

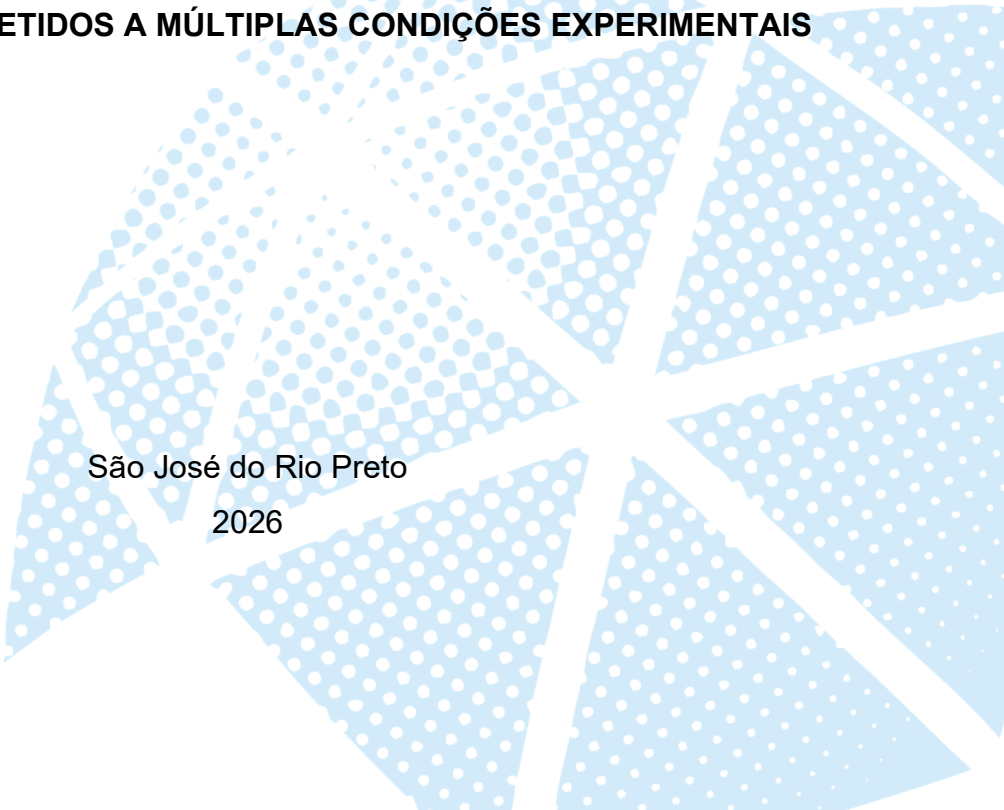
UNIVERSIDADE ESTADUAL PAULISTA – UNESP
Instituto de Biociências, Letras e Ciências Exatas - Campus de São José do
Rio Preto

STELLA BICALHO SILVA PAVANI

IMPACTOS DA EXPOSIÇÃO A DESREGULADORES ENDÓCRINOS NA
PRÓSTATA E NOS TESTÍCULOS DE GERBILOS DA MONGÓLIA (*Meriones*
***unguiculatus*) SUBMETIDOS A MÚLTIPLAS CONDIÇÕES EXPERIMENTAIS**

São José do Rio Preto

2026



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Tese apresentada à Universidade Estadual Paulista (UNESP), Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto, para obtenção do título de Doutor em Biodiversidade.

Orientadora: Prof.^a. Dr.^a. Patrícia Simone Leite Vilamaior

Coorientadora: Prof.^a. Dr.^a. Fernanda Cristina Alcantara dos Santos

São José do Rio Preto

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IMPACTO POTENCIAL DESTA PESQUISA

A presente pesquisa possui elevado potencial científico e técnico, ao alertar sobre o uso crescente de desreguladores endócrinos e ao ressaltar sua interferência na próstata e nos testículos. Ademais, fornece referencial científico que subsidia a elaboração de possíveis estratégias de prevenção e de políticas públicas de saúde humana e ambiental.

POTENTIAL IMPACT OF THIS RESEARCH

The present study has significant scientific and technical potential. It highlights the growing use of endocrine disruptors and emphasizes their impact on the prostate and testes. Additionally, the study provides a scientific foundation for developing prevention strategies and public policies that protect human and environmental health.

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Dedico este trabalho a Deus, que é o autor da vida e mestre de todo o conhecimento.

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*“O caminho muda, e muda o caminhante
É um caminho incerto, não o caminho
errado.*

*Eu, caminhante, quero o trajeto terminado
Mas no caminho, mais importa o durante”.*

Estevão Queiroga – A Partida e o Norte

RESUMO

Desreguladores Endócrinos (DEs) são substâncias que afetam o funcionamento do sistema endócrino e de órgãos hormônio-responsivos como a próstata e os testículos. A crescente exposição a estas substâncias tem provocado distúrbios no eixo reprodutivo, os quais podem prejudicar a fertilidade. Este estudo explorou os impactos causados pela exposição a múltiplos DEs na morfofisiologia da próstata e dos testículos de gerbilos da Mongólia (*Meriones unguiculatus*). No primeiro capítulo, foram avaliados os impactos da exposição a metais potencialmente tóxicos (alumínio, arsênio, cádmio, cromo, chumbo, mercúrio e níquel), revelando seus mecanismos de toxicidade na próstata de modelos murinos. Os resultados incluem desequilíbrio hormonal e dano oxidativo associados a alterações morfológicas, que variam de acordo com o metal, tempo de exposição, concentração e idade. No segundo capítulo, foram investigados os efeitos da exposição às formulações comerciais de glifosato, atrazina e 2,4-D na próstata ventral de gerbilos da Mongólia. Os resultados mostraram que a exposição individual ou combinada a baixas concentrações dos herbicidas induziu desequilíbrio hormonal e alterações morfológicas, além de insultos proliferativos na próstata. No terceiro capítulo, foram avaliados os efeitos da exposição perinatal ao Bisfenol A (BPA) em gerbilos adultos sob condições normais e com hiperplasia prostática, através da avaliação morfofisiológica e molecular da glândula. A exposição ao BPA durante um estágio crítico do desenvolvimento reprogramou a próstata adulta, tornando-a suscetível a desordens teciduais e moleculares, sobretudo em gerbilos com hiperplasia prostática. No quarto capítulo, foram investigadas as repercussões metabólicas decorrentes da exposição perinatal ao BPA nos testículos de gerbilos, associadas ao possível efeito atenuante do β -cariofileno (BCP). O estudo mostrou que o BPA induziu distúrbios em vias metabólicas essenciais. Entretanto, o tratamento com BCP atenuou essa disfunção metabólica testicular. Sumariamente, os estudos mostram que a exposição a DEs em diferentes fases da vida, em diferentes doses ou concentrações, provoca danos morfofisiológicos latentes que, a longo prazo, prejudicam a função reprodutiva. Tomados juntos, estes estudos refletem um contexto real de exposição humana, destacando a próstata e os testículos como órgãos-alvo de múltiplos DEs.

Palavras-chave: Histopatologia; metabolismo; morfologia; reprodução; toxicologia.

ABSTRACT

Endocrine-disrupting chemicals (EDCs) are substances that affect the functioning of the endocrine system and hormone-responsive organs such as the prostate and testes. Increasing exposure to these substances has caused morphophysiological disturbances in the reproductive axis, which may impair fertility. This study explored the impacts of exposure to multiple endocrine-disrupting chemicals (EDCs) on the morphophysiology of the prostate and testes of Mongolian gerbils (*Meriones unguiculatus*). In the first chapter, the effects of exposure to potentially toxic metals (aluminum, arsenic, cadmium, chromium, lead, mercury, and nickel) were investigated, revealing their mechanisms of toxicity in the prostate of murine models. The results include hormonal imbalance and oxidative damage associated with morphological alterations, which vary according to the metal, exposure time, concentration, and age. In the second chapter, the effects of exposure to commercial formulations of glyphosate, atrazine, and 2,4-D were investigated on the ventral prostate of Mongolian gerbils. The results showed that individual or combined exposure to low concentrations of these herbicides induced hormonal imbalance and morphological alterations, as well as proliferative insults in the prostate. In the third chapter, the impacts of perinatal exposure to Bisphenol A (BPA) were investigated in adult gerbils under normal conditions and with prostatic hyperplasia, through morphophysiological and molecular assessment of the gland. Exposure to BPA during a critical stage of development reprogrammed the adult prostate, making it more susceptible to tissue and molecular disorders, especially in gerbils with prostatic hyperplasia. In the fourth chapter, the metabolic repercussions induced by perinatal exposure to BPA were investigated in the testes of gerbils, associated with the possible attenuating effect of β -caryophyllene (BCP). This study showed that BPA disrupted essential metabolic pathways. However, treatment with BCP mitigated this testicular dysfunction. In summary, the studies demonstrate that exposure to EDCs at different stages of life, doses, or concentrations induces latent morphophysiological damage that, in the long term, impairs reproductive function. Taken together, these studies reflect a real-life context of human exposure, highlighting the prostate and testes as target organs for the action of multiple endocrine-disrupting chemicals (EDCs).

Keywords: Histopathology; metabolism; morphology; reproduction; toxicology.

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LISTA DE ABREVIATURAS E SIGLAS

ABP	Androgen-Binding Protein/Proteína de Ligação a Andrógenos
AMH	Hormônio Antimülleriano
AR	Androgen Receptor/Receptor de Andrógeno
BCP	β -caryophyllene/ β -cariofileno
BPA	Bisphenol A/Bisfenol A
DEs	Desreguladores Endócrinos
DHT	Di-hidrotestosterona
EDCs	Endocrine-disrupting Chemicals
EPA	Environmental Protection Agency/Agência de Proteção Ambiental
ER α	Estrogen Receptor Alpha/Receptor de Estrógeno Alfa
ER β	Estrogen Receptor Beta/Receptor de Estrógeno Beta
EROs	Espécies Reativas de Oxigênio
Fig.	Figura
FSH	Hormônio Folículo Estimulante
GnRH	Hormônio Liberador de Gonadotrofinas
HPA	Hipotálamo-Pituitária-Adrenais
HPB	Hiperplasia Prostática Benigna
HPG	Hipotálamo-Pituitária-Gônadas
LD	Lobo Dorsal
LDH	Desidrogenase Láctica
LH	Hormônio Luteinizante
LL	Lobo Lateral
LV	Lobo Ventral
PAP	Prostatic Acid Phosphatase/Fosfatase Ácida Prostática
PSA	Prostate Specific Antigen/Antígeno Prostático Específico
UGS	Urogenital Sinus/Seio Urogenital

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1. INTRODUÇÃO

1.1 DESREGULADORES ENDÓCRINOS

A Agência de Proteção Ambiental (EPA) estadunidense define os Desreguladores Endócrinos (DEs) como substâncias que afetam o funcionamento regular do sistema endócrino por interferir na síntese, secreção, transporte, ligação ou eliminação de hormônios naturais no organismo, responsáveis pela homeostase, reprodução, desenvolvimento e comportamento dos seres vivos (KAVLOCK *et al.*, 1996). O termo “endocrine disruptor” foi formalmente introduzido pela primeira vez no início da década de 1990, por meio da emissão da declaração denominada “Wingspread Statement”, em que Theo Colborn e colaboradores alertaram a comunidade científica local sobre os efeitos dos DEs na saúde humana e ambiental (YILMAZ *et al.*, 2020). Deste período em diante, estudiosos de diferentes partes do mundo vêm se dedicando a compreender as complexidades e os mecanismos de ação desempenhados por essas substâncias.

De acordo com KABIR, RAHMAN e RAHMAN (2015), dentre os tipos mais comuns de DEs, estão os compostos químicos naturais (ex.: fitoestrógenos), pesticidas (ex.: herbicidas, fungicidas, inseticidas), produtos químicos sintéticos usados como solventes e seus subprodutos (ex.: bifenilas policloradas, bifenilas polibromadas), hormônios sintéticos (ex.: pílulas anticoncepcionais), produtos usados na fabricação de plásticos (ex.: BPA), derivados do plástico (ex.: microplásticos), ftalatos, metais pesados, entre outros. Devido às aceleradas e crescentes ações antropogênicas, diferentes ecossistemas têm recebido montantes diários de DEs, os quais são atingidos por descargas de efluentes industriais e de tratamento de água, resíduos de atividades de mineração, além de escoamento de áreas urbanas e agrícolas (VARTICOVSKI *et al.*, 2022). Em 2018, a Comissão Lancet sobre Poluição e Saúde apontou que a poluição e contaminação ambiental foram responsáveis por 9 milhões de mortes prematuras, representando um valor superior ao triplo de mortes associadas ao HIV/AIDS, tuberculose e malária, com o impacto mais acentuado em países em desenvolvimento (LANDRIGAN *et al.*, 2018). Neste contexto, as vidas humanas e selvagens se tornam diariamente ameaçadas e expostas aos DEs por meio de diferentes rotas e vias de exposição que incluem inalação de material particulado, ingestão de água e alimentos contaminados e contato dérmico (ARETZ *et al.*, 2021; KANG; LEE; LIM, 2022; REHMAN *et al.*, 2018).

Estudos *in vivo* demonstram que a vida selvagem vem sendo profundamente afetada pela exposição a DEs, com impactos em diferentes classes de animais e plantas que exercem funções ecológicas relevantes e participam de cadeias tróficas importantes (HAYES *et al.*, 2011; KAMINER BITTON; ZUCKER; GRUNTMAN, 2025; LEÃO *et al.*, 2024; MUÑOZ-PENUELA *et al.*, 2024; SALLA *et al.*, 2024). Similarmente, a vida humana vem sofrendo os efeitos adversos da exposição desenfreada a DEs, com prejuízos não apenas direcionados àqueles primariamente expostos, mas também com interferências no desenvolvimento das próximas gerações (HEINDEL *et al.*, 2017; LAPEHN; PAQUETTE, 2022). Particularmente, dentre os efeitos adversos no sistema endócrino, podem ser citados disfunções na tireoide que resultam em desordens metabólicas (CALSOLARO *et al.*, 2017), inflamação e fibrose pancreática (SUN *et al.*, 2024), bem como desregulação da glicose e interrupção da síntese hormonal no eixo hipotálamo-pituitário-adrenal (HPA) (YANG *et al.*, 2022). A exposição a DEs também está relacionada à neurotoxicidade em diferentes espécies (SANDOVAL-HERRERA *et al.*, 2023; TORRES-RUIZ *et al.*, 2023; WANG *et al.*, 2025). Adicionalmente, devido à importância da integridade do eixo reprodutivo para o sucesso da reprodução, parte dos estudos tem se dedicado à avaliação e compreensão dos efeitos causados pela exposição a DEs nos parâmetros reprodutivos de animais e seres humanos (CHAM *et al.*, 2024; DUTTA; BANU; AROSH, 2023; GERALD *et al.*, 2023; SVANHOLM *et al.*, 2023).

O impacto da exposição a DEs sobre a saúde reprodutiva tem despertado crescente interesse científico, principalmente devido à disponibilidade e persistência desses compostos em ambientes naturais, domésticos, ocupacionais e industriais (DING *et al.*, 2022; LAUTAN *et al.*, 2025). Substâncias como o BPA, ftalatos, pesticidas, poluentes orgânicos persistentes e metais potencialmente tóxicos possuem a capacidade de mimetizar, antagonizar ou modular a ação de hormônios endógenos, além de alterar a expressão de receptores celulares e enzimas esteroidogênicas, resultando em alterações morfofisiológicas intrínsecas (BARAKAT *et al.*, 2019; CHITAKWA *et al.*, 2024; GUARNOTTA *et al.*, 2022). Muitos estudos que investigam a ação de DEs têm se concentrado em órgãos hormônio-responsivos, tais como as mamas (RUIZ *et al.*, 2021), ovários (RUIZ *et al.*, 2023a) e adrenais (GUERRA *et al.*, 2025). Entretanto, a próstata e os testículos representam órgãos-chave por desempenhar papéis centrais na função reprodutiva (LIU, 2025). A integridade morfofuncional de ambos é indispensável para a fertilidade e para a manutenção da

homeostase do eixo reprodutivo como um todo (VERZE; CAI; LORENZETTI, 2016). A exposição a DEs é capaz de comprometer o funcionamento da próstata e dos testículos, por meio de mecanismos que envolvem desequilíbrios hormonais, estresse oxidativo, alterações epigenéticas e disfunções celulares que podem levar à infertilidade (AQUINO *et al.*, 2019; BROUARD *et al.*, 2022; MANIKKAM *et al.*, 2012; SILVA *et al.*, 2024). Portanto, tornam-se imprescindíveis a expansão, investigação e ampliação do conhecimento acerca dos mecanismos subjacentes aos efeitos da exposição a DEs sobre a próstata e os testículos, utilizando modelos *in vivo* que possibilitem maior translação dos achados para o contexto real de exposição humana.

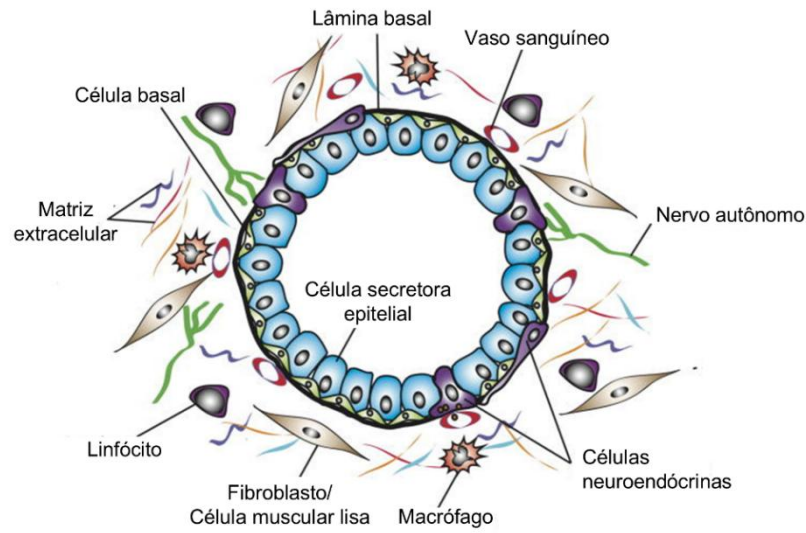
1.2 A PRÓSTATA

1.2.1 Aspectos histológicos e anatômicos da glândula prostática

A próstata é uma glândula acessória do sistema reprodutivo de mamíferos que pode ser encontrada em machos e fêmeas, incluindo humanos (BEDOLO *et al.*, 2023; TOMALTY *et al.*, 2023). Histologicamente, a próstata é caracterizada por uma glândula exócrina composta, com classificação epitelial simples ou pseudoestratificada, túbulo-alveolar, dividida em três compartimentos principais: epitelial, estromal e luminal, que se comunicam através de importantes vias de sinalização para promover a manutenção homeostática da glândula (ROCHEL *et al.*, 2007; UNTERGASSER; MADERSBACHER; BERGER, 2005). O epitélio prostático apresenta quatro tipos distintos de células (secretores luminiais, basais, intermediárias e neuroendócrinas). As células secretoras luminiais são as mais frequentes no compartimento epitelial e são responsáveis pela síntese e secreção de proteínas, tais como o antígeno prostático específico (PSA) e a fosfatase ácida prostática (PAP), lançadas no lúmen (MARKER *et al.*, 2003). As células epiteliais basais atuam como fonte progenitora das células secretoras (EL-ALFY *et al.*, 2000). No epitélio ainda podem ser encontradas escassas células neuroendócrinas que produzem neuropeptídeos, como a serotonina, bem como células intermediárias e células do sistema imunológico, como macrófagos e linfócitos (MARKER *et al.*, 2003). O compartimento estromal é composto pela matriz extracelular rica em fibras colágenas que se interpõe entre os ácinos secretores. Há também a presença de células musculares lisas, miofibroblastos e fibroblastos que exercem comunicação intrínseca com o epitélio por vias de interação e sinalização. No estroma também são

encontrados células endoteliais, fibras nervosas autonômicas e gânglios associados, além de telócitos e componentes do sistema imunológico como macrófagos e linfócitos (BARRON; ROWLEY, 2012) (Figura 1).

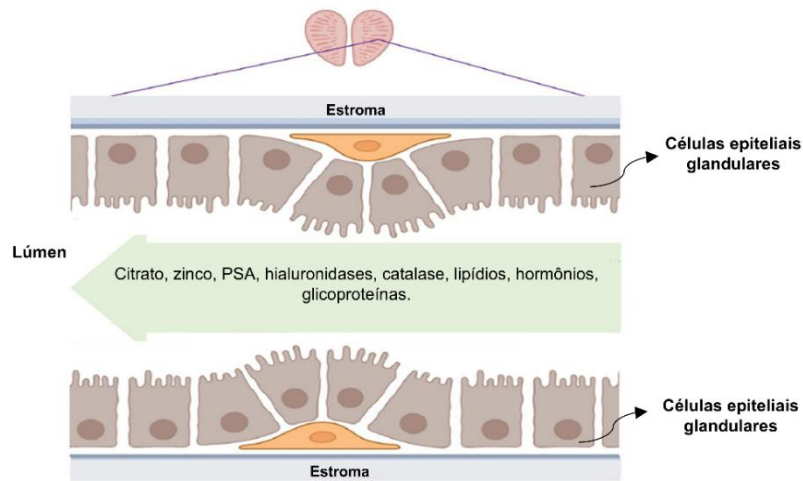
Figura 1. Representação esquemática de um ácino prostático evidenciando os principais componentes celulares do epitélio e do estroma da próstata.



