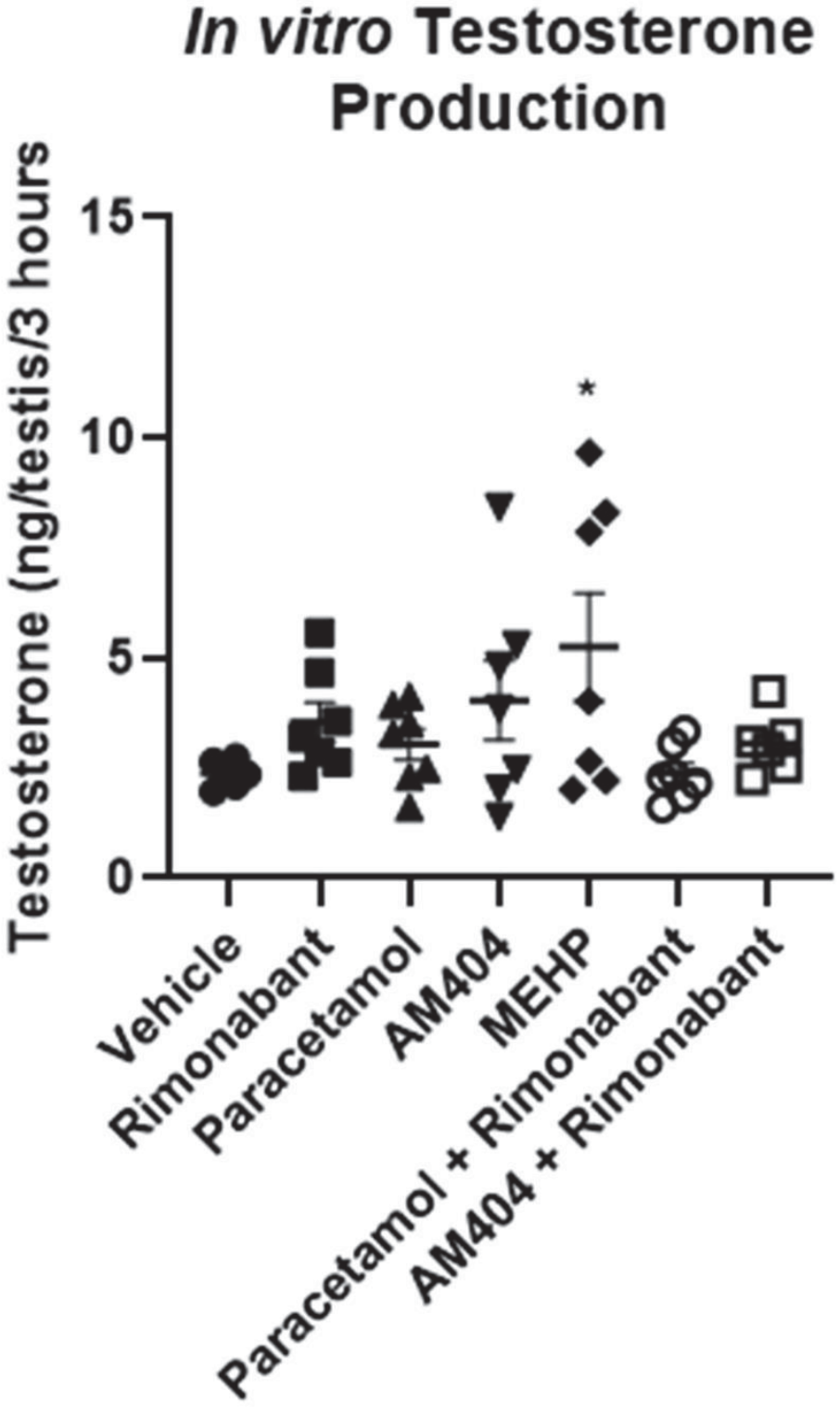

Figure 7 - Testosterone production by GD18 testes cultured *in vitro* during 3 hours. The sample size ranged from 6 to 7 litters/group, as indicated in the scatter plots. Data expressed as means ± SEM. One-way ANOVA followed by Dunnett's post-test. *Significantly different from the control group ($p \leq 0.05$).