Fonte: Extraído e adaptado de Barron *et al.* (2012)

A principal função da próstata é produzir e secretar o fluido prostático, rico em fosfatases alcalinas, que nutre e confere viabilidade aos espermatozoides (SILVA *et al.*, 2023). As principais secreções produzidas pelas células epiteliais glandulares da próstata incluem: citrato, zinco, fosfatases, PSA e PAP (HUA *et al.*, 2025) (Figura 2).

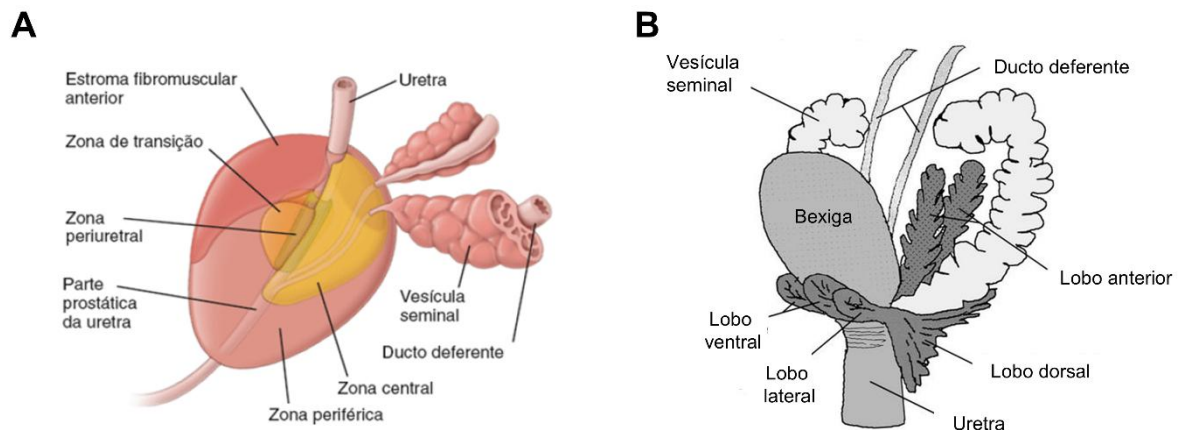
Figura 2. Representação esquemática da estrutura histológica da próstata, apresentando os compartimentos de epitélio e estroma, bem como os principais fluidos secretados.



Fonte: Extraído e adaptado de Hua *et al.* (2025)

A próstata humana apresenta quatro zonas anatomicamente e clinicamente distintas: central, periférica, periuretral e de transição. A zona central envolve os ductos ejaculadores e se projeta sobre a base da bexiga. A zona periférica constitui a maior porção da glândula, posterior e lateral da próstata. A zona periférica envolve a uretra proximal aos ductos ejaculadores e é o principal sítio de desenvolvimento do câncer prostático. A zona de transição é o principal sítio de desenvolvimento de lesões proliferativas como a Hiperplasia Prostática Benigna (HPB) (ROSS; PAWLINA, 2016; VERZE; CAI; LORENZETTI, 2016) (Figura 3A). A próstata de roedores difere anatomicamente da próstata humana, sendo formada por quatro lobos bilaterais distintos e simétricos que circundam a uretra na base da bexiga, designados como lobo ventral (LV), lobo lateral (LL), lobo dorsal (LD) e o lobo anterior ou glândula coaguladora, proximamente ligada às vesículas seminais (OLIVEIRA *et al.*, 2016). Devido à proximidade e fusão dos lobos dorsal e lateral, este é por vezes referido como dorsolateral (ROCHEL *et al.*, 2007) (Figura 3B). Apesar de intensas discussões, de acordo com o Consenso de Bar Harbor de 2001, não há evidências seguras que sustentem uma relação direta entre os lobos específicos da próstata de roedores e as zonas específicas da próstata humana (AARON; FRANCO; HAYWARD, 2016; SHAPPELL *et al.*, 2004).

Figura 3. Representação esquemática da próstata humana indicando a sua localização relativa, com base em cores distintas, as quatro zonas da próstata, bem como o estroma fibromuscular anterior (A). Representação esquemática da próstata de roedores com vista lateral (B).



Fonte: Extraído e adaptado de Ross e Pawlina (2016); Toivanen e Shen (2017)

Dentre os lobos prostáticos de roedores, o lobo ventral vem sendo amplamente utilizado em estudos morfológicos, devido à alta responsividade hormonal e por ser um importante sítio de desenvolvimento de lesões proliferativas (FALLEIROS-JÚNIOR *et al.*, 2016). Além disso, apresenta maior potencial de incidência de lesões pré-malignas e malignas em indivíduos adultos, com um período de latência tumoral mais longo do que os demais lobos (CAMPOS *et al.*, 2008; GONÇALVES *et al.*, 2013).

1.2.2 Desenvolvimento da próstata

O crescimento e o desenvolvimento da próstata começam na vida fetal e se completam na maturidade sexual. A morfogênese da próstata se inicia no seio urogenital (UGS), definido como uma subdivisão da cloaca (MARKER *et al.*, 2003). O UGS surge em camundongos machos e fêmeas por volta do 13º dia pós-concepção e em humanos por volta da 7ª semana de gestação. A partir de então, na fase de diferenciação, surgem brotos epiteliais e ramificações que obedecem a um padrão preciso e originam os diferentes lobos da próstata (MARKER *et al.*, 2003). Neste período, ocorre também a ramificação dos brotos, sendo eles estimulados por sinalizações parácrinas, adquirindo características típicas de cada lobo da próstata do roedor, sendo essa fase finalizada 15 a 30 dias após o nascimento. Portanto, o crescimento e desenvolvimento da próstata continuam ocorrendo após o nascimento, e a morfogênese ramificada está quase totalmente completa em camundongos com duas semanas de idade. Neste período, há um ganho de peso da glândula devido aos níveis de testosterona que também aumentam conforme os testículos se desenvolvem (MARKER *et al.*, 2003). Assim, nas fases pré-natal e pós-natal, o crescimento e

desenvolvimento da próstata são dependentes dos andrógenos circulantes produzidos pelos testículos, os quais são ainda necessários para o completo desenvolvimento da glândula (CAMPOS *et al.*, 2006). Observações comparativas do desenvolvimento prostático em roedores e humanos demonstram que a morfogênese ocorre de maneira análoga em ambos os grupos, com vários conjuntos distintos de brotos epiteliais crescendo a partir da uretra (MEEKS; SCHAEFFER, 2011).

1.2.3 O gerbilo da Mongólia como modelo experimental

O gerbilo da Mongólia (*Meriones unguiculatus*) é uma pequena espécie de roedor pertencente à família Muridae e à subfamília Gerbillinae, naturalmente encontrada na Mongólia, China e Rússia (LEE; LEE; JEON, 2021). Esses animais apresentam um comportamento dócil, micção infrequente, são menores que os ratos e maiores do que os camundongos, apresentam fácil manuseio e adaptação em laboratório e têm comportamento monogâmico, tornando-os bons modelos experimentais (ROCHEL *et al.*, 2007). O gerbilo da Mongólia foi introduzido na pesquisa científica em 1960, sendo utilizado em pesquisas de neurofisiologia, imunologia e cultura celular (PINTO-FOCHI *et al.*, 2014). Essa espécie de roedor consolidou-se como um modelo experimental valioso para o estudo da próstata e do eixo reprodutivo por reunir particularidades anatômicas e endocrinológicas que facilitam análises mecanísticas em animais imunocompetentes (RUIZ *et al.*, 2023b). Além disso, a espécie desenvolve neoplasias espontâneas associadas ao envelhecimento e demonstra alta responsividade a tratamentos hormonais (CAMPOS *et al.*, 2008) (Figura 4).

Figura 4. Fotografia representativa de um espécime de gerbilo da Mongólia (*Meriones unguiculatus*).



Fonte: Extraído de Ruiz *et al.* (2023b)

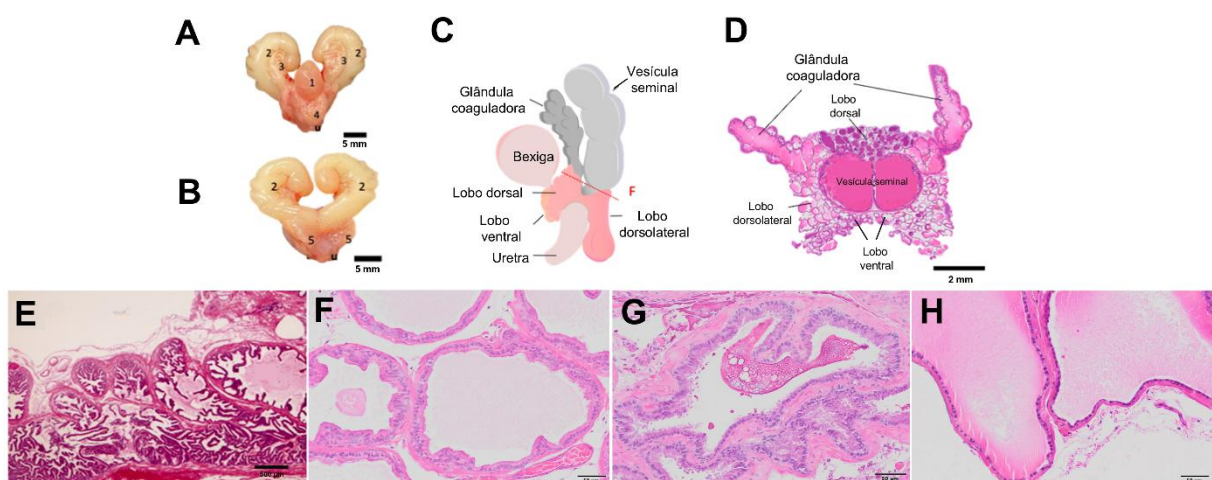
Ademais, a gestação na espécie *Meriones unguiculatus* dura de 24 a 26 dias e o desmame ocorre 3 semanas após o nascimento (LEONEL *et al.*, 2017). O desenvolvimento do gerbilo compreende as fases impúbere (1–14 dias pós-natais), pré-puberal (14–42 dias pós-natais) e púbere (a partir de 42 dias pós-natais), atingindo a maturidade sexual aos 90 dias pós-natais (PINTO-FOCHI *et al.*, 2014). A compreensão detalhada desses estágios é fundamental, pois cada um deles corresponde a janelas de suscetibilidade, entendidas como períodos específicos do desenvolvimento nos quais o organismo apresenta maior sensibilidade a estímulos, agentes externos ou fatores ambientais (WRIGHT, 2017). Nestes períodos, ocorrem diferentes eventos biológicos cruciais, como mudanças dinâmicas nas taxas de crescimento, regulação hormonal e metabólica, diferenciação celular e tecidual, bem como o desenvolvimento do sistema imunológico (WRIGHT, 2017). Considerando estes aspectos, quaisquer perturbações nestes estágios podem gerar impactos permanentes.

1.2.4 A próstata do gerbilo da Mongólia

O complexo prostático do gerbilo da Mongólia apresenta uma organização estrutural singular, composto por quatro lobos bilaterais simétricos, anatomicamente e histologicamente distintos (ventral, lateral, dorsal e anterior) (GOES *et al.*, 2007). O lobo anterior é também chamado de glândula coaguladora (Figura 5 A-D). Os lobos apresentam arquitetura histológica similar, mas diferem em alguns aspectos. O lobo

anterior apresenta ácinos e ductos organizados em túbulos ramificados com lúmen amplo (Figura 5E) (ROCHEL *et al.*, 2007). O lobo ventral se distingue dos demais por apresentar uma organização mais frouxa do tecido estromal e células musculares lisas mais finas (Figura 5F). As estruturas acinares do lobo dorsal apresentam menor diâmetro quando comparadas aos demais lobos e são circundadas por um estroma denso, que apresenta numerosas células musculares lisas e abundante secreção luminal (Figura 5G). O lobo lateral ou dorsolateral é envolto por estroma fibromuscular relativamente fino, rico em fibras colágenas e musculatura lisa, que contribui para a contratilidade e a ejeção do fluido prostático (5H) (ROCHEL *et al.*, 2007). Estruturalmente, a próstata do gerbilo da Mongólia apresenta-se como uma glândula túbulo-alveolar, com ácinos compostos por um epitélio simples e cúbico circundado por uma camada de músculo liso, vasos sanguíneos e fibras reticulares e colágenas que compõem o estroma fibromuscular (GOES *et al.*, 2007).

Figura 5. Imagens representativas da vista ventral (A) e dorsal (B) do complexo glandular seminal de gerbilos da Mongólia machos: 1, bexiga urinária; 2, vesícula seminal; 3, glândula coaguladora; 4, lobo ventral da próstata; 5, lobo dorsal da próstata; u, uretra. Figura esquemática representando as regiões da glândula desta espécie em uma vista lateral (C); vista panorâmica de um corte histológico do complexo prostático (D). Secções histológicas representativas dos lobos anterior (E), ventral (F), dorsal (G) e dorsolateral (H) de gerbilos da Mongólia machos. Coloração: Hematoxilina-Eosina (HE). Barras de escala: 500 µm (E), 50 µm (F, G, H).



Fonte: Extraído e adaptado de Ruiz *et al.* (2023b) (A-D); extraído e adaptado de Rochel *et al.* (2007) (E); arquivos pessoais (F, G, H).

Além da próstata masculina, a espécie apresenta próstata feminina funcional altamente responsiva a esteroides sexuais, o que amplia as possibilidades experimentais para investigar plasticidade tecidual, envelhecimento e dimorfismo sexual no eixo reprodutivo (BEDOLO *et al.*, 2023; SANTOS *et al.*, 2006). A próstata do gerbilo possui alta responsividade hormonal, além de responder precisamente à ação de DEs, exibindo alterações morfofuncionais reprodutíveis que apoiam seu uso em estudos de regulação endócrina e de modulação hormonal (BIANCARDI *et al.*, 2012; GRIGIO *et al.*, 2024; ZUCÃO *et al.*, 2024). No contexto patológico, o modelo apresenta desenvolvimento espontâneo e induzido de lesões associadas ao envelhecimento (CAMPOS *et al.*, 2008). Além disso, a alta sensibilidade da próstata a exposições a DEs em períodos críticos do desenvolvimento, torna essa espécie um modelo valioso para compreender as consequências de perturbações hormonais em diferentes fases da vida (DE SOUZA *et al.*, 2025; LIMA *et al.*, 2015; SANCHES *et al.*, 2017).

1.2.5 Fisiologia e regulação hormonal da próstata

Fisiologicamente, a organogênese, o desenvolvimento, o crescimento e a função prostática são mediados por hormônios esteroides, principalmente por andrógenos como a testosterona e di-hidrotestosterona (DHT) (BEREKETOGLU *et al.*, 2021). A testosterona disponível é convertida em DHT pela ação das isoformas 1 e 2 da enzima 5 α -redutase, expressas pelas células epiteliais da próstata (SWERDLOFF *et al.*, 2017). O DHT é mais potente que a testosterona, sendo o principal andrógeno ativo na próstata (MARKS; MOSTAGHEL; NELSON, 2008; PARK *et al.*, 2022). A função androgênica na próstata é mediada pela ligação ao receptor de andrógeno (AR), um fator de transcrição dependente de ligante que pertence à superfamília de receptores nucleares (ERDMANN *et al.*, 2022). O AR exerce importante influência sobre a patofisiologia do câncer de próstata. Após a ligação do ligante, o AR sofre alterações conformacionais que promovem sua translocação do citoplasma para o núcleo, interação com elementos responsivos ao andrógeno no DNA e recrutamento de cofatores transcricionais, culminando na ativação de genes responsáveis por processos de proliferação, sobrevivência celular, metabolismo e secreção prostática (ZHOU; BOLTON; JONES, 2015). A ativação da via androgênica é determinante para a transformação neoplásica inicial, sustentando a proliferação das células malignas e promovendo a progressão tumoral, o que, em alguns casos,

leva à adoção de terapias antiandrogênicas na tentativa de tratar e controlar neoplasias prostáticas (SEVERSON *et al.*, 2022).

Embora os andrógenos sejam os principais responsáveis pela regulação molecular e fisiológica da próstata, os estrógenos e as vias estrogênicas também desempenham um papel crucial no controle hormonal da próstata. Os receptores de estrógeno alfa (ER α) e beta (ER β) são distribuídos de diferentes formas no epitélio e no estroma prostáticos. O ER α pode ser encontrado em maior abundância no estroma, mas pode ser detectado nas células epiteliais, sobretudo em condições de proliferação celular e hiperplasia (SHEN *et al.*, 2019). Seu papel oncogênico está bem descrito na literatura, uma vez que atua mediando efeitos proliferativos, inflamatórios e tumorigênicos no tecido prostático (YEH *et al.*, 2014). O ER β localiza-se principalmente nas células epiteliais (basais e luminais), tanto na próstata saudável quanto em condições patológicas (CHRISTOFOROU; CHRISTOPOULOS; KOUTSILIERIS, 2014). O papel do ER β está direcionado à pró-diferenciação, exercendo também atividades anti-inflamatórias e antiproliferativas. Algumas isoformas funcionais estão diretamente associadas a efeitos supressores de tumor, induzindo apoptose e reduzindo invasividade celular (CHEN; LI; OU-YANG, 2022).

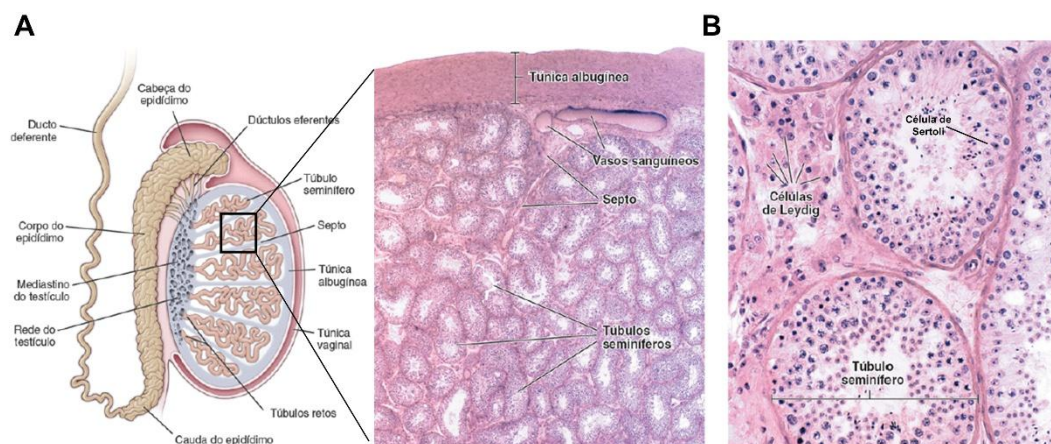
1.3 OS TESTÍCULOS

1.3.1 Aspectos anatômicos e histológicos dos testículos

Nos mamíferos, os testículos constituem as gônadas masculinas responsáveis pela produção de gametas e hormônios esteroides, exercendo funções fundamentais na reprodução e no dimorfismo sexual (THARMALINGAM; JORGENSEN; MITCHELL, 2018). Anatomicamente, os testículos são órgãos pares, ovais, localizados no interior do escroto, ligados ao epidídimo pelos ductos eferentes. Histologicamente, são envolvidos por uma cápsula de tecido conjuntivo denso denominada túnica albugínea, da qual partem septos que compartimentalizam o parênquima em lóbulos (ROSS; PAWLINA, 2016). Cada lóbulo contém de 1 a 4 túbulos seminíferos, estruturas altamente enoveladas onde ocorre a espermatogênese, processo que envolve a diferenciação progressiva de células germinativas desde espermatogônias até espermatozoides maduros (ROSS; PAWLINA, 2016) (Figura 6A). O epitélio seminífero é formado principalmente por células germinativas em diferentes estágios e pelas células de Sertoli, que sustentam, nutrem e regulam o microambiente intratubular,

além de comporem a barreira hematotesticular (GOMES-SILVA *et al.*, 2023). No interstício testicular localizam-se as células de Leydig, responsáveis pela síntese de testosterona, hormônio essencial para a manutenção da espermatogênese e para o desenvolvimento dos caracteres sexuais secundários (ROSS; PAWLINA, 2016) (Figura 6B). A interação coordenada entre células germinativas, de Sertoli e de Leydig, mediada pelo eixo hipotálamo-pituitário-gônadas (HPG), garante a produção eficiente dos gametas, bem como a homeostase endócrina nos machos (FISCHER; EHLERT; AMIEL CASTRO, 2019). Assim, os testículos constituem órgãos centrais para a perpetuação da espécie e para a manutenção da função reprodutiva masculina.

Figura 6. Representação esquemática do testículo humano, indicando as principais estruturas e compartimentos (A). Fotomicrografia representativa do testículo humano, evidenciando células de Leydig e de Sertoli (B).



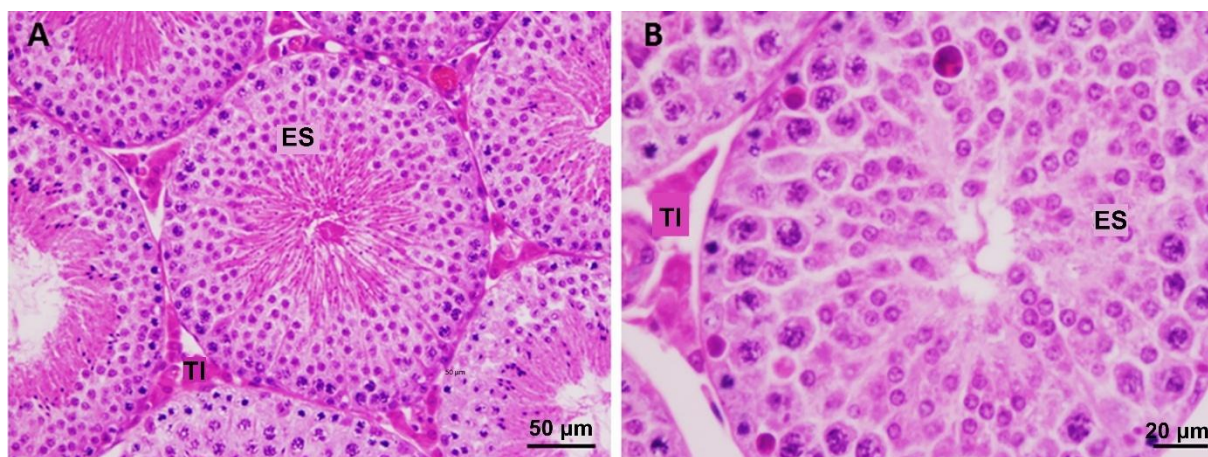
Fonte: Extraído e adaptado de Ross e Pawlina (2016)

1.3.2 Os testículos do gerbilo da Mongólia

A morfologia testicular de *M. unguiculatus* apresenta semelhanças com outros modelos murinos. Em roedores desta espécie, o desenvolvimento testicular se inicia com a formação do broto gonadal a partir da crista urogenital e a subsequente diferenciação das células somáticas, que se diferenciam em células de Sertoli e células de Leydig intersticiais (SEGATELLI *et al.* 2002). Durante o período impúbere, os testículos dos gerbilos apresentam predominância de gonócitos, os quais deixam de ser visíveis no 14º dia pós-natal. O período pré-puberal ocorre entre os dias 14 e 21. Contudo, a partir do 21º dia, notam-se os primeiros espermatócitos, período no qual a meiose começa a ocorrer (PINTO-FOCHI *et al.*, 2016). A espermatogênese

inicia-se na segunda semana de vida e a maturidade sexual ocorre aos 90 dias pós-nascimento (PINTO *et al.*, 2010). A duração média de cada ciclo do epitélio seminífero é determinada em $10,6 \pm 1,0$ dias, e a duração total da espermatogênese é de aproximadamente 47,5 dias. No entanto, a espermiogênese dos gerbilos da Mongólia é dividida em 12 estágios, sendo mais curta do que em ratos e camundongos, que possuem 19 e 16 estágios, respectivamente (SEGATELLI *et al.*, 2002). O início da puberdade ocorre no 42º dia, quando as células de Leydig maduras que expressam 17 β -HSD estão presentes (RUIZ *et al.*, 2023b). Em indivíduos adultos, o epitélio seminífero do gerbilo da Mongólia é composto por camadas concêntricas de espermatogônias, espermatócitos e espermátides. Os túbulos seminíferos são compostos de epitélio germinativo limitado basalmente por uma camada de células mioides e organizados por um tecido conjuntivo frouxo que se conecta à túnica albugínea (SEGATELLI *et al.*, 2002) (Figura 7).

Figura 7. Secções histológicas da organização estrutural dos testículos de *Meriones unguiculatus* coradas com HE em visão panorâmica (A) e detalhada (B), evidenciando o epitélio seminífero (ES) e o tecido intersticial (TI).



Fonte: Extraído e adaptado de Negrin *et al.* (2018)

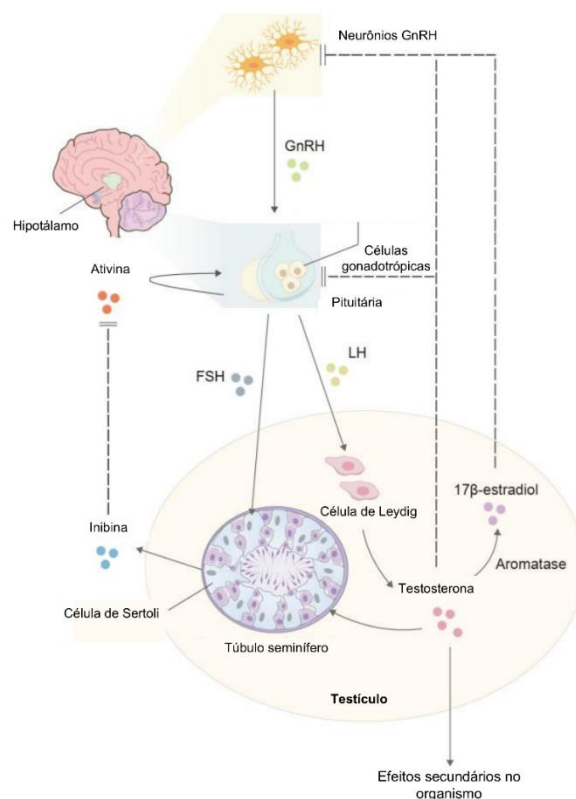
Os estudos que investigam o desenvolvimento testicular de *M. unguiculatus* têm destacado a sensibilidade desse roedor à exposição perinatal a DEs, tornando-o um modelo relevante para compreender os efeitos dessas substâncias sobre a biologia reprodutiva. Pesquisas experimentais têm demonstrado que compostos como ftalatos, óleos vegetais e outros agentes com ação estrogênica ou antiandrogênica podem interferir de modo significativo na diferenciação das células germinativas, na organização dos cordões seminíferos e na esteroidogênese fetal e neonatal (NEGRIN

et al., 2018). Além disso, alterações como atraso na migração e maturação dos gonócitos, remodelamento anormal do epitélio seminífero, alterações no número e na função das células de Sertoli e Leydig, além de modificações em vias hormonais dependentes de andrógenos, têm sido observadas nesse modelo experimental (CHRISTANTE *et al.*, 2018). Em conjunto, esses achados reforçam que o gerbilo da Mongólia apresenta vulnerabilidade particular às perturbações ambientais, permitindo a identificação de janelas críticas de desenvolvimento testicular e contribuindo para a elucidação dos mecanismos pelos quais desreguladores endócrinos comprometem a reprodução masculina em mamíferos.

1.3.3 Controle hormonal nos testículos

A reprodução envolve a integração de sinais hormonais que atuam em vários sistemas para gerar uma resposta fisiológica sincronizada. O controle hormonal testicular ocorre sob influência do eixo HPG. Os neurônios hipotalâmicos secretam o hormônio liberador de gonadotrofinas (GnRH), que estimula a adeno-hipófise a liberar os hormônios folículo estimulante (FSH) e luteinizante (LH) (SILVERTHORN, 2017). O LH atua sobre os receptores LHCGR localizados nas células de Leydig, promovendo a produção de testosterona via sinalização cAMP/PKA e esteroidogênese. Após ser sintetizada, a testosterona é parcialmente ligada à proteína de ligação ao andrógeno (ABP) para transporte, ligando-se posteriormente ao AR nas células de Sertoli e outras células somáticas testiculares como células de Leydig e células mioides peritubulares, sendo essencial para espermatogênese, diferenciação germinativa e integridade da barreira hematotesticular (LEI; YANG; YANG, 2025; SILVERTHORN, 2017). Já o FSH liga-se ao receptor FSHR expresso nas células de Sertoli, estimulando sua proliferação. As células de Sertoli também secretam o hormônio antimülleriano (AMH), que desempenha um papel fundamental na regressão das estruturas reprodutivas femininas durante o desenvolvimento masculino (LI *et al.*, 2024; SILVERTHORN, 2017). Desequilíbrios nesses hormônios podem levar à redução da fertilidade, à infertilidade ou a doenças do sistema reprodutivo. Esses hormônios regulam, ainda, um sistema de retroalimentação negativa, uma vez que a testosterona, produzida pelas células de Leydig em resposta ao LH, e a inibina B, secretadas pelas células de Sertoli em resposta ao FSH, suprimem, respectivamente, GnRH/LH e FSH, garantindo a manutenção e homeostase do eixo HPG (SILVERTHORN, 2017) (Figura 8).

Figura 8. Controle hormonal do eixo HPG e da função reprodutiva masculina. O hipotálamo regula a síntese e a secreção dos hormônios adeno-hipofisários LH e FSH através do GnRH. O LH induz as células de Leydig a secretar testosterona, o que reduz a produção de GnRH e LH por meio de feedback negativo. O FSH atua nas células de Sertoli para induzir a secreção de inibina, que, por sua vez, inibe a produção de FSH. As células de Sertoli, sob a ação combinada da testosterona e do FSH, estimulam a proliferação e a maturação das células germinativas. A espermatogênese normal depende da ação coordenada de todos esses hormônios.



Fonte: Extraído e adaptado de Li *et al.* (2024)

1.3.4 Metabolismo testicular

O metabolismo e a bioenergética testicular são considerados um conjunto de processos bioquímicos responsáveis pelo fornecimento energético e de substratos essenciais para o funcionamento das células somáticas e germinativas dos testículos, a fim de garantir a manutenção da esteroidogênese e espermatogênese (RATO *et al.*, 2012; SILVA *et al.*, 2022). Particularmente, o metabolismo testicular apresenta singularidades que o diferenciam de outros órgãos. Transportadores específicos,

conhecidos como GLUTs, permitem a entrada de glicose através da bicamada lipídica da célula, que sofre uma série de conversões bioquímicas até formar o piruvato (DIAS *et al.*, 2014). Nas células de Sertoli, a via glicolítica assume papel central para sustentar a maturação das células germinativas, uma vez que, em condições fisiológicas, o piruvato gerado nesse processo é convertido em lactato pela enzima lactato desidrogenase (LDH) (CARRAGETA *et al.*, 2024). Esse lactato é então liberado para o fluido luminal, onde atua não apenas como substrato energético essencial para as células germinativas em desenvolvimento, mas também como um modulador com efeito antiapoptótico. Paralelamente, os lipídios exercem funções igualmente relevantes, fornecendo a principal fonte de energia para as células de Sertoli, além de contribuir como precursores estruturais indispensáveis à formação das membranas das células germinativas (CARRAGETA *et al.*, 2024). A integridade lipídica é, portanto, essencial para a saúde reprodutiva (JARAK *et al.*, 2018). As vitaminas e os aminoácidos exercem importantes papéis na defesa antioxidante testicular para controlar níveis excessivos de espécies reativas de oxigênio (EROs), formadas pelo metabolismo celular aeróbio (CABRITA *et al.*, 2011). As EROs, por sua vez, podem causar efeitos celulares e moleculares adversos, como alteração da fluidez das membranas, danos à integridade do DNA, oxidação de proteínas, danos mitocondriais, os quais resultam em prejuízos à motilidade espermática e à fertilidade (BUCAK *et al.*, 2007; CABRITA *et al.*, 2011). Considerando os aspectos mencionados, a regulação coordenada desses processos depende de sinais hormonais e de fatores endógenos intrínsecos, e quaisquer alterações nesse equilíbrio podem comprometer a espermatogênese e resultar em prejuízos à fertilidade masculina.

1.4 LIMITAÇÕES E JUSTIFICATIVA

A exposição desenfreada a DEs tem sido uma das causas que levam à diminuição da saúde e do potencial reprodutivo de uma ou mais gerações, afetando, sobretudo, órgãos hormônio-responsivos como os testículos e a próstata. O gerbilo da Mongólia (*Meriones unguiculatus*) vem se mostrando um modelo experimental sólido em estudos envolvendo a ação de DEs, por apresentar características morfofisiológicas que guardam notável semelhança com os humanos, incluindo a organização estrutural da próstata e a dependência hormonal dos tecidos reprodutivos. A espécie também demonstra elevada responsividade a variações nos níveis de andrógenos e estrogênios, o que a torna particularmente útil para avaliar os efeitos de compostos

desreguladores endócrinos sobre a espermatogênese e a homeostase prostática. Assim, o emprego desta espécie em estudos morfofisiológicos possibilita uma ampla compreensão dos mecanismos de toxicidade reprodutiva, permitindo também a extrapolação de potenciais riscos da exposição a DEs em diferentes estágios da vida de humanos. Apesar dos esforços, há uma carência de estudos na literatura que investiguem os efeitos da exposição a DEs em estágios críticos do desenvolvimento, em múltiplas gerações, e que considerem a ocorrência de misturas destas substâncias no ambiente. Neste contexto, o presente trabalho objetiva investigar os impactos da exposição a diferentes DEs na próstata e nos testículos de gerbilos da Mongólia submetidos a múltiplas condições experimentais, através da utilização de técnicas moleculares, morfológicas, imunohistoquímicas e metabolômicas. O estudo morfofisiológico da próstata e dos testículos nos permitirá compreender os mecanismos de toxicidade envolvidos na exposição a DEs, bem como os impactos na função prostática e testicular. Com os achados deste estudo, esperamos agregar conhecimento consistente que possibilite compreender e avaliar os efeitos sobre a saúde reprodutiva, além de estabelecer relações dose-resposta, fornecendo subsídios para políticas públicas, regulamentações ambientais e estratégias preventivas que visem proteger a saúde reprodutiva das populações expostas.

2. ARTIGO 1: IMPACTS OF HEAVY METAL EXPOSURE ON THE PROSTATE OF MURINE MODELS: MECHANISMS OF TOXICITY

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O presente artigo segue as normas do periódico.

Impacts of heavy metal exposure on the prostate of murine models: mechanisms of toxicity

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Abstract

Heavy metals are elements found into the environment mainly due to anthropogenic activities. Naturally occurring and higher released doses cause disorders in the prostate, which depends on appropriate hormonal regulation, and exposure to heavy metals may impair prostate homeostasis. The current work highlighted the main mechanisms of toxicity of different environmental heavy metal contaminants, such as aluminum, arsenic, cadmium, chromium, lead, mercury, and nickel, and their impacts found in the prostate morphophysiology in murine models. The repercussions triggered by heavy metals on the prostate include hormonal imbalance and oxidative damage, leading to morphological alterations, which can vary according to the chemical properties of each element, exposure time and concentration, and age. The information of altered biological pathways and its impacts on the prostate of exposed murines are related to human outcomes being useful in the real context of human exposure.

Keywords: Heavy metal contamination; Endocrine-disrupting metals; Morphology; Prostate development.

Highlights

- The prostate of murine models is a target gland for heavy metal toxicity;
- Exposure to heavy metals induces mitochondrial dysfunction and inflammatory process;
- Heavy metal exposure increases the lipid peroxidation in prostate cells of murines;
- Expression of sex steroid receptors is altered during heavy metal exposure.

1. Introduction

1.1. Heavy metal contamination and toxicity

Heavy metals are defined as chemical inorganic elements which have a density greater than 5 g/cm³ [1]. They play important roles in living organisms, acting in oxygen transport, metabolic reactions, and vitamin synthesis, and their deficiency in the body can negatively impact biological systems [2]. Due to these properties, many of these metals are considered essential, such as iron, calcium, and zinc [3,4]. The current concern is restricted to non-essential and toxic heavy metals, such as aluminum, arsenic, cadmium, chromium, lead, mercury, and nickel, widely used by the mining and smelting industries, through mineral processing and metallurgical extraction [5,6]. For this reason, several non-target species have been affected due to heavy metal environmental contamination [7–10].

Particulate matters containing several heavy metals are frequently released into the environment through discharge of industrial and agricultural effluents, process derived from anthropogenic activity [7,11]. In addition, these particulate matters can accumulate and persist for a long time in the air, soil, and water [7,12–14]. The most important routes of exposure occur through inhalation of heavy metal contaminated-particles, skin contact, and the major sources of exposure are drinking water and consumption of contaminated food [9]. The molecular, morphophysiological and oxidative effects caused by heavy metal exposure on prostate and other tissues vary by chemical element, exposure time, bioavailability, excretion rate, and their toxicity depends on the concentration [15,16].

The cell toxicity of these chemicals initially occurs by the inactivation of antioxidant processes, after the excessive increase in reactive oxygen species (ROS) generation, oxidizing biomolecules and disturbing cell redox homeostasis [16,17]. The disturbance of the redox state, in turn, favors the occurrence of inflammatory processes that lead

to the appearance of tissue morphological alterations, proliferation, and finally, cell death [2,10].

Heavy metals act as endocrine disrupting chemicals (EDCs), because it has similar chemical structure with hormones, which enables interaction with specific receptors and activates endocrine signaling pathways [18–20]. Since the endocrine system responds strictly even at low hormone levels, any interferences in hormone and receptor synthesis and metabolism caused by exposure to EDCs are easily recognized and generate important physiological responses [21]. Thus, endocrine alterations can impair prostate cell homeostasis, leading to morphophysiological disturbances in the gland [22,23].

Heavy metals exposure also induce the transcription of genes that encode metallothioneins (MT's), an important group of cysteine-rich proteins found in the prostate and other peripheral tissues as MT-1 and MT-2 isoforms [24–26]. MT's isoforms are highly expressed in the dorsal and lateral lobes of the prostate, and their role is related to heavy metal detoxification, scavenging free radicals and ROS through binding of cysteine groups to metals, in order to maintain homeostatic control of the prostate [27–29].

Regarding repair and detoxification systems, the main changes generally caused by non-essential heavy metals in the male reproductive system of murine models include changes in sex steroid levels, loss of gametes, increase in sperm abnormality, decrease in sperm motility, damage to epithelial cells of seminiferous tubules, and infertility [23,30–32]. Exposure to heavy metals is also known to cause testicular damage, as these elements are easily permeated across the blood-testis barrier [33]. In females, endocrine-disrupting metals exposure is associated with an increase in the number of breast cancer cases [34] and deficiencies in the content of essential metals

during gestation [3]. Although studies provide evidence of the specific mechanisms of action of heavy metals and how they induce adverse effects on the reproductive axis, prostate studies are still scarce, despite of its medical-scientific interest.

Some studies have evaluated heavy metal toxicity in humans [35–37]. However, due to the many ethical limitations, there are few investigations in the prostate [38]. In animal research, canines are suitable in prostate studies for being the only mammal known to develop spontaneous prostate cancer that can metastasize to bone, as seen in humans [39]. Therefore, the use of murine models, a widely investigated group among mammals, stands out in scientific research due to their short life cycle, rapid sexual maturation, and morphological, physiological, and genetic similarities to humans, providing accurate information about human conditions [40].

1.2. The Prostate

The prostate is an accessory gland, which secretes an alkaline fluid to transport and provide viability to sperm, contributing to reproductive success [41–43]. Anatomically, the prostate of murine models is divided into the anterior, ventral, lateral, and dorsal, (or dorsolateral lobes), embedded in a connective tissue that make up the stromal component [39]. Histologically, the murine prostate is similar to that of humans, which is composed of acini, ducts, and the epithelium contains columnar luminal secretory, basal, and neuroendocrine cells that perform specific functions in the gland [44,45]. Great attention has been directed to the prostate due to increasing oncogenic transformation, which favors the emergence of neoplasia and cancer when compared to other organs, making prostate cancer one of the most commonly diagnosed cancers worldwide [46,47]. The increase in the incidence of prostate cancer in highly industrialized countries, suggests a correlation with the overuse of technology sources, inappropriate eating habits, and exposure to environmental contaminants [48].

The development and growth of the prostate are provided by paracrine epithelial-stromal interactions, with a high dependence on androgen signaling [49–51]. The androgenic hormones, especially testosterone and with strongest effects, dihydrotestosterone (DHT), bind to a specific androgen receptor (AR) and promote a variety of secretion products, besides playing an important role in prostate growth, contributing especially to maturation and adult reproductive maintenance [52]. On the other hand, estrogens play different roles in the prostate, mainly related to exaggerated cell proliferation and tissue disorganization [53]. Estrogenic effects on the prostate are mediated by the estrogen receptors alpha (ER α) and beta (ER β) [54]. ER α is primarily localized in the stroma, whereas ER β is localized in the epithelium of murine prostate, and both are related to cell proliferation and differentiation [55]. For these reasons, different outcomes were observed in the prostate after prolonged exposure to these hormones [56,57]. Therefore, contact with endocrine disruptors such as heavy metals, at different stages of development, may trigger a variety of intracellular signaling pathways, causing hormonal imbalance in androgen and estrogen activities, and harming normal gland functionality [58].

Based on the above, the current study reviewed the main impacts caused by non-essential heavy metal exposure (Aluminum, Arsenic, Cadmium, Chromium, Lead, Mercury, Nickel: elements that do not have a well-defined biological function and have a high degree of toxicity even at low concentrations), to investigate whether these chemicals interfere in the prostate morphophysiology in murine models subjected to different experimental conditions. Thus, we aim to provide recent information regarding the mechanisms of toxicity related to heavy metals and their impact on the murine prostate. Due to highly conserved and well-known prostate morphophysiology of these mammals, the exposure to heavy metals has similar effects applicable for humans.

2. How do different non-essential heavy metals affect the murine prostate?

2.1. Aluminum (Al)

Aluminum is the most abundant metal in the Earth's crust and is one of the major sources of contamination due to its high prevalence in the environment [59]. In general, exposure to aluminum occurs mainly through the consumption of food, cookware, use of antiperspirants and vaccines, which contain residues and specific aluminum salts in the composition [60–62]. The primary route of entry into cells occurs through cell membranes or epithelial cells via ion channels, endocytosis, or receptor-mediated, and its toxicity is similar for humans and other mammals [63,64]. In 2007, the tolerable weekly intake of Al for humans was adjusted to 1 mg/kg body weight [65]. Exposures to Al at 7 and 34 mg/kg were sufficient to cause inadequate development of prostate alveoli, due to a high level of pro-inflammatory cytokines, which promote leukocyte degranulation in the extracellular medium, and alter the development of the epithelium and stroma [66].