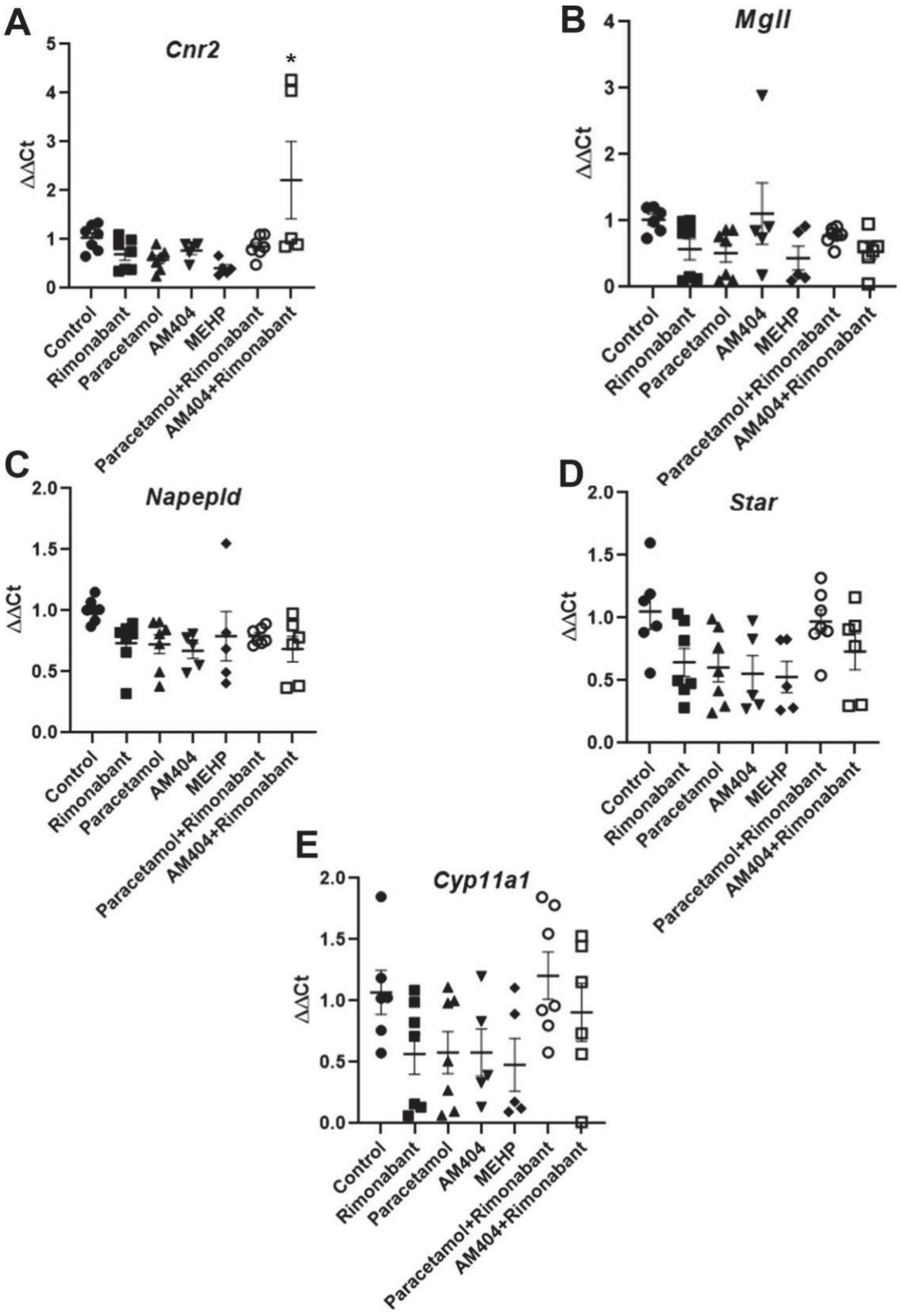

Figure 8 - Relative gene expression in GD18 testes cultured *in vitro* during 3 hours for *Cnr2* (A), *Mgll* (B), *Napepld* (C), *Star* (D) and *Cyp11a1* (E). The sample size ranged from 5 to 7 litters/group, as indicated in the scatter plots. Data expressed as means ± SEM. One-way ANOVA followed by Dunnett's post-hoc test. *Significantly different from the control group ($p \leq 0.05$).

SUPPLEMENTARY MATERIAL

Table S1 - Forward and reverse primer sequences of the selected genes.