Regarding the prostate redox balance, Al acts as a pro-oxidant, since it has affinity for other metals, such as iron, through the catalytic Fenton reaction, which occurs in the presence of hydrogen peroxide (H_2O_2) and potentiates ROS production [59]. Overexposure to Al and its metabolites impairs the antioxidant activity, leading to a redox imbalance. Al contributes to cellular function decline and favors the onset of chronic diseases and pathologies derived from oxidative stress [67,68], despite powerful enzymatic and non-enzymatic antioxidant systems to neutralize ROS.

Exposure to aluminum chloride (AlCl_3) may also affect the prostate in an indirect cascade. AlCl_3 intoxication can promote the formation of nitric oxide (NO) in the prostate, which at low concentrations may act as a pro-tumorigenic agent, activating factors related to angiogenesis, migration, and cell invasion [69]. In the testis,

increasing tissue levels of nitric NO, an important mediator of testicular function, promote a decrease in testosterone production [70,71]. These findings reflect in prostate development and function, due to their dependence on androgen signalization, especially in the early stages of development [66]. AlCl_3 interrupted the ductal branching pattern, mainly in male prostate, and increased abnormal cell proliferation [72]. The main mechanism affected by AlCl_3 is the expression of AR and $\text{ER}\alpha$ receptors in male and female prostate of the Mongolian gerbil. According to the authors, perinatal exposure modulates the expression of AR and $\text{ER}\alpha$, modifying receptor expression, and changing patterns of prostate development [52,72]. Furthermore, these impacts demonstrate a sex-specific response after AlCl_3 exposure, leading to permanent changes that remain until adulthood [52]. These effects demonstrate a metalloestrogenic action to this metal, mimicking the action of estrogen, leading to a process of cell proliferation and inflammation in the Mongolian gerbil prostate at different stages of life [52,72,73] (Figure 1).

2.2. Arsenic (As)

Arsenic is a metalloid endocrine-disrupting chemical element, naturally found in more than 200 mineral forms, including arsenites and oxides, and widely released into the environment due to uncontrolled mining and smelting [74,75]. Inorganic arsenite (iAs) is the main arsenic-derived contaminant found in drinking water, and the two most toxic oxidation states are trivalent [iAs(III)] and pentavalent [iAs(V)] [76]. As a result, the Food and Agriculture Organization (FAO) has established a maximum safe level of 0.1 mg/kg of arsenic in mineral and natural waters [77].

Arsenic initially enters the cell via membrane transporters, and can accumulate in tissues, especially in the prostate, if there is an imbalance in its excretion [78]. One of the main effects of cellular damage in the prostate is related to increased levels of

malondialdehyde (MDA), an important marker of lipid peroxidation, which suggests direct damage to membrane lipids. In addition, the metabolic processes involving the reduction in iAs(V) deplete reduced glutathione (GSH), an important xenobiotic detoxifying molecule, impairing the activity of proteins and enzymes that control DNA repair mechanisms [79]. Regarding a genotoxic assignment, As exposure promotes epigenetic changes, such as DNA methylation, sister chromatid exchange, telomere dysfunction, and DNA strand breaks [78]. All of these reported DNA alterations are associated with genomic instability and increased vulnerability to cancer development [80,81] (Figure 2).

Sodium arsenite (NaAsO_2) exposure in early life (from the 23rd to the 53rd day of life) decreases the expression of AR, as well as prostatein (protein dependent on androgen and important for the maintenance of prostate function) in male Wistar rats. The lowest tested concentration of NaAsO_2 (0.01 mg/L) was able to reduce collagen fibers, cell proliferation, and secretory epithelium height. These findings indicate impairment in prostate function, which may lead to infertility in pubescent males [82]. Thus, the difficulty of prostate homeostasis resumption increases the chance of the emergence of pathologies associated with exposure to As [83].

2.3. Cadmium (Cd)

Cadmium is a metal that is widely found naturally in the earth's crust combined with other heavy metals, at concentrations of 0.1-1 ppm [84]. Exposure occurs through drinking water, industrial and agricultural residues, smoking, contaminated food intake, and environmental contamination [85]. After exposure and absorption, Cd is retained in the liver and kidneys, where it can remain for a long time, as its long biological half-life can range from 10 to 30 years [86]. The established maximum level for Cd in mineral and natural waters is 0.003 mg/kg, according to the FAO [77]. However,

plasma Cd levels in the human population range from 0.4 to 4 µg/L and >10 µg/L in areas with high environmental contamination [87].

The main toxicological effects of Cd in murine prostate include changes in the oxidative status through increased production of ROS and free radicals, leading to disturbances in the enzymatic antioxidant system [88]. Cd interacts with membranes and disturbs the mitochondrial and peroxisome functions, by increasing lipid peroxidation and suppression of the antioxidant enzymes, especially from the glutathione family, which form complexes, inducing oxidative stress [89]. In addition, exposure to 0.2 mg/kg of cadmium chloride (CdCl₂) increased thiobarbituric acid reactive substances (TBARS) in Wistar rat prostate, an important product formed during lipid peroxidation [88]. Antioxidant machinery was also depleted by Cd, promoting a decrease in antioxidant enzyme activity, such as superoxide dismutase (SOD), catalase (CAT), and GSH, which impacts in tissue antioxidant protection [90,91]. In addition, ROS generation is related to DNA degradation, as well as harming the synthesis of proteins and nucleic acids [92].

Besides the oxidative damage, morphological alterations in the prostate were observed in Wistar rats exposed to a non-carcinogenic dose (15 ppm). The decrease in the epithelial cell height and the presence of invaginations in the lumen were associated with morphological alterations after Cd exposure [16]. These features were compared to castrated prostate and were related to an increase in IGF-B5 expression, which inhibits prostatic growth factor (IGF-I) activity and promotes gland involution [16]. Furthermore, the histological lesions and alterations found in the murine prostate exposed to Cd are associated with inflammatory response and its mediators [51,93]. The release of chemokines, such as cyclooxygenase-2 COX-2 and 5-lipoxygenase

(LOX), and the production of inflammatory subproducts, as well as prostaglandins and leukotrienes, resulted in inflammatory process progression [94].

Cd exposure is also associated with genotoxicity and mutagenicity, which can induce epigenetic changes, modification in gene expression, strand breaks and impaired DNA repair [95,96]. In addition, Cd exposure is related to DNA hypermethylation by up-regulation of methyltransferases, enzymes that control DNA methylation during the process of Cd-induced carcinogenesis [97].

Cd access to cells is favored by a mimicking property of this metal in the binding site of zinc (Zn) transporters (ZnT1-ZnT10; SLC30A), impairing zinc homeostasis [98]. Zn is an essential metal for the prostate and other male reproductive organs [99], involved in the genetic expression of steroid receptors [100]. Thus, Zn deficiency due to Cd exposure induces proliferation of malignant cells, causing mitochondrial disturbances, which provide an energy resource for these cells, in addition to altering the expression of sex steroid receptors that control gland development [100,101] (Figure 3).

2.4. Chromium (Cr)

Chromium is widely used in steel and alloy production, and contamination is mainly through consumption of contaminated drink and food, and occupational exposure [102,103]. The United States Environmental Protection Agency (US EPA) established the maximum allowed safe value of 100 µg/L total Cr in drinking water [104]. However, previous studies have shown that humans are exposed to higher concentrations of Cr, posing a reproductive health concern [105,106].

Cr has trivalent [Cr(III)] and hexavalent [Cr(VI)] oxidized forms that have important biological properties, as they interact with intracellular macromolecules, and cause carcinogenesis and genotoxicity effects [107]. CrVI presents high cellular permeability

and oxidizing potential, initiating exaggerated production of ROS, which releases secondary messengers that activate cellular mechanisms, such as apoptosis and antioxidant defense mediated by NF- κ B, AP-1, and Nrf2 [2,108].

Cr exposure can also cause structural alterations in the membrane, disrupt cellular functions and integrity, react with histones, and finally cause DNA damage [109]. Once inside the cell, CrVI can be reduced to CrIII by reacting with the superoxide radical to form the hydroxyl radical, which reacts with guanine residues and creates a variety of potentially DNA damaging radical adducts [110]. Although some studies indicate that Cr alters the weight of the rat prostate, causing an increase in prostate weight on exposure at 1 and 2 mg/kg [111], and a decrease at a dose of 0.5 mg/kg [112], the data available in the literature on the mechanisms of toxicity of this metal in the prostate are still scarce (Figure 4).

2.5. Lead (Pb)

The main route of Pb exposure occurs occupationally through the manufacture of ammunition, batteries, and military and surgical equipment [113]. After exposure, Pb binds to erythrocytes and is distributed through tissues, having a great affinity for bone, where it accumulates easily, remaining there for up to 30 years [114]. The mechanisms of Pb toxicity include the substitution of important bivalent cations (e.g. Mg^{2+} and Fe^{2+}), associated with cell biological processes, such as cell division, protein crosslinking, enzyme regulation, and neurotransmitter release [2]. Although the maximum limit for Pb in natural and mineral waters is 0.001 mg/kg [77], there is evidence of Pb accumulation in toenails and hair (3.8-10.1 μ g/g), and breast milk (36 mg/L) [115].

In the reproductive system, even at low concentrations, Pb exposure is also associated with male reproductive dysfunction related to the hypothalamic-pituitary-gonadal axis (HPG) [116]. Although the mechanisms related to prostate exposure to

Pb are unclear and scarce, indirect alteration in reproductive-related organs could promote significant repercussions in this organ. Mice [117] and Wistar rats [118] exposed to high doses of lead acetate (PbAc), showed a decrease in ventral prostate weight, related to a decrease in systemic testosterone levels. In addition, combined exposure to more than one type of heavy metal can result in different changes in the prostate. Exposure to PbAc and NaAsO₂ in adulthood promotes a drastic decrease in prostate weight, suggesting possible fertility impairment [118].

A mechanistic explanation for this finding is that a synthetic molecular response to this alteration could be related to imbalance of androgens and estrogens in murine models exposed to Pb. Its exposure can decrease the mRNA expression of some cytochrome P450 enzymes, such as *cyp19*, increasing androgen levels [94]. As previously mentioned, estrogens are responsible for health homeostasis of the prostate in murines by contrasting the androgenic pathological response and promoting a paracrine signalization mechanism of epithelial-stromal interaction [119,120]. Thus, the imbalance of androgen/estrogen sensitization in the prostate, related to its own receptors, should be investigated to better understand its modulation in the prostate (Figure 5).

2.6. Mercury (Hg)

Mercuric chloride (HgCl₂) is considered a toxic form of Hg due to its ability to form complexes with biomolecules, such as GSH, and it is therefore a substance widely used for scientific research purposes [31]. Although the mechanisms underlying the reproductive toxicity of mercury are not well understood, it is already known that exposure, at a concentration similar to 1 mg/kg/day, causes oxidative changes through increased production of ROS, followed by depletion of antioxidant enzymes and

histopathological alterations in rat testis, in addition to impairment in the neuroendocrine system [30,121].

In the prostate, Hg exposure appears to cause the depletion of thiols, that trigger the production of ROS and promote the inactivation of enzymes involved in the antioxidant defense system [122]. Changes in oxidative balance were reported at low concentrations of HgCl₂ (4.6 µg/kg and 0.7 µg/kg) by an increase in MDA levels, followed by an increase in SOD antioxidant enzyme activity in rats [31]. Thus, damage to membrane lipids and prevention of the activation of an important enzyme of the first line of antioxidant defense have been linked to Hg exposure. However, the increase in SOD can be interpreted as an attempt to mitigate this redox imbalance. In part, these effects may be related to other male reproductive changes found in this study [31]. Furthermore, Hg promoted inactivation of antioxidant cytochrome P450 aromatase enzymes which interferes in the biosynthesis and signaling pathway of sex hormones that regulate prostate activity [30] (Figure 6).

2.7. Nickel (Ni)

Nickel, like other metals, is an environmental and occupational toxicant, which humans are exposed to through contact with materials containing Ni in their composition, such as jewelry, cooking utensils, and surgical prosthetics [105,123]. The estimated human exposure dose is 0.3-0.6 mg [124]. Although some research points to the essential role of Ni in growth, development, and reproductive performance [23,125,126], excessive exposure can cause several cellular and tissue disorders in the murine reproductive system [105]. The toxicity of nickel, as well as its effects on cancer development, are based on its ability to block calcium channels. Differences in calcium concentrations in the intra- and extracellular environment alter gene expression levels that control cell growth and apoptosis processes [110].

Concentrations below half of the maximum effective concentration (5 mg/kg) caused important changes in the prostate morphology of male Wistar rats. Hyperplasia in basal and epithelial cells and epithelial degeneration were associated with exposure to nickel oxide nanoparticles (NiONPs) and nickel oxide microparticles (NiOMPs) [127]. However, according to the authors, these data are not comparable, since there are no similar studies. In contrast, exposure to 5 and 10 mg/kg/day of nickel sulfate in adult male albino mice caused a reduction in the absolute and relative weight of the prostate but did not cause any histopathological changes in the gland [128]. Despite demonstrating conflicting data, the authors suggest that the absence of these alterations is explained by the degree of nickel sulfate accumulation in these animals, which occurs as follows: Epididymis > Testis > Seminal vesicle > Prostate (Figure 7). In this way, Ni repercussions in the prostate molecular pathways remain unclear until now.

3. Data sources and search strategy

To assess the state of knowledge about the exposure of different heavy metals in the prostate of murine models, we performed a bibliographic search on the main platforms (PubMed, Scholar Google, Scielo, Scopus), using several keywords: (endocrine dysregulation, heavy metal, murine, prostate, reproduction, reproductive dysfunction, toxicology) and their combinations. We selected articles (N=17) that met the main criteria = 1: written in English, 2: published in the last 11 years, between 2012-2023, and 3: that studied the effects of heavy metals on the prostate of murine models. This review aims to analyze recently published studies, highlighting the general outcomes, as shown in table 1.

4. Final remarks and conclusion

The papers presented in this review point to the impacts caused by exposure to non-essential heavy metals (Al, As, Cd, Cr, Mg, Pb, and Ni) on the prostate of murine models, which provide recent theoretical support for the risks of human exposure. In addition, the doses employed in the studies, for the most part, are environmentally relevant and match the real human exposure context. We note that although other organs of the reproductive system are also affected, the prostate is an important target gland for toxicity of these metals and their metabolites, with attention to the damages related to reproduction and development.

The present review included recent studies that evaluated the impacts caused by exposure to different non-essential heavy metals on the prostate of murine models, which presented a correlation with histological alterations, oxidative imbalance, and endocrine dysregulation. These effects were verified in the male and also in the female prostate, both with important functions for this mammalian group, and the impacts caused by heavy metals differed according to the sex and life stage.

Table 1 shows that some heavy metal studies have focused on critical developmental windows, such as perinatal. The accumulation and transfer of heavy metals across blood-placental barrier, mediated by specific transporters present in the syncytiotrophoblast, reach the fetus and cause anomalies that may extend into adult life [129–131]. The development of the prostate of murine begins late in the fetal period, and heavy metal exposure at this critical stage can cause disturbances in the maternal hormonal environment, leading to structural changes and malignant transformations in the fetal prostate [51,132].

Herein, we showed that the general outcomes found in the prostate presented similarity among the animals used in the studies. It is worth note that all the studies reviewed were focused on the ventral prostate, although murine models have three

other histologically distinct regions [39]. This lobe is highly responsive to exposure to heavy metals and other endocrine disruptors, being the main site of the development of proliferative lesions[42,133]. On the other hand, the dorsolateral anterior lobes are related to prostate cancer [134]. In addition, the ventral lobe has more premalignant and malignant lesions in adult subjects, with a longer tumor latency period than the dorsolateral lobe [135].

According to the majority of the studies analyzed, we observed that the main alterations found in the prostate of murine models are morphological. In several studies, exposure to heavy metals was shown to alter body and prostate weight. The decrease in body weight may be associated with a systemic biological effect [66,136] and is an important indicator of the harmful effects of heavy metal exposure on the animal's health [52]. On the other hand, the decrease in prostate weight frequently observed after different exposures may be related to a local effect exerted by metals, which in turn may also correlate with antiandrogenic activity, possibly caused by a decrease in testosterone and DHT levels, another common finding in the evaluated studies, which leads to a consequent reduction in gland weight [137].

Each heavy metal was shown to exert a different effect on prostate biology, although the majority cause an imbalance in hormone regulation, which is important and necessary for normal gland function. Most of the studies evaluated the impacts caused by heavy metals on reproductive hormones. Some metals like copper also alter the structure and form complexes with GnRH and neurokinin B, a neuropeptide that indirectly controls GnRH release. In turn, there are increase in the levels of LH and FSH released by the gonads [138]. On the other hand, other heavy metals can inhibit hypothalamic GnRH secretion by binding paracrine prostaglandin (PGE_2) to EP1 and

EP2 receptors on GnRH neurons, consequently decreasing LH and FSH secretions [139].

In this sense, several studies demonstrated that exposure to heavy metals, at different stages of development, tested concentrations, and routes of exposure, provided a favorable environment for endocrine dysregulation via the HPG axis, promoting fluctuation in serum androgen levels and the expression of their receptors in males and females. Low testosterone serum levels are associated with androgen deficiency, which may reflect in other aspects, such as decreased prostate weight, indicating a possible involution process and gland atrophy [140]. On the other hand, high testosterone levels may alter prostate branching morphogenesis patterns and cause androgenization [72].

A common finding noted in table 1 is with respect to the frequency of positive cells for steroid sexual hormone receptors, such as AR, and ER α /ER β , which can be explained by the similarity between heavy metals and sex hormones [140]. Cd has a high affinity for the hormonal binding site of ER α , inducing an increase in the proliferation of estrogen-dependent cells and transcription of target genes. These characteristics make it a metalloestrogenic agent [141]. Similar properties have been reported for Al [73,142,143].

Important alterations were also reported in the morphology of the prostate. Incidences of hyperplasia, inflammation, cell proliferation, and changes in the proportions of prostate compartments, such as epithelium, lumen, and stroma, were frequently reported. As a defense strategy, exposure to heavy metals involves humoral and cellular components through leucocyte activation and the release of inflammatory mediators by the prostate, such as pro-inflammatory cytokines, resulting in adaptive or permanent alterations, such as the emergence of neoplasia [51,144,145]. Although it

is important to reestablish cellular homeostasis, tissue repair is a biological process with a high metabolic cost, capable of generating high oxygen consumption, which may increase ROS production and further promote inflammation [146]. The exaggerated metabolism to produce ATP and oxygen favors the mitochondrial dysfunction and formation of reactive oxygen and nitrogen species, promoting damage to essential biomolecules, such as lipids, proteins, and nucleic acids, seen in lipid peroxidation and protein oxidation assays [147,148]. These processes can be controlled by the antioxidant activity of some enzymes, such as SOD, CAT, and GSH. However, in more serious injuries, the activity of the antioxidant enzymes seems to be inefficient, favoring cellular redox imbalance [88]. Moreover, the mitochondrial dysfunction occasioned by the uncontrolled production of ROS, can impair other processes that require a high energy cost, such as spermatogenesis and fertilization [149]. Taken together, all the reported changes may also favor the development of prostate cancer.

We need to highlight the existence of very few recent studies involving exposure to heavy metals in murine models. In the last 11 years, studies have focused on commonly reported metals, with relevant information about Al, Cd, As, and Hg. However, there are a wide variety of heavy metals found individually in the environment or through mixtures with other metals, which in synergy can cause even greater damage, needing further study.

Considering all aspects mentioned, we conclude that exposure to non-essential heavy metals can induce the emergence of tissue pathologies, endocrine dysregulation, and oxidative imbalance in the prostate of murine models, through different mechanisms of toxicity. All findings reported here indicate that non-essential heavy metals induced prostatic insults that impair male fertility, in addition to predisposing the animals to more severe injuries, such as prostate cancer. These data,

although presented in murine models, reflect the care and importance of avoiding human contact and exposure to heavy metals, since these metals can have several harmful biological effects on the prostate. The doses employed in the studies refer to occupational, ecosystem-linked, diet-associated, and non-occupational sources of exposure and the environmentally relevant concentrations reflect a real scenario to which humans are exposed. We also reinforce here the importance of creating other studies to assess the risks of exposure and ensure safe concentrations that are not harmful to the prostate.