Gene	Forward sequence	Reverse sequence
Rpl19	5' CAATGAAACCAACGAAATCG3'	3' TCAGGCCATCTTTGATCAGCT5
Insl3	5'CGCGACCGGGAGCTG3'	3'GAGAAGCCTGGTGAGGAAGC5
Stra8	5'ACTCCATTAAACCAGGAACCAGA3	3'AGGTCTCCAGGCACTTCAAC5'
Star	5'ACCAAGCGTAGAGGTTCCAC3'	3'TTCAGCTCTGATGACACCGC5'
Cyp11a1	5'GGAGCTGGTATCTCCTCTACCA3'	3'TTGCCCAGCTTCTCCCTGTAA5
Lgr5	5'TCATACTGTCACTGTGAGCTGG	3'ATTTCCAGCAAGACGCAACTCT5
Cnr2	5'CTCCAAGGTGCCAGACAGAG	3'GCCCCTTGAAGGCATCTTCT5'
Faah	5'GTTCACCTTGGACCCTACCG	3''AGAAGGGAATCAGCGTGTGG5
Napepld	5'GCTCCTTCGTCCCAGTATCG	3'TTCCGTGGGAAGTCGAAACC5'
Mgll	5'CGGAACAAGTCGGAGGTTGA	3'TGTCCTGACTCGGGGATGAT5'

F = Forward; R = Reverse.

Table S2 - Maternal and gestational data from the fetal experiment. Dams were treated from gestation day (GD) 15 to 18 and euthanized on GD18 for the collection of fetuses and maternal organs. Data expressed as means ± SEM. Statistical analysis was performed using One-Way-ANOVA followed by Dunnett's post-hoc test. *Significantly different from the control group ($p \leq 0.05$).

Parameters	Control (N = 7)	P50 (N = 8)	P250 (N = 6)	DEHP750 (N = 5)
Bodyweight	332.5 ± 6.73	315.6 ± 10.89	317.7 ± 10.24	305.3 ± 8.11
Ovary (g)	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.02
Ovary (%)	0.05 ± 0.003	0.05 ± 0.003	0.05 ± 0.006	0.05 ± 0.005
Adrenal (g)	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
Adrenal (%)	0.03 ± 0.002	0.03 ± 0.002	0.03 ± 0.003	0.03 ± 0.003
Kidney (g)	0.97 ± 0.03	0.95 ± 0.05	0.98 ± 0.03	0.89 ± 0.03
Kidney (%)	0.29 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.30 ± 0.01
Liver (g)	14.72 ± 0.72	13.69 ± 0.67	14.42 ± 0.93	14.13 ± 0.65
Liver (%)	4.42 ± 0.16	4.33 ± 0.12	4.52 ± 0.17	4.62 ± 0.11
Spleen (g)	0.81 ± 0.04	0.79 ± 0.03	0.74 ± 0.03	0.64 ± 0.02*
Spleen (%)	0.24 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	0.21 ± 0.01*
Litter size (n)	11.00 ± 0.31	10.00 ± 0.65	10.29 ± 0.81	10.50 ± 0.76
Male fetuses (n)	5.00 ± 0.53	4.38 ± 0.71	4.89 ± 0.70	4.33 ± 0.67
Female fetuses (n)	6.29 ± 0.57	5.63 ± 0.82	5.29 ± 0.71	6.17 ± 0.79
Male fetuses' weight	1.38 ± 0.04	1.35 ± 0.03	1.37 ± 0.02	1.37 ± 0.02
Female fetuses' weight	1.35 ± 0.03	1.31 ± 0.02	1.29 ± 0.03	1.33 ± 0.03
Early reabsorption	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	0.17 ± 0.17

Table S3 - Maternal and gestational data from the postnatal experiment. Dams were treated from gestation day 15 to 18 and euthanized along with their pups on postnatal day 7. Data expressed as means ± SEM. Statistical analysis was performed using One-Way ANOVA followed by Dunnett's post-hoc test.

Parameters	Control (N = 7)	P50 (N = 6)	P250 (N = 5)	DEHP750 (N = 4)
Bodyweight	297.1 ± 11.35	284.5 ± 5.14	299 ± 10.42	310.6 ± 7.25
Ovary (g)	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.05	0.24 ± 0.10
Ovary (%)	0.04 ± 0.004	0.05 ± 0.005	0.05 ± 0.02	0.08 ± 0.03
Uterus (g)	0.53 ± 0.03	0.54 ± 0.04	0.56 ± 0.04	0.63 ± 0.08
Uterus (%)	0.18 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.03
Adrenal (g)	0.11 ± 0.01	0.12 ± 0.02	0.12 ± 0.03	0.18 ± 0.04
Adrenal (%)	0.04 ± 0.004	0.04 ± 0.01	0.04 ± 0.10	0.06 ± 0.01
Kidney (g)	1.04 ± 0.05	0.99 ± 0.04	1.04 ± 0.05	1.11 ± 0.06
Kidney (%)	0.35 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.36 ± 0.02
Liver (g)	13.96 ± 1.01	12.23 ± 0.27	14.34 ± 0.96	15.45 ± 0.63
Liver (%)	4.69 ± 0.28	4.30 ± 0.06	4.80 ± 0.18	4.97 ± 0.16
Spleen (g)	0.73 ± 0.06	0.80 ± 0.06	0.79 ± 0.06	0.77 ± 0.09
Spleen (%)	0.25 ± 0.02	0.28 ± 0.02	0.27 ± 0.02	0.25 ± 0.03
Litter size (n)	11.43 ± 0.92	9.50 ± 0.76	11.80 ± 1.16	12.00 ± 1.08
Male pups (n)	4.43 ± 0.37	5 ± 0.63	5 ± 0.89	6.75 ± 1.44
Female pups (n)	7 ± 0.85	4.50 ± 0.72	6.80 ± 0.37	5.25 ± 1.38
PND1 male weight	6.35 ± 0.11	6.40 ± 0.11	6.23 ± 0.12	6.41 ± 0.25
PND7 male weight	12.65 ± 0.46	13.98 ± 0.60	13.02 ± 0.52	12.59 ± 0.51
PND1 female weight	6.04 ± 0.10	6.12 ± 0.14	5.78 ± 0.14	6.15 ± 0.25
PND7 female weight	12.35 ± 0.40	13.72 ± 0.48	12.66 ± 0.53	11.91 ± 0.58

4 DISCUSSÃO

Ao longo dos últimos anos, o sistema endocanabinoide têm ganhado relevância quanto ao desenvolvimento fetal do testículo e do ovário. As evidências discutidas no **artigo 1** apontam o seu envolvimento na proliferação celular de diversas linhagens celulares gonadais (Dochez-Arnault et al., 2023), diferenciação celular (Cacciola et al., 2008), influência na síntese de hormônios (Dalterio, 1980; Dalterio; Bartke, 1981; Dalterio et al., 1984; Dochez-Arnault et al., 2023), entrada na meiose (De Domenico et al., 2017) e possivelmente ser um dos fatores etiológicos que promove o câncer de células germinativas (Barchi et al., 2020). Não obstante, distúrbios na função do sistema endocanabinoide durante as janelas críticas de formação das gônadas e de diferenciação celular podem desencadear diversos efeitos deletérios e duradouros que possivelmente impactarão a saúde reprodutiva e endócrina do animal afetado. Dado ao crescente aumento de desordens do sistema reprodutor e aumento pela busca por tratamentos de reprodução assistida (Skakkebaek et al., 2022), destaca-se a importância de elucidar todos os principais mecanismos de sinalização que estão envolvidos na gonadogênese. Além disso, conforme demonstrado no **artigo 2**, desreguladores endócrinos como o exemplo do plastificante DEHP, podem interagir com o sistema endocanabinoide. Embora não tenha sido demonstrada uma relação causal entre a interação molecular entre o sistema endocanabinoide e a redução da produção de testosterona frente à exposição ao DEHP, é plausível sugerir que essa via possa estar envolvida nos mecanismos de toxicidade associados.

Uma das principais correntes atuais do campo da Toxicologia envolve a busca por eventos moleculares inicializadores (do inglês *Molecular Initiating Events*, MIEs) e vias de efeitos adversos (do inglês *Adverse Outcomes Pathways*, AOPs) (Browne et al., 2017). Os MIEs podem ser entendidos como “a interação inicial entre a molécula exógena com o biossistema ou biomolécula que pode ser causalmente ligada à via de efeito adverso” e, AOP seria uma sequência lógica de eventos ou processos dentro de sistemas biológicos que podem ser usados para entender os efeitos adversos e refinar as práticas atuais de avaliação de risco dentro da Toxicologia (Allen et al., 2014).