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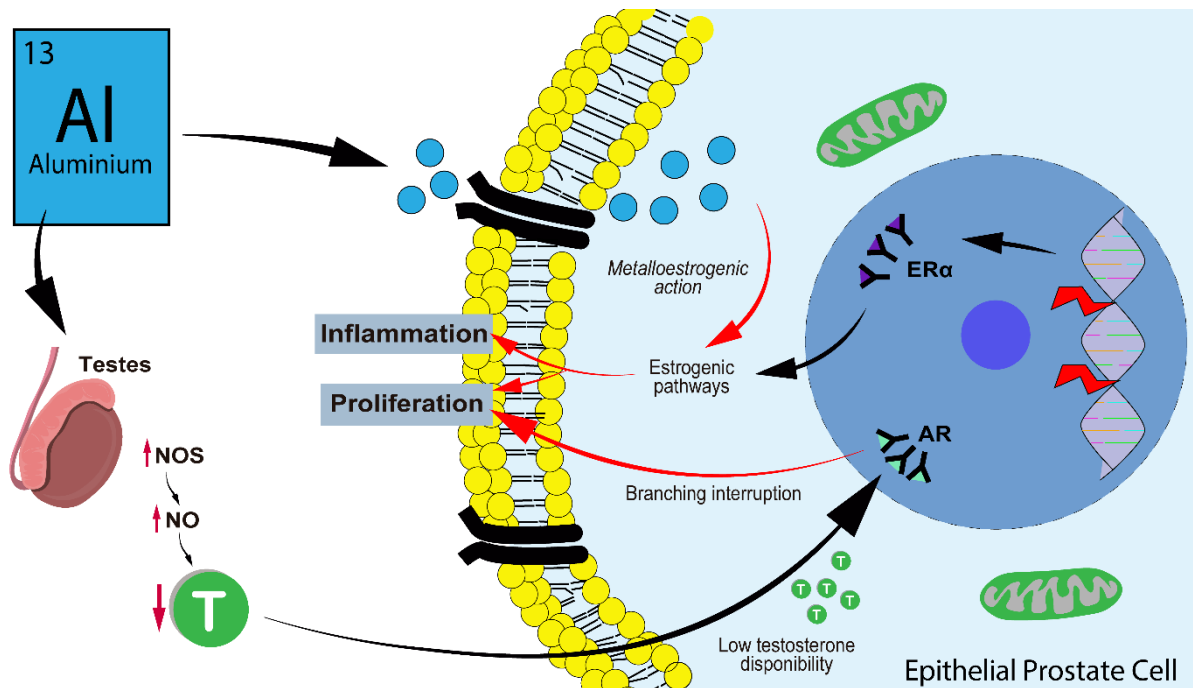
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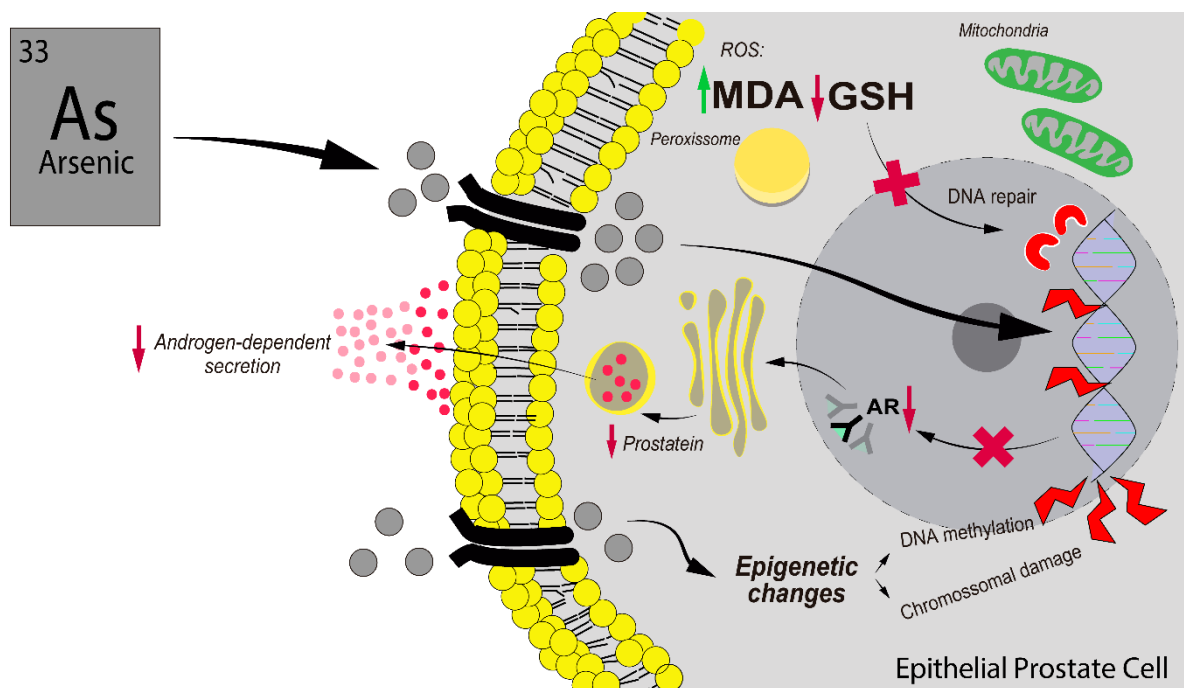
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Figure 1



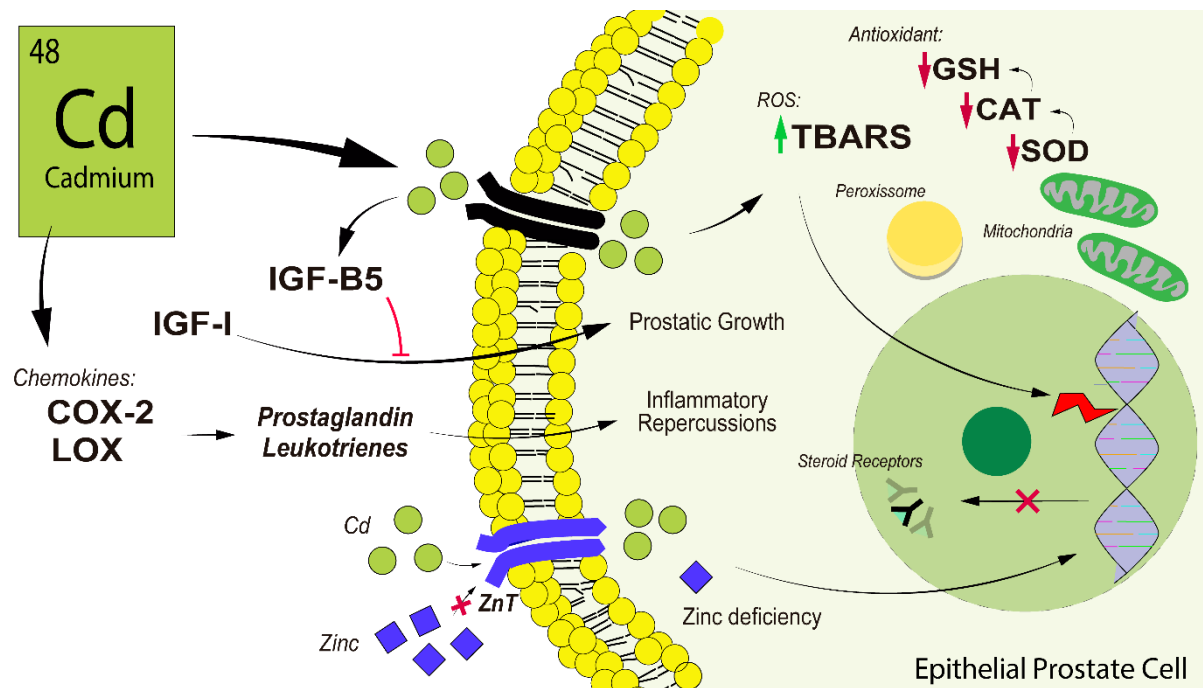
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Figure 2



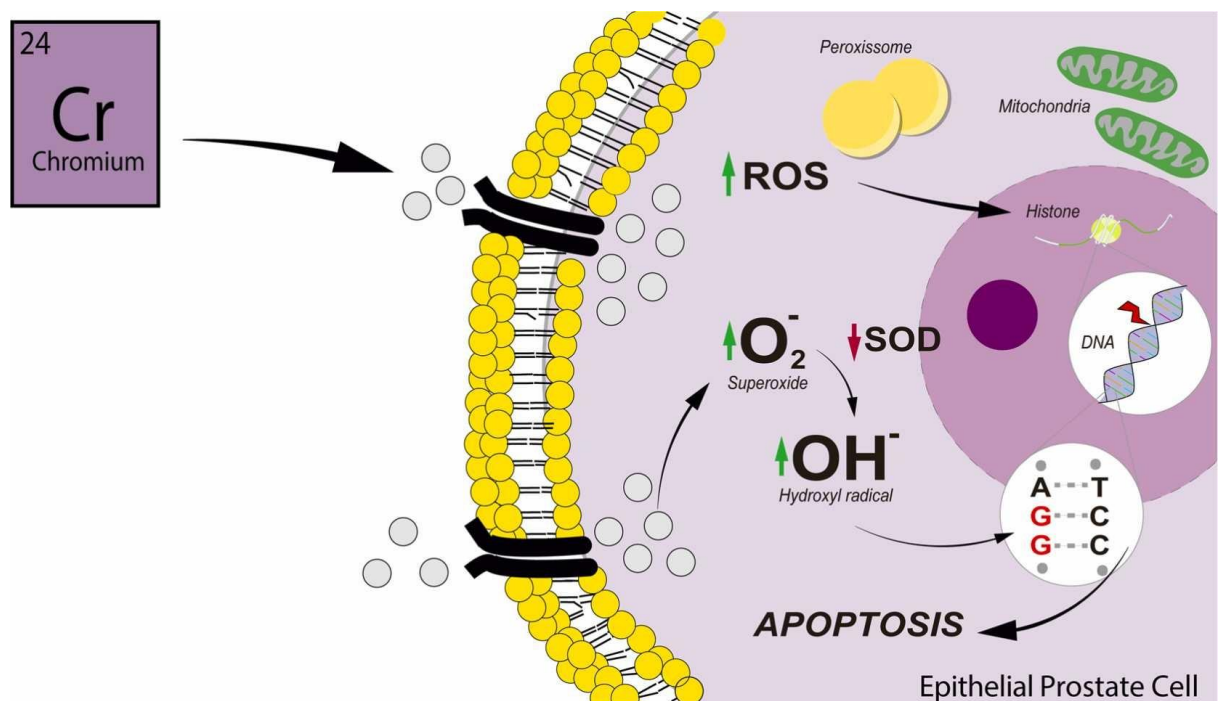
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Figure 3



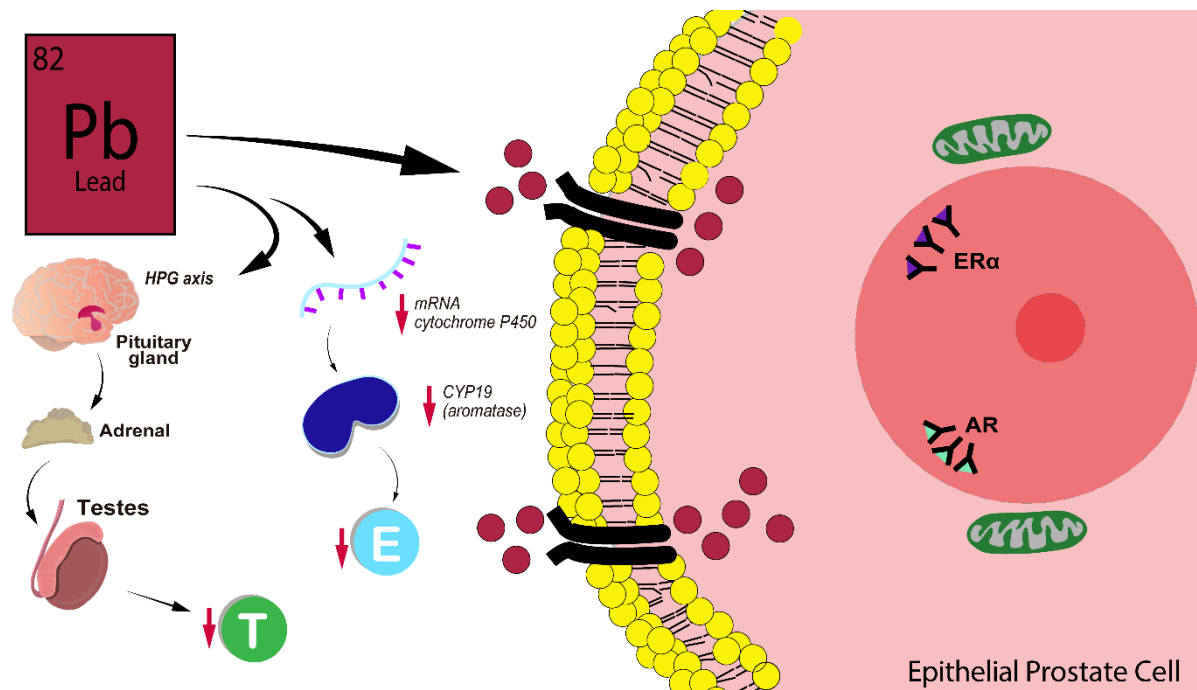
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Figure 4



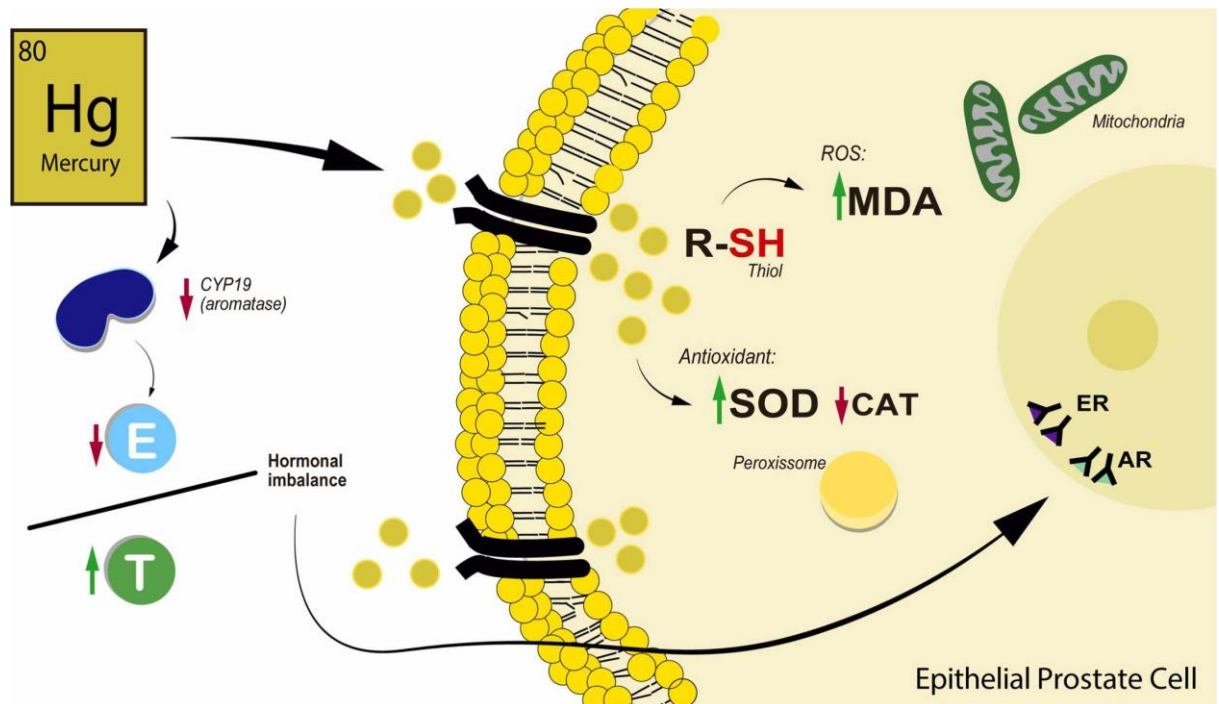
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Figure 5



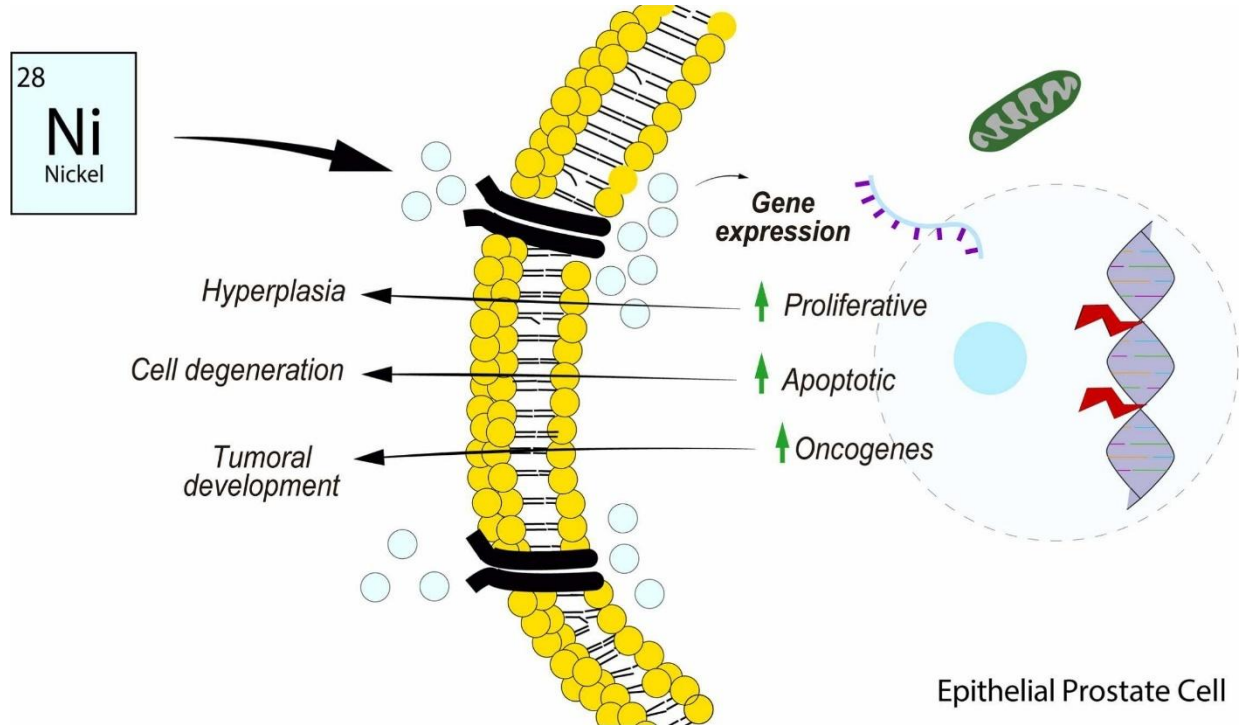
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Figure 6



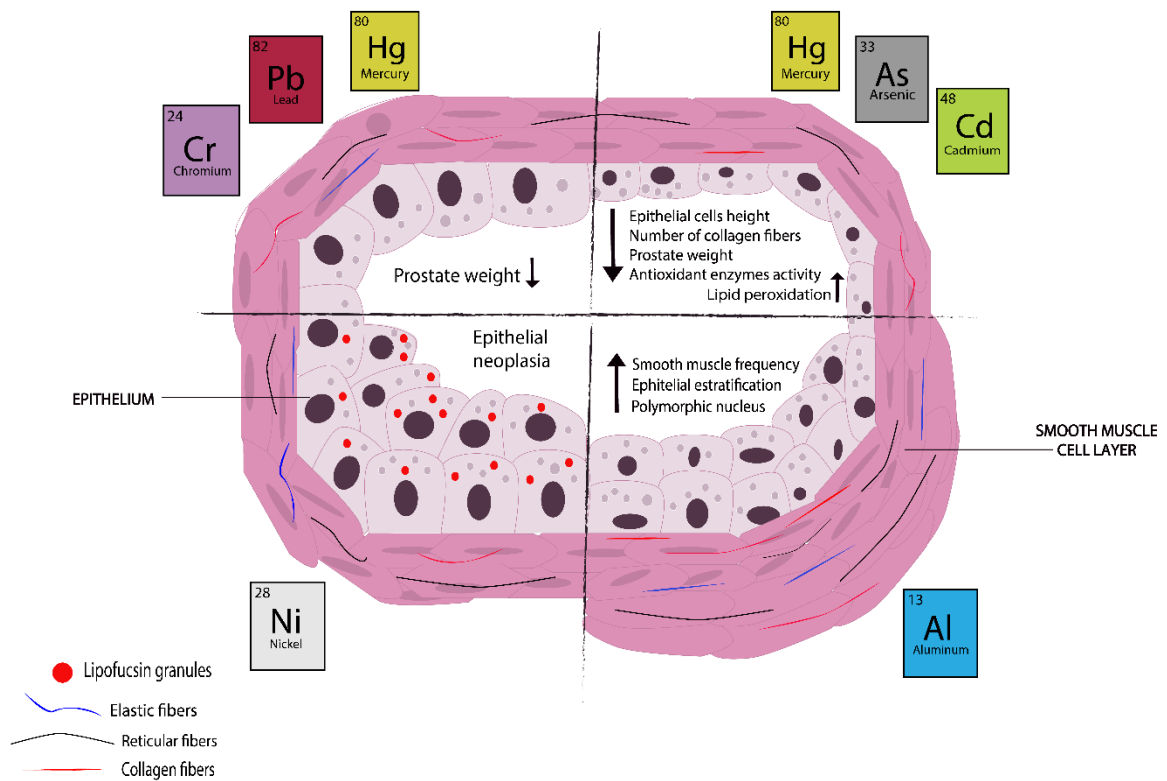
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Figure 7



Source: Extracted from Silva *et al.* (2023)

Figure 8



Source: Extracted from Silva *et al.* (2023)

Table 1: Summary of papers that evaluated the effects caused by exposure to heavy metals on the prostate of murine models, including the animal/sex, type of compound and dose, route of administration, life stage, analysis employed, main outcomes, and reference number.