No exemplo desta tese, o possível aumento de expressão dos genes *Cnr2* e *Napepld*, que codifica o receptor CB2 e a enzima que sintetiza a anandamida,

respectivamente, frente à exposição ao DEHP poderia ser um MIE, uma vez que o CB2 é um receptor acoplado à proteína G inibitória, que por sua vez, reduz a síntese de monofosfato de adenosina cíclico (abreviado em inglês por cAMP), o que diminui a ação da proteína cinase A (PKA, do inglês *protein kinase A*). A síntese de qualquer hormônio esteroide, incluindo a testosterona, envolve a mobilização do colesterol para dentro da mitocôndria, pela ação da proteína reguladora aguda da esteroidogênese (STAR, do inglês *Steroidogenic acute regulatory protein*), a qual precisa ser fosforilada pela PKA para realizar esta função (Andric; Kostic, 2019). Além da conhecida redução da expressão gênica da STAR mediada pelo DEHP (Sree et al., 2023), o possível aumento da expressão do CB2, poderiam resultar juntos na síntese reduzida de andrógenos. As consequências destes MIEs, ou ainda, os AOPs, seriam o desenvolvimento dos sintomas da SDT, que incluem a presença de hipospádias, criptorquidismo, baixa produção de espermatozoides e câncer testícular de células germinativas (Wohlfahrt-Veje et al.; 2009; Wang; Qian, 2021; Willson, 2021). Visto que não tenham havido grandes sinais de toxicidade materna, conforme os dados representados nas tabelas suplementares S2 e S3 do **artigo 2**, é provavelmente que os todos os efeitos mediados pelo DEHP observados tenham sido resultado do seu potencial de agir como desregulador endócrino. Dentro do nosso conhecimento, é a primeira vez que foi relatada uma alteração da expressão de genes do sistema endocanabinoide em gônadas de ratos mediadas pelo DEHP. O estudo mais semelhante observado foi no modelo de zebrafish, onde um outro ftalato (o diisononil ftalato, DINP) também alterou o perfil de expressão gênica do sistema endocanabinoide nas gônadas. É provável que outros ftalatos com desregulação endócrina conhecida também utilizem o sistema endocanabinoide como MIE, portanto, são necessários mais estudos para avaliar esta possível relação.

Quanto ao MEHP, que é o metabólito ativo do DEHP responsável pelos seus efeitos anti-androgênicos, paradoxalmente aumentou a concentração da testosterona, assim como demonstrado por outros estudos (Lehraiki et al., 2009; Zhao et al., 2012). Este efeito foi observado no modelo de produção de testosterona *ex vivo*, onde o testículo fetal é mantido em cultura à 37°C pelo período de 3 horas. É provavel que este efeito pró-androgênico tenha sido resultado de uma resposta compensatória de curto prazo, uma vez que a testosterona seja um hormônio produzido “sob demanda”, a maquinaria enzimática já expressa acelerou a sua

cinética enzimática. O período de 3 horas seria muito curto para que houvessem alterações na expressão gênica, com a consequente redução da testosterona. Porém, era esperado que se houvesse uma interação com o CB2, como por exemplo o seu agonismo direto, haveria uma resposta rápida de inibição da PKA e da atividade da STAR, com a consequente redução da síntese de testosterona. Porém, a nossa hipótese não possa ser totalmente descartada, visto que talvez o DEHP e não o MEHP possa interagir com o CB2, ou ainda, outros mecanismos moleculares podem estar envolvidos. Além disso, há também a limitação de que esta cultura foi testada somente no curto prazo, sendo que a exposição prolongada pudesse culminar em outros resultados.

Outra limitação deste estudo foi a não detecção da expressão do *Cnr1*, que codifica o receptor CB1. Este gene é pouco expresso no testículo fetal, portanto, a técnica de transcrição reversa seguida de reação em cadeia da polimerase em tempo real (RTqPCR) não seria o principal método de detecção, a qual a PCR digital seria o mais indicado (Morley, 2014). Também, é possível que o DEHP pudesse ter modificado a produção de canabinoides endógenos como a AEA e o 2-AG, contudo, estes metabólitos também não foram mensurados. Além disso, no experimento *in vitro*, foi testado apenas o rimonabant que é um antagonista do CB1. Seria importante que estudos futuros avaliassem a inibição e ativação do CB2, uma vez que este receptor é mais expresso em gônadas fetais (De Domenico et al., 2017; Dochez-Arnault et al., 2023) e, como demonstrado neste estudo, está ligado com a toxicidade do DEHP.