Animal /sex	Type of compound and dose	Route of administration	Life stage	Analysis	Main outcomes	Reference
Wistar rat (male)	Sodium arsenite (NaAsO ₂) 0.01 mg/L and 10.0 mg/L	Drinking water	Prepubertal	Evaluation of water, food consumption, and physical status. Weight prostate determination, serum testosterone level analysis, morphological and stereological analysis, relative volume collagen determination, immunohistochemistry, extraction, and quantification of protein, western blotting, GSH measurement, SOD, CAT, and MDA determination	Decrease in epithelial height and collagen fibers amount. Increase in metalloproteinase 2. Reduction in cell proliferation and protein associated with antioxidant response. Increase in DNA methylation and apoptosis occurrence. Decrease in the androgen receptor, prostatein, and IL-10 expression. Increase in MDA levels.	[65]
Mongolian gerbil (male)	Cadmium chloride (CdCl ₂) 5 µmol/kg	Subcutaneous injection	Pubescent	Biometric, hormone level determination, cytochemistry, stereological, morphometric, karyometric, multiplicity and incidence of lesion determination, immunocytochemistry, detection	Increase in epithelial height and thickness, increase in nuclear area and cellular perimeter, reduction in secretory activity of the acinar epithelium, incidence of inflammatory infiltrate cells in the lumen and foci of prostate lesions, increase in the amount of collagen and reticular fibers in the stroma,	[112]

				of DNA fragmentation, immunostained cells quantification	decrease in stromal AR- positive cells.	
Mongolian gerbil (female and male)	Aluminum chloride (AlCl ₃) 100 mg/kg/day	Intrauterine	Newborn and adult	Biometrical, histological, serum testosterone dosage, three-dimensional reconstruction, stereological, immunohistochemical, AR, ER α , and PCNA quantification	Newborn females and males presented decreased body weight. Adult females and males presented an increase in serum testosterone levels. Female: Newborns presented a reduction in the anogenital distance (AGD), an increase in paraurethral mesenchyme (PaM), and epithelial buds (EB). Adults presented increased body weight and a reduction in AR-positive cells in stroma and epithelium and a reduction in ERα in the stroma. Newborn and adult females presented ERα and PCNA-positive cells. Males: Newborn and adult males presented an incidence of PCNA-positive cells. Newborns presented an increase in smooth muscle (SM) and relative frequency of EB, a decrease in the mesenchymal compartment, and AR-positive cells. Adults presented prostates with	[38]

higher epithelial compartments, an increase in the epithelial and SM relative frequency in the ventral prostate.						
Mongolian gerbil (female and male)	Aluminum chloride (AlCl ₃) 10 mg/kg/day	Oral	Newborn	Biometrical, histological, serum testosterone dosage, three-dimensional reconstruction, morphological, morphometrical, immunohistochemical, immunostaining quantification	Females and males presented a disruption of postnatal prostate morphogenesis, increased in AR and PCNA-positive cells. Females: Increased serum testosterone levels, reduction in epithelial bud length, presence of ERα-positive markings. Males: Decreased body weight, acceleration of the ductal canalization process, increase in the perialveolar smooth muscle frequency	[57]
Rat (male)	Cadmium chloride (CdCl ₂) 0.2 mg/kg	Subcutaneous injection	Adult	Antioxidant enzyme activity and lipid peroxidation determination	Increase in lipid peroxidation. Reduction in SOD, POD, CAT, and GSR activity.	[71]
Wistar rat (male)	Cadmium chloride (CdCl ₂) 15 mg/L	Oral	Adult	Hormone level determination, measurement of the cadmium concentration in prostate	Increase in cadmium accumulation in prostatic lobes.	[122]

Wistar rat (male)	Aluminum chloride (AlCl ₃) 7 mg/kg and 34 mg/kg	Intraperitoneal injection	Peripubertal period	Food and water consumption determination, body and ventral prostate weights, plasma testosterone level determination, mast cell count, myeloperoxidase, NAG activity and IL-6 level determination, immunohistochemistry, proliferation index determination	Decrease in food and water consumption and body weight. Increase in the prostatic stromal compartment and decrease in the luminal compartment. Increase in the positive stromal leucocyte frequency. Increase in myeloperoxidase activity. Decrease in the percentage of AR in epithelial cells.	[51]
Mongolian gerbil (female and male)	Aluminum chloride (AlCl ₃) 10 mg/kg/day	Oral	Adult	Body weight, absolute and relative weight of prostate. Histochemistry, morphometric, histopathological, ultrastructural analysis. Biochemical assays	Males: Increase in thickness of secretory epithelium and frequency of total collagen and smooth muscle cells. Incidence of prostatic hyperplasia, and glandular inflammation. Stratification of secretory epithelium. Presence of polymorphic nucleus and prostatic epithelial cells. Presence of lipofuscin granules in epithelial cell cytoplasm. Decrease in SOD and CAT activity. Female: Increase in frequency of collagen and smooth muscle. Glandular hyperplasia, perialveolar inflammation, intraepithelial neoplasia. Nuclear and cellular polymorphism, epithelial degeneration, autophagic vacuoles, and accumulation of lipofuscin granules.	[123]
Mongolian gerbil (female and male)	Aluminum chloride (AlCl ₃) 10 mg/kg/day	Oral	Adult	Body and prostate complex weight. Morphological, morphometrical,	Males: Increase in the relative frequency of the epithelium and smooth muscle, and decrease in the luminal compartment. Cellular	[124]

				ultrastructural, immunohistochemical analysis and cell quantification	anabolism, increase in the area of PAS-positive secretion. Increase in AR expression in the lumen. Cell proliferation in epithelium and stroma. Females: Hyperplasia and increased secretory activity, great epithelial development. Higher frequency of AR-positive cells. Frequency of ER α -positive stromal cells. Increased cell proliferation.	
Rat (male)	Chromium IV (potassium dichromate) 1 or 2 mg/kg	Intraperitoneal injection	Juvenile	Prostate weight, histological analysis, serum hormone determination (Testosterone, LH, and FSH)	Decrease in epithelial cell height. Incidence of secretion in the alveolar lumen. Decrease in serum testosterone and LH levels. Increase in FSH levels.	[92]
Wistar rat (male)	Mercuric chloride (HgCl ₂) 4.6 μ g/kg and 0.07 μ g/kg	Intramuscular injection	Adult	Prostate weight, lipid peroxidation evaluation, SOD, and CAT activity determination	MDA level elevation. Increase in SOD activity.	[22]
Albino Wistar rat (male)	Aluminum chloride (AlCl ₃) 34 mg/kg	Oral	Adult	Serum testosterone level determination, epididymal spermatozoa analysis, MDA levels determination, DNA fragmentation analysis. Histopathological examination	Decrease in serum testosterone levels. Decrease in sperm motility. Elevation in DNA fragmentation. Incidence of multiple calcified materials in the lumen from the prostate gland.	[55]

Mongolian gerbil (male and female)	Aluminum chloride (AlCl ₃) 10 mg/kg	Oral	Newborn	Prostate complex weight, Morphological, morphometrical, and ultrastructural analysis, immunohistochemistry and immunostaining quantification	Adult males: Increase in prostate relative weight, growth of the epithelium and the smooth muscle layer. Increase in cell proliferation in the epithelium. Senile males: Intense growth in the epithelium, increase in AR-positive cells in the epithelium. Decrease in AR-positive cells in the stroma, epithelial and stromal proliferation. Adult females: Decrease of epithelial and stromal AR immunostaining, reduction in the ERα frequency. Increased cell proliferation in epithelium and stroma. Senile females: Reduction in the frequency of AR-positive cells in the stroma. Increase in ERα. Increased cell proliferation in epithelium and stroma.	[50]
Rat (male)	Cadmium acetate (C ₄ H ₆ CdO ₄) 20 μ g/kg	Intraperitoneal injection	Adult	Prostate weight. Prostatic acid phosphatase level determination. LPO and GSH level determination. Histological examination. Relative genes expression evaluation. Immunohistochemistry	Increase in prostate weight. Decrease in GSH and increase in LPO levels. Increase in the number of acini and mitotic figures. Increase in number and irregular acinar growth pattern. Increase in size of the secretory epithelial cells and cytoplasm. Basal cell proliferation. Increased expression of AR and ERα along with decreased expression of ER-β. Increased epithelial cell proliferation.	[125]

Rat (male)	Cadmium acetate ($C_4H_6CdO_4$) 10 mg/L	Gestational and lactational	Adult	Prostate weight determination, serum testosterone assay, histopathological analysis, morphometric-stereological analysis, immunohistochemistry, <i>TUNEL</i> assay, proliferation and apoptosis index determination, AR semiquantitative analysis	Inflammatory foci in the stromal compartment, presence of stratification, and epithelial dysplasia. Presence of reactive epithelial atypia. Changes in distribution and compaction of collagen and reticular fibers.	[37]
Wistar rat (male)	Methylmercury (MeHg) 0.5, 1.0 and 3.0 mg/kg	Oral	Adult	Body and organ weight determination, mercury level determination, histopathological, morphometric, and stereological analysis, AR-semiquantitative analysis, cell proliferation determination	Decrease in the absolute and relative weights of the ventral prostate. Increase in mercury levels in the prostate. Incidence of inflammatory foci and increase in blood vessels in the stroma and stratified focal hyperplasia. Increase in the glandular lumen. Increase in the epithelial and luminal components. Decrease in the stromal component. Increase in numbers of Ki67-positive cells.	[111]
Wistar rat (male)	Nickel oxide nanoparticles (NiONPs) and Nickel oxide microparticle (NiOMPs) 5 mg/kg	Oral	Adult	Body and prostate weight determination, histopathological analysis	Epithelial and basal hyperplasia. Epithelial degeneration	[108]

Source: Extracted from Silva *et al.* (2023)

3. COMPILADO DE ARTIGOS DA MESMA TEMÁTICA

3.1 INDIVIDUAL AND COMBINED EFFECTS OF COMMERCIAL GLYPHOSATE, ATRAZINE AND 2,4-D HERBICIDES ON THE GERBIL VENTRAL PROSTATE

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O presente artigo segue as normas do periódico.

Individual and combined effects of commercial glyphosate, atrazine and 2,4-D herbicides on the gerbil ventral prostate

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CRedit author statement

Stella B. Silva, Conceptualization, Investigation, Formal analysis, Writing – original draft. **Vitor Grigio**, Conceptualization, Formal analysis. **Thalles F. R. Ruiz**, Conceptualization, Formal analysis. **Marilia F. Calmon**, Resources, Validation. **Paula Rahal**, Resources. **Fernanda C. A. dos Santos**, Validation, Supervision. **Sebastião R. Taboga**, Funding acquisition, Validation, Supervision. **Patricia S. L. Vilamaior**, Conceptualization, Supervision, Project administration, Writing – review & editing. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that have no competing interests.

Data availability

Data will be made available on request.

Abstract

Exposure to pesticides, individually or in a mixture, in drinking water is one of the main sources of human contamination, which causes adverse effects on the reproductive system. Our study aimed to investigate, the effects of a 90-day exposure to low concentrations of glyphosate (GLY), atrazine (ATZ), and 2,4-dichlorophenoxyacetic acid (2,4-D), in commercial formulations, on morphological, molecular, and hormonal parameters of the ventral prostate of gerbils (*Meriones unguiculatus*). The animals were exposed via drinking water to individual concentrations of GLY: 700 µg/L, ATZ: 3 µg/L, and 2,4-D: 70 µg/L, as well as to their mixture (MIX). Our findings showed an increase in prostatic complex relative weight in ATZ-exposed animals. Stereological and morphometric techniques indicated an increase in the percentage and thickness of muscular stroma, following an increase in the amount of collagen and reticular fibers in the MIX group. Histopathological analysis showed a decrease in the incidence of epithelial atrophy, subepithelial inflammation, and microacini in the MIX. On the other hand, ATZ-exposed animals showed an increase in hyperplasia and total prostatic intraepithelial neoplasia (PIN). The expression of caspase-3 decreased and estrogen receptor alpha (ER α) increased in the 2,4-D and MIX. Western blotting showed an increase in estrogen receptor beta (ER β) expression in MIX-exposed animals. Testosterone levels decreased in animals from the GLY, ATZ and 2,4-D groups. Our findings provide evidence that individual or

combined exposure to herbicides causes hormonal imbalance and morphological alterations, besides favoring the incidence of proliferative lesions in the prostate, predisposing the gland to more severe injuries.

Keywords: Agrochemicals; DMA® 806 BR; Pesticide Mixture; Reproduction; Roundup WG®.

Highlights

- The mixture of herbicides induced tissue alterations in gerbil ventral prostate;
- ATZ-exposed animals showed prostatic hyperplasia and intraepithelial neoplasia;
- 2,4-D and MIX decreased the expression of caspase-3, an apoptotic marker;
- 2,4-D and MIX promoted up-regulation of the estrogen receptor alpha in the prostate;
- Individual exposure to herbicides decreased testosterone levels.

1. Introduction

In modern agriculture, herbicides are relevant compounds for controlling pests and weeds and for enhancing crop yield, accounting for approximately 50% of all pesticides (Gensch et al., 2024; Ma et al., 2023). In 2021, the total amount of herbicides used in agriculture worldwide reached 1.7 million tons of active ingredients, representing an accelerated increase in recent decades (FAO, 2023). Despite their contribution to food production, herbicide residues can accumulate and contaminate water resources through leaching, absorption, and runoff processes, resulting in a high ecotoxicological impact (Akash et al., 2022; Salaro et al., 2023; Tudi et al., 2021).

Water resources, including drinking water, consistently receive quantities of organic pollutants (Alagan et al., 2023), herbicide residues (Monticelli Barizon et al., 2022), and industrial discharges (Chowdhary et al., 2020). Herbicides can occur

individually or can form complex mixtures in water bodies, interacting synergistically or antagonistically to produce unintended effects on exposed organisms (Alehashem et al., 2024; Choung et al., 2013; Gustavsson et al., 2017). Even at low concentrations, ingesting water contaminated with herbicides can lead to a range of adverse effects, such as developmental toxicity and male reproductive disorders (Cassault-Meyer et al., 2014; Ghorbani Nejad et al., 2024; Wan et al., 2021).

Glyphosate (N-[phosphonmethyl]glycine, GLY) is a broad-spectrum herbicide used in weed control, through inhibition of plant respiration and development (López-Chávez et al., 2021; Soares et al., 2019; Xu et al., 2022). GLY is the primary active compound in Roundup®, a commercial chemical formulation widely used around the world in agriculture, forestry and urban areas (Telahigue et al., 2021). Despite its action on target plants, a range of adverse effects have been reported in males exposed to GLY, such as alterations in androgenic and estrogenic pathways (Gorga et al., 2021), increases in aromatase mRNA levels (Cassault-Meyer et al., 2014), and decreases in total sperm production (Dai et al., 2016). Glyphosate concentrations ranging from 1.8 to 427 µg/L have previously been detected in surface water in countries such as Argentina and United States, posing a risk to the aquatic ecosystem (Geng et al., 2021).

Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine, ATZ) is an environmental persistent herbicide, frequently reported in drinking water (De Caroli Vizioli et al., 2023; Zhao et al., 2024). In countries such as Brazil and the United States, ATZ values detected in drinking water samples were 7 µg L⁻¹ and 27.5 µg L⁻¹, respectively, exceeding the limits allowed by regulatory agencies (Nogueira Neto et al., 2024; Perkins et al., 2022). Exposure to ATZ via drinking water is related to an increased risk of neonatal complications (Ghorbani Nejad et al., 2024) and endocrine

dysregulation (Cleary et al., 2019). ATZ is also related to ventral prostate injuries (Martins-Santos et al., 2018), in addition to increasing the risk of cancer development when associated with other herbicides (Panis et al., 2022).

The 2,4-dichlorophenoxyacetic acid (2,4-D) is a persistent herbicide that is used in weed control and in public and private aquatic systems (Dehnert et al., 2018; Gaaied et al., 2022). In Galicia, Spain, concentrations ranging from 62-207 ng L⁻¹ were detected in drinking and surface water, representing a severe concern for human and ecosystem health (Islam et al., 2018). Despite its efficiency in agricultural control, low concentrations of 2,4-D have been linked to kidney toxicity (Aydin et al., 2005), damage to spermatogenesis (Alves et al., 2013), imbalance in gonadal hormone levels, and decreased androgen receptor expression (Marouani et al., 2017). For these reasons, and due to its association with different organs of the reproductive system, 2,4-D has been considered an endocrine disruptor (Rocha et al., 2023).

The prostate is a main secretory gland of the male reproductive tract, which secretes an alkaline fluid to provide viability to sperm (Aquino et al., 2023). A relation has recently been observed between exposure to environmental contaminants, and the incidence of prostate disorders (Lima et al., 2020; Silva et al., 2023). However, in vivo studies relating the prostate and individual herbicide exposure are scarce and the effects of mixing these compounds are even less researched.

Considering these aspects, we hypothesized that individual exposure to herbicides interferes in prostate morphophysiology, and that the effects will be amplified when exposed to the mixture. In order to test this hypothesis, we aimed to investigate the individual and combined effects of GLY, ATZ, and 2,4-D, contained in commercially available formulations, on the prostate morphophysiology, molecular aspects, and hormone secretion in Mongolian gerbils.

2. Material and methods

2.1. Herbicide preparation

Commercial formulations of the herbicides Glyphosate (GLY; Roundup WG® 720 g/kg⁻¹ of glyphosate and 207.5 g/kg⁻¹ of other ingredients, Monsanto do Brasil Ltda), Atrazine (ATZ, Atrazina Atanor® 50 SC, 50.0 g/L⁻¹ of atrazine and 58.4 g/L⁻¹ of other ingredients, SCA, Buenos Aires, Argentina), and 2,4-dichlorophenoxyacetic acid (2,4-D, DMA® 806 BR, 80.6 g/L⁻¹ of 2,4-D and 42.0 g/L⁻¹ of other ingredients, Dow Agrosiences Ltda, Brazil) were obtained from specialized manufacturers. We chose to use commercial formulations, as these are frequently used and reported in water samples, including drinking water. The commercial herbicide formulations were prepared weekly and diluted in dechlorinated water to the desired test concentrations of the following active ingredient: 700 µg/L (GLY), 3 µg/L (ATZ), and 70 µg/L (2,4-D). The chosen concentrations were established according to the maximum contaminant levels (MCL) for drinking water, according to the United States Environmental Protection Agency (EPA, 2019), considered as the no-observed-adverse-effect levels of herbicides.

2.2. Animals and experimental design

Fifty male Mongolian gerbils (*Meriones unguiculatus*), 6 months of age, were randomly divided into 5 experimental groups (n=10/group): GLY - animals that received filtered water containing 700 µg/L of GLY; ATZ - animals that received filtered water containing 3 µg/L of ATZ; 2,4-D - animals that received filtered water containing 70 µg/L of 2,4-D; MIX - animals that received filtered water containing the aforementioned concentrations of the three herbicides: 700 µg/L + 3 µg/L + 70 µg/L; and Control (CTL): animals that received filtered water free of herbicides. For the different experimental

groups, water was provided weekly in glass bottles with drinking fountains. The animals were kept separately in polysulfide isolators, on racks equipped with air exhaustion at a temperature of 22 ± 2 °C, and fed *ad libitum* with a standard diet for rats and mice at the Animal Maintenance Center of the Department of Biological Sciences of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State University (UNESP). Weekly measurements were taken of the volume of water ingested by each animal, in order to assess the water consumption among the experimental groups. All procedures involving the animals were in accordance with the guidelines of the National Council for Animal Control and Experimentation (CONCEA, Brazil) and were approved by the Ethics Committee on the Use of Animals (CEUA, IBILCE, UNESP), protocol number 244/2023.

2.3. Sample collection

After 90 days of exposure, the animals were euthanized by anesthetic overdose (Isoflurane 3%) and decapitated, and blood was immediately collected, centrifuged, and stored at -80 °C for hormonal analysis. The complex prostatic, which comprises the different prostatic lobes, was removed, and the ventral prostate was identified, removed, and fixed whole in 4% paraformaldehyde for 24 h, dehydrated in alcohol, processed, and embedded in paraffin in a Leica Semi Enclosed System (Histosec, Merck, Darmstad, Germany). The samples were sectioned at a thickness of 4 µm for morphological and immunohistochemical analysis (n=6/group). The ventral prostate was also flash-frozen in liquid nitrogen and stored at -80 °C for molecular analysis (n=4/group). This lobe was chosen due to its strong response to foreign compounds, and its tendency to develop proliferative lesions. Body fat was also removed and weighed (retroperitoneal, epididymal, visceral and subcutaneous).

2.4. Morphological, stereological, and morphometric analysis

The histological sections were stained with picosirius red for collagen analysis and the Gömöri's reticulin technique was used to evaluate the reticular fibers. Periodic Acid Schiff (PAS) staining was also carried out to identify mucous cells. Stereological and morphometric analyses were performed on histological sections stained with Hematoxylin-Eosin (HE). The collagen and reticular fiber distribution and the relative volumes of prostatic compartments (epithelium, lumen, muscular stroma, non-muscular stroma, and blood vessels) were evaluated. In total, 60 ventral prostate fields for each experimental group (10 fields/animal) were randomly scanned with a BX61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120® Virtual Microscopy Slide Scanning System (VS120-S5), and analyzed using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA). The relative frequency was estimated using the M130 multipoint test (Weibel, 1963) applied to the prostate (Huttunen et al., 1981).

Morphometric analyses were performed to determine the height of the epithelium and the smooth muscle layer (SML) thickness. For each analysis, 300 measurements were obtained from each experimental group (50 measurements/animal). The evaluations were performed under light microscopy and the histological images were digitized with a BX61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120® Virtual Microscopy Slide Scanning System (VS120-S5), using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA).

2.5. Histopathological analysis

We evaluated the incidence and multiplicity of lesions in the prostate fragments of the different experimental groups, using the HE-stained sections. The lesions were classified according to previous studies (Gonçalves et al., 2010; Shappell et al., 2004), that included gerbils (Gonçalves et al., 2013). The following lesions were investigated: epithelial atrophy, epithelial atypia (flat and papillary), hyperplasia, prostatic intraepithelial neoplasia (PIN), such as low-grade PIN (LGPIN), high-grade PIN (HGPIN), and total PIN, adenocarcinoma, microinvasive carcinoma, mucinous metaplasia, microacini, and inflammation (periductal, intraluminal, subepithelial). The incidence was expressed as the number of animals exhibiting each type of lesion (percentage) and multiplicity was expressed by quantifying the number of lesions found in each histological image analyzed (average number of lesions/group).

2.6. Immunohistochemistry

Immunohistochemistry techniques were used to evaluate α -smooth muscle actin (α -sma, mouse monoclonal, 1A4, 1:100, sc-32251, Santa Cruz Biotechnology), phosphohistone-H3 (PHH3, rabbit polyclonal, 1:75, Ser10, 9701, Cell Signaling), and activated caspase-3 (caspase-3, rabbit polyclonal, 1:75, NB100-56113, Novus Biological), as well as to determine the relative frequency of androgen receptor (AR, polyclonal rabbit, anti-AR, 1:50, sc-816, Santa Cruz Biotechnology, CA, USA), estrogen receptor alpha (ER α , mouse monoclonal, 1:50, sc-8005, Santa Cruz Biotechnology) and estrogen receptor beta (ER β , rabbit polyclonal, anti-ER β , 1:50, sc-8974, Santa Cruz Biotechnology, CA, USA). Briefly, histological sections of the ventral prostate were subjected to deparaffinization, rehydration, and antigen retrieval at 96 °C for 45 min, using 10 mM citrate buffer (pH 6.0). The endogenous peroxidase activity was blocked with 10% hydrogen peroxide in methanol for 20 min. Subsequently, nonspecific proteins were blocked in 5% skimmed milk for 60 min. The sections were

incubated with primary antibodies diluted in BSA 1% overnight at 4 °C. We also used BSA 1% as a negative control. The histological sections were then incubated with a specific polymer kit (NovolinkTM polymer detection system 1, Leica Biosystems Newcastle Ltd.) for 1 h at room temperature or at 37 °C. The reactions were revealed with diaminobenzidine (DAB), and counterstained with hematoxylin. Immunoreactive epithelial and stromal cells were counted using one histological section per animal (n=6/group) and the percentage of positive cells was calculated using QuPath[®] software (Version 6.0).

2.7. Western Blotting

In order to quantify the expression of steroid receptors, frozen prostate samples were homogenized at 4 °C using an extraction buffer containing protease inhibitor (Protease Inhibitor Cocktail, P8340, Sigma Aldrich, St. Louis, MO, USA). Total protein was quantified by the BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23, 227, ThermoFisher Scientific) and the samples were separated by SDS-PAGE on 10% and 12% polyacrylamide TRIS-glycine gels and transferred to nitrocellulose membrane. The membranes were incubated with 5% dry milk in TBST for 1 h at room temperature (RT) and then incubated with primary antibodies: Anti-AR (sc-7305; 1:300, mouse monoclonal, Santa Cruz Biotechnology), Anti-ER α (sc-8005; 1:1000, mouse monoclonal, Santa Cruz Biotechnology) and Anti-ER β (PA1-310B; 1:500, rabbit polyclonal, Invitrogen, Thermo Fisher), or β -actin (13E5; 1:1000, Cell Signaling) overnight at 4 °C, and incubated with secondary antibodies (mouse Anti-mouse IgG, HRP-linked Antibody, #7076, 1:2000, Cell Signaling or Anti-rabbit IgG, HRP-linked Antibody, #7074, 1:2000, Cell Signaling) for 2 h at room temperature. The membranes were incubated with Luminol-Enhancer solution (Cod. XLS142L, Cyanagen, Italy) and peroxide solution (Cod. XLS142P, Cyanagen, Italy) and were revealed in an imaging

system machine (ChemiDoc MP, BioRad, Hercules, CA, USA). The density of the bands was quantified using Image Lab software (Bio-Rad Laboratories, Inc.) and was normalized using endogenous protein β -actin.

2.8. Hormone determination

Total testosterone and estradiol levels were measured using serum samples (n=6/group), through specific commercial kits, following the manufacturer's instructions (USA Diagnostics).

2.9. Statistical analysis

Data distribution was determined using the Shapiro-Wilk test. Parametric data were submitted to unifactorial one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons. Non-parametric data were subjected to Dunn's test. Results are expressed as means and standard error of the mean (mean $\pm$ SEM) considering $p < 0.05$. All statistical analyses were carried out using the GraphPad Prism (version 8.0, Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1. Water ingestion

The volumes of water ingested containing the corresponding concentrations of the herbicides and their mixture were similar among the treated groups. These data ensure that all treated groups ingested very similar amounts of herbicides (Fig. 1).

3.2. Biometry

The biometric data are presented in Table 1. Relative prostatic complex weight showed an increase in ATZ and MIX compared to CTL ($p=0.0004$ and $p=0.0006$). Ventral lobe absolute and relative weights increased in ATZ compared to MIX ($p=0.0003$). There were no statistical differences in body weight or percentage of body fat among the groups.

3.3. Morphological, stereological and morphometric investigation

Collagen and reticular fiber distribution was analyzed using picrosirius and Gömöri's reticulin stains, revealing damage in the pattern of organization of these fibers (Fig. 2 A-J). There was an increase in the percentage of collagen area in the MIX group compared to CTL ($p=0.0003$) (Fig. 2K), followed by an increase in the percentage of reticular fibers in the MIX group compared to CTL ($p=0.0063$), ATZ ($p=0.0141$) and 2,4-D ($p=0.0027$) (Fig. 2L).

The stereological analysis showed differences in the relative frequency of prostate tissue components in the different experimental groups. There was an increase in the percentage of muscular stroma in the MIX group compared to CTL ($p=0.0368$). The stromal compartment was increased in GLY compared to the ATZ group ($p=0.0480$). The morphometric analysis demonstrated an increase in the epithelium height in the MIX group compared to the CTL ($p=0.0343$), GLY ($p=0.0248$), and ATZ ($p=0.0283$) groups. The MIX group also presented an increase in the thickness of muscular stroma when compared to all other groups ($p=0.0004$) (Table 2).

3.4. Herbicide exposure induces histological lesions in gerbil prostate

The ventral prostate of the experimental groups showed several acini composed of a simple and cubic epithelium surrounded by a layer of muscle, blood vessels, and

reticular and collagen fibers that make up the fibromuscular stroma (Fig. 3 A-T). The luminal compartment of the ventral prostate also contained prostate fluid. Multiplicity of prostatic lesions showed a decrease in epithelial atrophy in the MIX and 2,4-D groups compared to CTL ($p=0.0187$). Regarding proliferative disorders in the epithelial and stromal compartments, we showed an incidence of benign hyperplasia in ATZ compared to CTL ($p=0.0167$). There was an increase in low-grade prostatic intraepithelial neoplasia (LGPIN) in the ATZ ($P=0.0154$), 2,4-D ($p=0.0077$) and MIX ($p=0.0368$) groups compared to CTL. Prostatic intraepithelial neoplasia (PIN total) was also observed in animals from the ATZ ($p=0.0121$) and 2,4-D ($p=0.0286$) groups. On the other hand, malignant lesions, such as adenocarcinoma, were present in the prostate of 2,4-D-exposed animals when compared to CTL ($p=0.0005$) and GLY ($p=0.0012$). A reduction in the incidence of microacin was observed in animals exposed to MIX compared to CTL ($p=0.0277$). Foci of intraluminal, periductal, and subepithelial inflammation were observed in all experimental groups, being decreased in the MIX group compared to CTL ($p=0.0087$) (Table 3).

3.5. 90-day exposure to herbicides modulates cell death and proliferation as well as estrogen receptors

Positive cells for caspase-3 (Fig. 4 A-G) and PHH3 (Fig. 4 H-N) were quantified and comparisons of the experimental groups showed that 2,4-D and MIX treatments led to decreased expression of activated caspase-3 in the epithelium compared to GLY ($p=0.0038$) (Fig. 4F). Activated caspase-3 also decreased in the stroma from the 2,4-D and MIX groups compared to CTL, GLY, and ATZ ($p=0.0005$) (Fig. 4G). Regarding PHH3 immunostaining, there was no significant difference among groups (Fig. 4M, N). The activated caspase-3/PHH3 ratio showed a higher rate of cell death in the different experimental groups, in contrast to proliferation (Fig. 4O).

Regarding hormone receptors, the epithelial and stromal expression of AR was not altered among the experimental groups (Fig. 5 A-C). Although ER α expression is common in the prostate stroma, in our experimental model, there was an increase in the expression of ER α in the epithelium of the MIX group compared to CTL ($p=0.0146$) and GLY ($p=0.0015$). ER α expression also increased in the epithelium of 2,4-D-exposed animals compared to GLY ($p=0.0079$). In addition, ER α expression was increased in the stroma of 2,4-D and MIX-exposed animals compared to CTL and ATZ ($p=0.0004$) (Fig. 5 D-F). The epithelial and stromal ER β expression was not altered in the different experimental groups (Fig. 5 G-I). Western blotting analysis revealed no differences in AR and ER α expression among the groups. However, there was an increase in ER β protein concentration in MIX compared to the other groups ($p=0.0155$) (Fig. 5 J-M).

3.6. Influence of herbicides on hormone levels

Serum total testosterone concentration was decreased in gerbils from the GLY ($p=0.0216$), ATZ ($p=0.0339$), and 2,4-D ($p=0.0248$) groups compared to CTL (Fig. 6A). Estradiol levels did not differ among the experimental groups (Fig. 6B). Testosterone/Estradiol ratio is summarized in Fig. 6C.

4. Discussion

This is the first study to investigate individual and combined exposure, via drinking water, to three widely used herbicides and their main morphophysiological, molecular, and hormonal alterations in Mongolian gerbil prostate, a well-known gland among rodents (Ruiz et al., 2023). Exposure to low concentrations of commercial formulations containing GLY, ATZ, and 2,4-D, caused alarming injuries to the prostate microenvironment by distinct mechanisms, since the changes were not the same in all

groups. In addition, even more severe lesions were found in the ventral prostate of the animals exposed to the mixture of herbicides.

Among the parameters evaluated, the stereology and morphometry pointed to different alterations in the prostate stromal compartments. The prostate of MIX-exposed males presented an increase in the frequency of muscular stromal compartment and epithelium height, and a consequent increase in SML thickness. These morphological alterations in prostatic tissue are in agreement with the percentage of reticular and collagen fibers also found in the prostate of MIX group. The spatial organization and remodeling of collagen is related to the development of malignant neoplasms, and the increase in these fibers may be associated with the tumor microenvironment formation (Guerra et al., 2024; Kader et al., 2023; Zadvorny et al., 2023). An increase in the deposition and area occupied by collagen and reticular fibers possibly explains the increases in the frequency of stromal compartments and corroborates the morphometric findings. The mixture of herbicides can promote remodeling of stroma, and alterations in stroma in a tumor microenvironment can contribute to prostate cancer (PCa) progression (Shiao et al., 2016). Studies involving other endocrine disruptors have reported similar effects in prostate using the same experimental model (Colleta et al., 2017; Silva et al., 2019).

After a 90-day exposure, several prostatic injuries were observed in the experimental groups. Epithelial prostate atrophy, which was reduced in 2,4-D and MIX-exposed animals, is characterized by a flattened acinar epithelium with reduced height and scarce cytoplasm, usually associated with low secretory activity (Tamarindo et al., 2021). On the other hand, individual exposure to ATZ caused hyperplasia, a benign proliferative disorder defined by the growth of the gland, as the result of an imbalance in the growth factors that regulate epithelial-stromal interactions (Castro et al., 2021).

Interestingly, prolonged exposure to a commercial formulation containing ATZ was associated with the appearance of prostatic injuries, also affecting the tissue homeostasis of efferent ductules in Wistar rats (Martins-Santos et al., 2018).

Prostatic intraepithelial neoplasia, such as LGPIN and HGPIN were observed in 100% of the animals in our study; the two subtypes differ in the number of cell layers and the form of the nuclei and nuclear atypia. PINs are the result of several processes, such as oxidative stress, inflammation, and cellular senescence, and may also be precursors to more severe injuries such as adenocarcinomas (Abu el Maaty et al., 2022; Shen and Abate-Shen, 2010). PINs have also been reported in Sprague-Dawley rats exposed to an insecticide, indicating that other classes of pesticides may induce neoplastic lesions on murine prostate (Winarni et al., 2021). We found areas affected by adenocarcinomas in animals exposed to the three herbicides and their mixture, with a significant incidence observed in 2,4-D-exposed animals. Although studies on the prostate are scarce, exposure to 2,4-D is known to cause reproductive dysfunction. A previous study showed that prolonged exposure to a commercial formulation containing 2,4-D, induced histological lesions in the testes of rats, including damage to the seminiferous tubules (Oakes et al., 2002). Therefore, our experimental model showed a histopathological gradient compatible with human prostate carcinogenesis, since the emergence of PINs can develop into adenocarcinomas. The α -sma immunoreaction in injured regions showed a rupture in smooth muscle fibers in the prostate of animals from GLY and ATZ groups, indicating higher invasiveness of epithelial cells (Hetzl et al., 2016). In general, the herbicides tested, even individually and at low concentrations, cause severe histopathological lesions on the prostate, which impair the gland functionality, and may result in metastatic processes.

Tissue homeostasis occurs through specific proliferation and cell death pathways, which require strict control to prevent the development of neoplasms. Imbalance in the interplay between proliferation and cell death is responsible for the onset of injuries, including cancer (Bianco et al., 2023; Medda et al., 2021; Peixoto et al., 2017). Although we did not observed alterations in the expression of PHH3, an important marker of cell proliferation, 2,4-D and MIX-exposed animals showed a decreased percentage of caspase-3 expression in the epithelium and stroma, also evidenced by cell death and the proliferation ratio. Caspase-3 is associated with the final control of apoptosis induction, which is widely expressed in prostate epithelial tissue under normal conditions (O'Neill et al., 2001). The loss of an intrinsic apoptotic control, in favor of cell survival, is responsible for the onset and progression of PCa, and is seen as a strategy to prevent cell death (Acar et al., 2021; D'Arcy, 2019; Zhong et al., 2022). In this instance, the reduction in caspase-3 and consequent inhibition of apoptosis by 2,4-D and MIX may impair the elimination of damaged cells, favoring cell survival in both compartments of the prostate, in addition to creating a pro-proliferative and dysfunctional microenvironment.

Androgens play fundamental roles in maintaining the functionality of the prostate, which is highly responsive to hormonal activity (Penning and Detlefsen, 2020; Zhang et al., 2024). Androgens, such as dihydrotestosterone (DHT) and testosterone, stimulate the production of prostatic secretion, in addition to regulating prostate growth (Lima et al., 2020; Oliveira et al., 2007). The reduction in serum testosterone levels seen in animals individually exposed to GLY, ATZ, and 2,4-D suggests that the herbicides have a direct impact on the testicular androgen biosynthesis pathway, which can impair sexual function and prostate functionality (Abdel-Razik et al., 2021; Lu et al., 2022). Previous studies showed that individual exposure to GLY, ATZ and 2,4-D

caused a reduction in testosterone levels, due to testicular damage and oxidative stress, which can result in Leydig cell dysfunction (Glover et al., 2022; Romano et al., 2010; Victor-Costa et al., 2010; Zhang et al., 2017). Interestingly, in our study, testosterone levels did not differ in animals exposed to the mixture, suggesting that the toxicodynamic and toxicokinetic interactions of the herbicides had an antagonistic effect on testosterone secretion (Hernández et al., 2013; Peng et al., 2022).

Estrogens regulate complex physiological processes in the prostate, and are mainly related to cell proliferation, tissue disorganization, and abnormal estrogen levels, which are linked to prostate carcinogenesis by binding to alpha ($ER\alpha$) and beta ($ER\beta$) receptors (Liu et al., 2020; Silva et al., 2023). Despite the absence of alterations in estradiol levels, we found a high percentage of $ER\alpha$ expression in epithelial and stromal cells of 2,4-D and MIX-exposed animals. This receptor is often related to inflammation induction, and the onset of potentially malignant lesions (Fleury et al., 2021), which corroborates our previously reported histopathological findings. As a result, high $ER\alpha$ immunoreactivity in the epithelium and stroma, suggests a high responsiveness to estrogenic activity in prostate tissue, which may also favor the proliferation of the gland, and could also disrupt the homeostatic balance of sex steroid hormones. Furthermore, the increase in $ER\beta$ expression in the MIX group, confirmed by western blotting, is attributed to a protective and compensatory role mediated by this receptor, since $ER\beta$ can negatively regulate $ER\alpha$ transcription capacity (Leonel et al., 2020). Hence, we suggest that both 2,4-D and MIX acted as endocrine disruptors, and the morphophysiological alterations reported in the prostate arose due to hormonal alterations, such as a decrease in testosterone levels and imbalance in $ER\alpha$ expression.

5. Conclusion

The current study presents comprehensive and novel data on the main impacts caused by commercial formulations containing glyphosate, atrazine, and 2,4-D herbicides, as well as their mixture, on prostate morphophysiology, by simulating a safe drinking water scenario. Although glyphosate exposure caused slight alterations in the prostate, its mixture with the other herbicides may cause more extensive impacts on the prostate. We conclude that prolonged ingestion of low concentrations of the herbicides tested, mainly 2,4-D and the mixture, is not safe for the prostate and may compromise reproductive function in the long term. Therefore, strict control of the quality of drinking water is essential, considering the risks to reproductive health.

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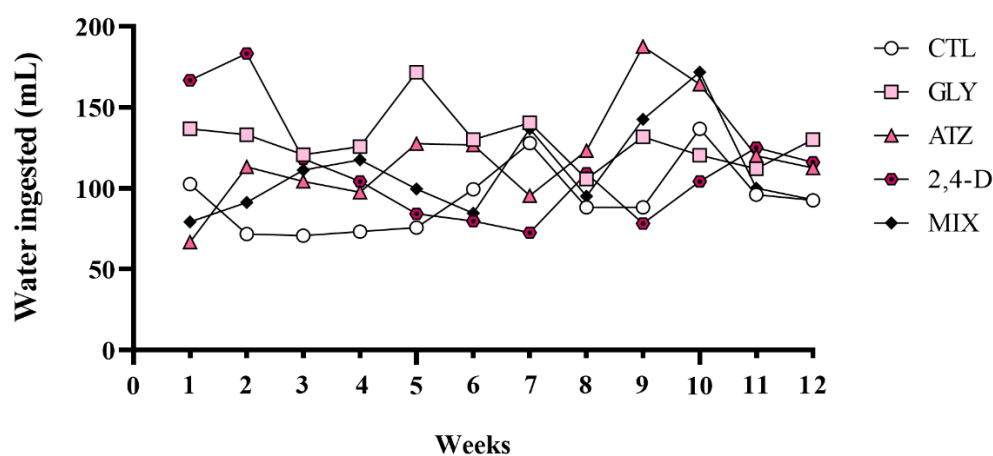
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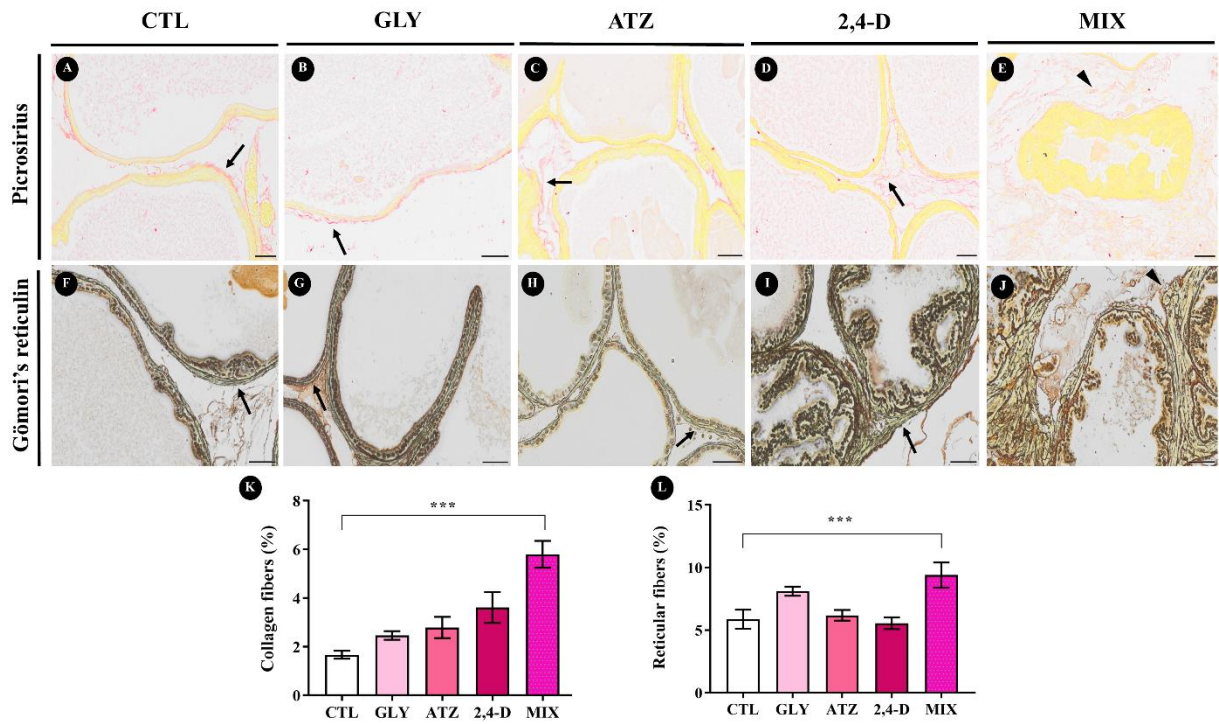
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Figure 1