O paracetamol não desencadeou nenhuma resposta tanto no estudo *in vivo* quanto o *in vitro*. Era esperado que este fármaco reduzisse a síntese de testosterona e diminuísse a distância anogenital (Kristensen et al., 2011; Kilcoyne; Mitchell, 2017), como resposta à alteração da expressão de genes do sistema endocanabinoides, ou ainda pela conversão do paracetamol em AM404 pela ação da enzima FAAH e ligação ao CB1, que eram as principais hipóteses deste trabalho. Embora a maioria dos estudos apontem para um efeito anti-andrôgenico (Mazaud-Guittot et al., 2013; Kristensen et al., 2011; Holm et al., 2015; Holm et al., 2016; Kilcoyne; Mitchell, 2017; Bauer et al., 2021), com o mecanismo de toxicidade também não sendo completamente conhecido, Gray et al. (2021) também não visualizaram este efeito. Dos estudos citados anteriormente que apresentam um efeito anti-androgênico, nenhum utilizou o modelo de rato Wistar, mas sim Sprague-

Dawley, testículos humanos e de camundongos. Uma possível explicação, que ainda precisa ser testada, seria uma provável resistência tanto sistêmica quanto localizada do testículo à toxicidade do paracetamol. Já foi sugerido em outro estudo que talvez o modelo de rato Wistar não seja o mais indicado para avaliar a toxicidade hepática do paracetamol e, ao que apontam as evidencias do **artigo 2** desta tese, também não seria o mais indicado para a toxicidade testicular. Contudo, seriam necessários estudos que comparasse diferentes modelo experimentais, incluindo dentre eles o rato Wistar, para avaliar se realmente trata-se de uma resistência à toxicidade do paracetamol.

Ainda assim, uma provável interação entre o paracetamol e o sistema endocanabinoide não pode ser descartada. Como mencionado anteriormente, o paracetamol também é metabolizado pela FAAH, uma enzima do sistema endocanabinoide (McCrae et al., 2018), o que resulta na sua conversão no metabólito AM404. O AM404 é capaz de ligar-se ao CB1, e aumenta a concentração extracelular de anandamida, que por sua vez, liga-se aos receptores CB1, CB2 e TRPV1, aumentando a sua atividade (Slattery; Klegeris, 2018). Dada a expressão testicular de CB1, embora em baixos níveis no testículo fetal, é provável que possa mediar alguns efeitos deletérios, como por exemplo reduzir a síntese de testosterona e alterar a função da célula de Sertoli e diferencição de gonócitos, uma vez que estas linhagens celulares o expressam (Dochez-Arnault et al., 2023). Além disso, o CB1 e o CB2 apresentam 44% de homologia entre as suas sequências de aminoácidos (Huang et al., 2020), portanto, seria importante também avaliar se o AM404 também é capaz de se ligar ao CB2 e caracterizar se essa ligação resultaria em agonismo ou antagonismo do receptor.

5 CONSIDERAÇÕES FINAIS

Em suma, o sistema endocanabinoide, ainda negligenciado, vem ganhando destaque como um mecanismo emergente envolvido na formação das gônadas fetais, podendo estar envolvido em processos como proliferação e diferenciação celular e síntese de testosterona. Contudo, este mecanismo pode ser alvo de desreguladores endócrinos, como o ftalato DEHP, que talvez possa utilizar o receptor CB2 como evento molecular inicializador, o que por sua vez, poderia explicar o aparecimento de desordens do trato reprodutor, como a Síndrome da

Disgenesia Testicular. No entanto, a relação causal entre a interação do DEHP com o sistema endocanabinoide e a redução da testosterona ainda deve ser melhor explorada em estudos posteriores. Apesar de não ter sido observado nenhum efeito mediado pela exposição ao paracetamol, é provável que o modelo de rato Wistar não seja o mais indicado para a sua avaliação em estudos de Toxicologia Reprodutiva e a interação entre o paracetamol e o sistema endocanabinoide não pode ser descartada.

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