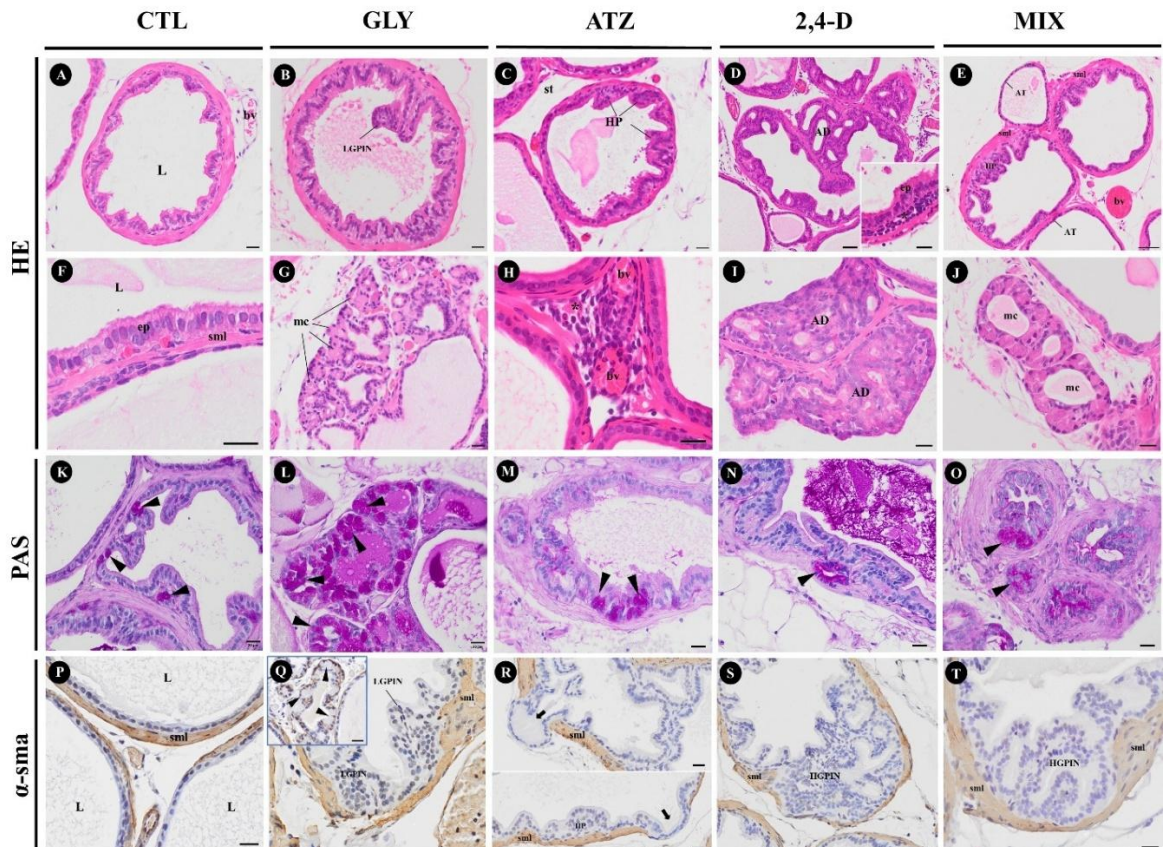
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Figure 2



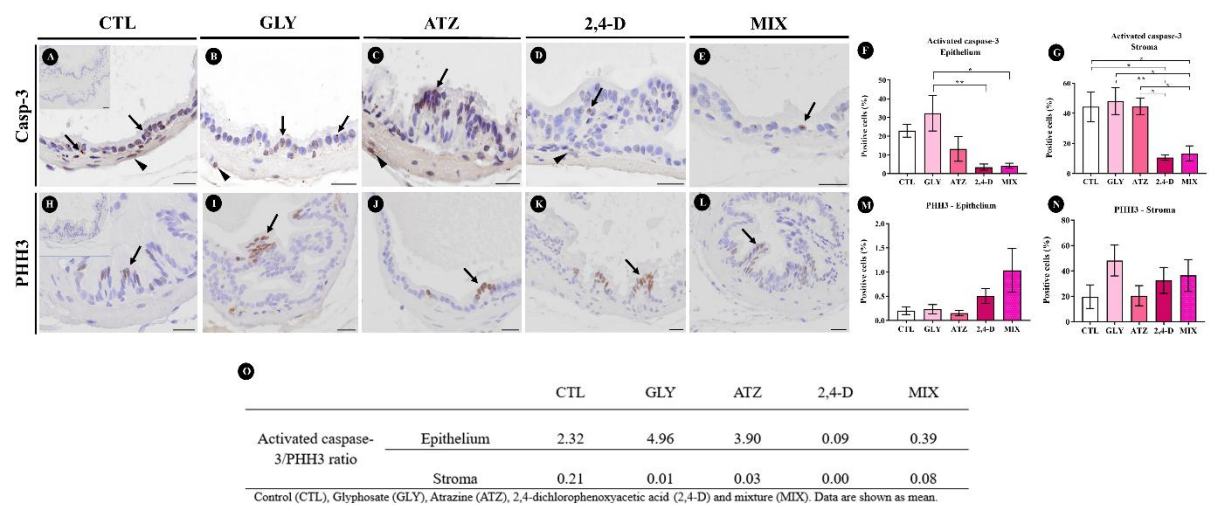
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Figure 3



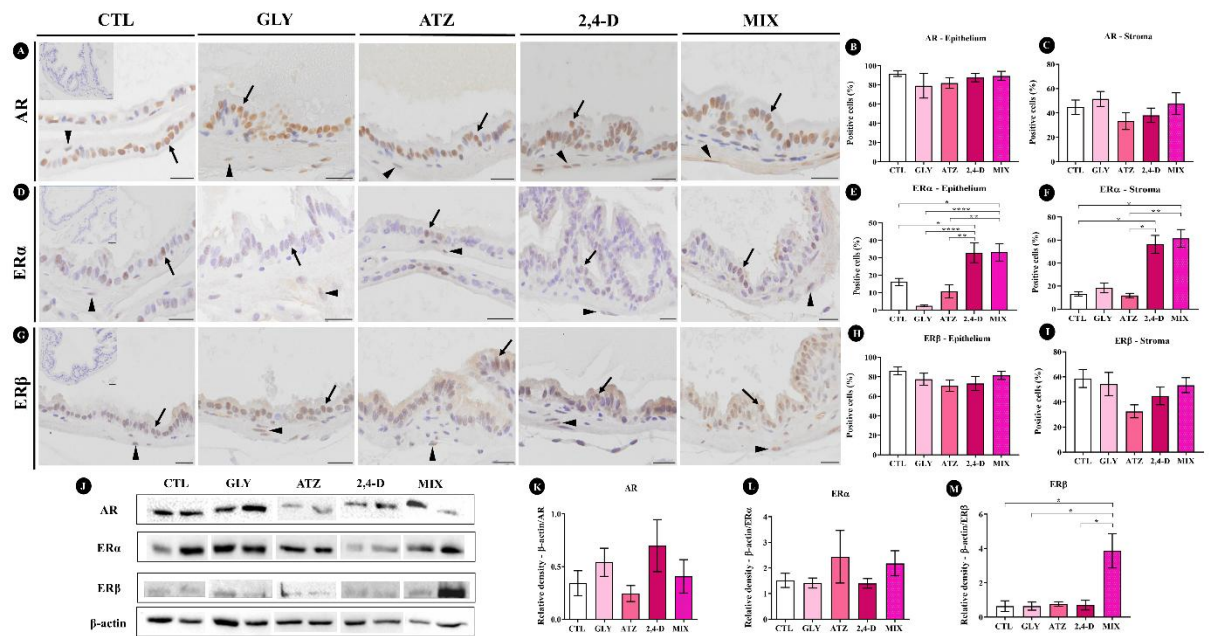
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Figure 4



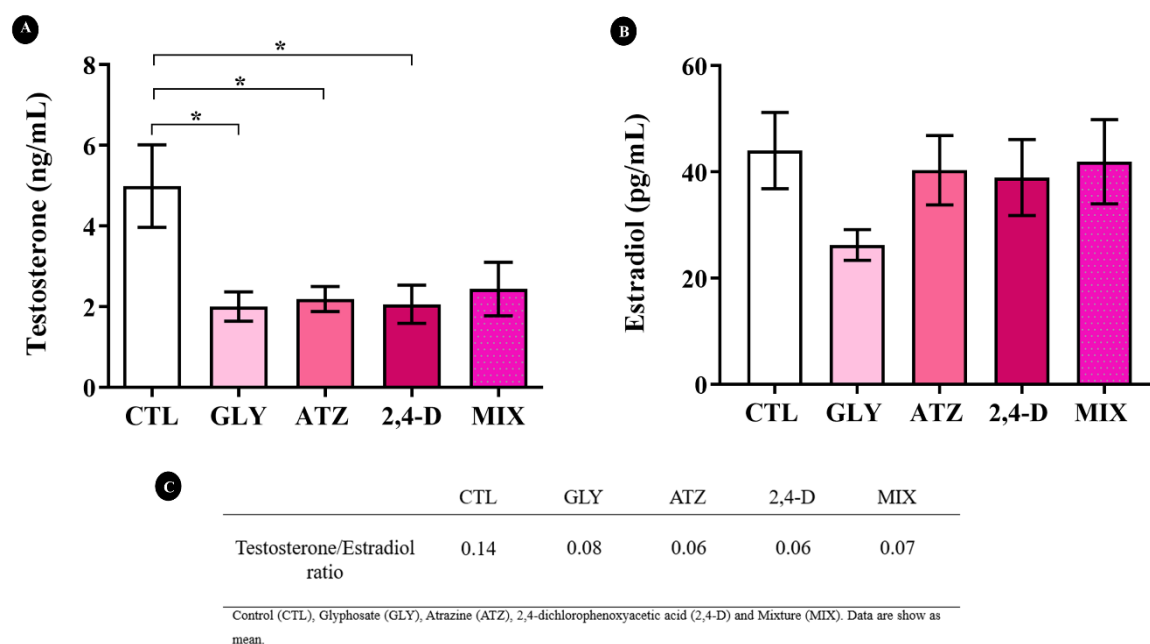
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Figure 5



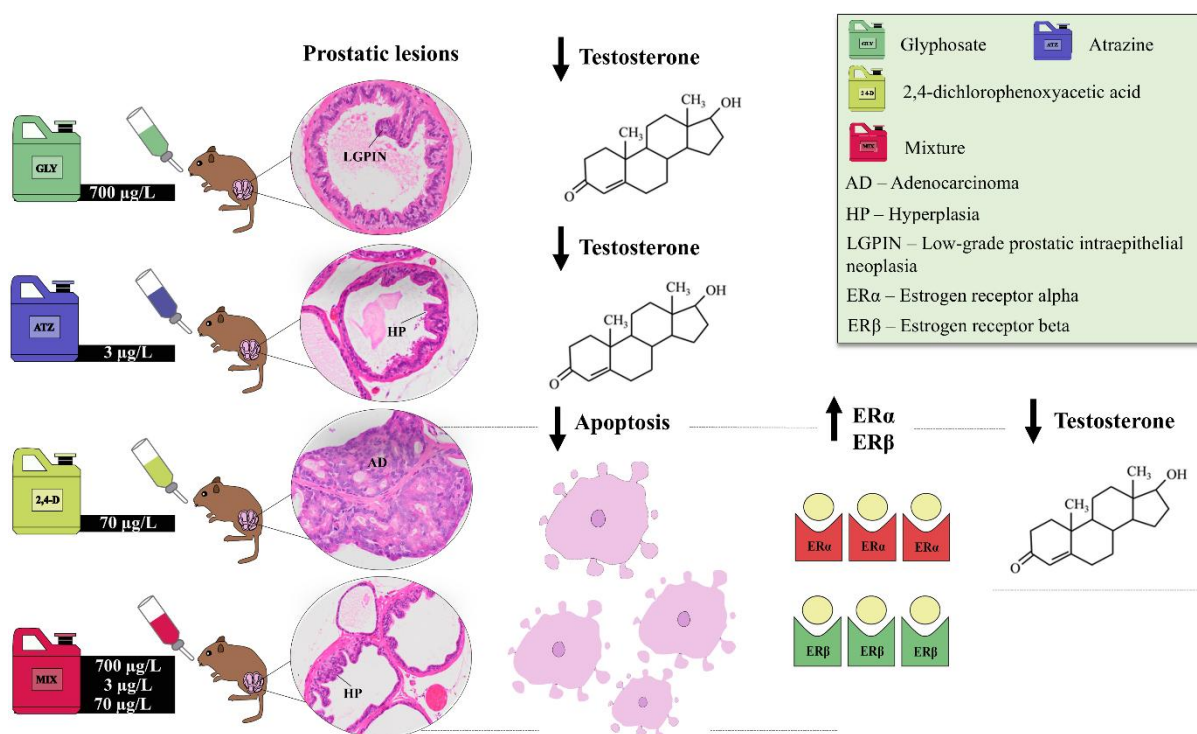
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Figure 6

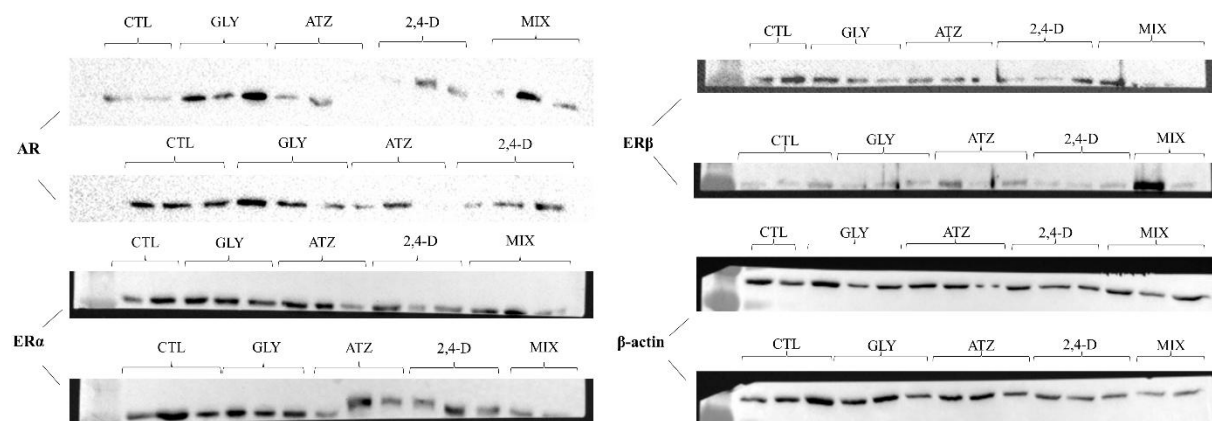


Source: Extracted from Silva *et al.* (2024)

Figure 7



Source: Extracted from Silva *et al.* (2024)

Supplementary Figure 1

Source: Extracted from Silva *et al.* (2024)

Table 1: Biometric data analyzed in Mongolian gerbils exposed to commercial formulations containing GLY, ATZ, 2,4-D, and their MIX.

	CTL	GLY	ATZ	2,4-D	MIX
Body weight (g)	77.60 ± 1.85	75.80 ± 1.98	76.70 ± 1.97	80.40 ± 4.12	77.64 ± 1.94
<i>Body fat</i>					
Absolute weight (g)	1.74 ± 0.25	1.76 ± 0.20	1.89 ± 0.23	2.64 ± 0.68	1.90 ± 0.40
Relative weight (g)	2.21 ± 0.02	2.32 ± 0.02	2.44 ± 0.02	2.55 ± 0.02	2.01 ± 0.04
<i>Prostatic complex</i>					
Absolute weight (g)	0.27 ± 0.00	0.29 ± 0.01	0.33 ± 0.02	0.31 ± 0.01	0.26 ± 0.00
Relative weight (x10 ³)	3.49 ± 0.07 ^a	3.94 ± 0.02 ^{ab}	4.40 ± 0.02 ^b	3.97 ± 0.01 ^{ab}	4.33 ± 0.01 ^b
<i>Ventral lobe</i>					
Absolute weight (g)	0.026 ± 0.001 ^{ab}	0.025 ± 0.002 ^{ab}	0.031 ± 0.002 ^a	0.026 ± 0.002 ^{ab}	0.020 ± 0.001 ^b
Relative weight (x10 ⁴)	3.34 ± 0.14 ^{ab}	3.63 ± 0.51 ^{ab}	4.11 ± 0.28 ^a	3.32 ± 0.23 ^{ab}	2.86 ± 0.23 ^b

Control (CTL), Glyphosate (GLY), Atrazine (ATZ), 2,4-dichlorophenoxyacetic acid (2,4-D), and Mixture (MIX). Data are shown as mean ± SEM. a,b Different letters indicate statistical differences among groups ($p \leq 0.05$).

Source: Extracted from Silva *et al.* (2024)

Table 2: Stereological and morphometric data analyzed in Mongolian gerbils exposed to commercial formulations containing GLY, ATZ, 2,4-D, and their MIX.

	CTL	GLY	ATZ	2,4-D	MIX
Stereological data (%)					
Epithelium	14.90 ± 1.38	15.27 ± 1.03	16.68 ± 2.25	18.41 ± 1.87	17.90 ± 2.18
Muscular stroma	6.91 ± 0.58 ^a	7.48 ± 0.79 ^{ab}	7.16 ± 0.47 ^{ab}	9.00 ± 0.65 ^{ab}	10.18 ± 0.99 ^b
Stroma	16.56 ± 1.47 ^{ab}	19.62 ± 2.70 ^a	10.43 ± 1.02 ^b	14.10 ± 2.02 ^{ab}	10.07 ± 1.22 ^{ab}
Lumen	60.24 ± 2.29	56.15 ± 4.50	65.16 ± 3.10	57.59 ± 1.91	53.34 ± 2.46
Blood vessel	1.38 ± 0.27	1.47 ± 0.31	0.56 ± 0.12	0.89 ± 0.19	1.29 ± 0.33
Morphometric data (µm)					
Epithelium height	18.20 ± 0.51 ^a	17.89 ± 0.80 ^a	18.01 ± 1.90 ^a	22.00 ± 1.49 ^{ab}	24.98 ± 2.26 ^b
SML thickness	11.07 ± 0.58 ^a	9.72 ± 0.84 ^a	9.55 ± 0.55 ^a	10.96 ± 0.69 ^a	15.09 ± 1.19 ^b

Control (CTL), Glyphosate (GLY), Atrazine (ATZ), 2,4-dichlorophenoxyacetic acid (2,4-D), Mixture (MIX), and Smooth muscle layer (SML). Data are shown as mean ± SEM. a,b Different letters indicate statistical differences among groups ($p \leq 0.05$).

Source: Extracted from Silva *et al.* (2024)

Table 3: Incidence and multiplicity of prostatic lesions of Mongolian gerbils exposed to commercial formulations containing GLY, ATZ, 2,4-D, and their MIX.

	Incidence (%)					Multiplicity				
	CTL	GLY	ATZ	2,4-D	MIX	CTL	GLY	ATZ	2,4-D	MIX
Epithelial atrophy	100	100	100	83.3	83.3	1-9 (3.9) ^a	0-12 (2.8) ^{ab}	0-8 (2.0) ^{ab}	0-4 (0.7) ^b	0-4 (0.8) ^b
<i>Epithelial atypia</i>										
Flat	100	100	100	100	100	0-6 (2.0)	0-6 (2.8)	0-6 (2.9)	0-5 (1.7)	0-5 (1.5)
Papillary	100	100	100	100	100	1-9 (5.8)	2-11 (6.1)	0-13 (6.1)	0-10 (4.5)	0-9 (3.6)
Hyperplasia	100	100	100	100	100	1-16 (7.1) ^a	2-19 (11.3) ^{ab}	5-25 (15.6) ^b	3-26 (13.2) ^{ab}	5-23 (11.8) ^{ab}
<i>PIN</i>										
LGPIN	100	100	100	100	100	0-8 (3.6) ^a	2-12 (5.9) ^{ab}	3-18 (11.3) ^b	3-24 (11.5) ^b	4-20 (10.6) ^b
HGPIN	100	100	100	100	100	0-8 (3.8)	0-7 (4.0)	1-13 (6.3)	0-10 (5.1)	0-6 (3.3)
Total	100	100	100	100	100	0-8 (7.5) ^a	0-12 (10.0) ^{ab}	1-18 (17.6) ^b	0-24 (16.6) ^b	0-20 (14.0) ^{ab}
Adenocarcinoma	0	16.6	83.3	100	83.3	0 (0) ^a	0-1 (0) ^a	0-2 (0.7) ^{ab}	0-3 (1.8) ^b	0-2 (0.8) ^{ab}
Microinvasive carcinoma	66.6	66.6	100	50	50	0-1 (0.4)	0-2 (0.5)	0-3 (0.9)	0-1 (0.2)	0-1 (0.1)
Mucinous metaplasia	50	33.3	66.6	33.3	66.6	0-4 (0.6)	0-17 (3.6)	0-2 (0.5)	0-1 (0.1)	0-2 (0.6)
Microacini	100	100	100	100	100	1-17 (6.8) ^a	0-13 (4.0) ^{ab}	0-18 (4.7) ^{ab}	0-13 (4.9) ^{ab}	0-5 (1.6) ^b
<i>Inflammation</i>										
Intraluminal	50	16.6	50	16.6	33.3	0-1 (0.25)	0-3 (0.55)	0-1 (0.27)	0-1 (0.05)	0-1 (0.16)
Periductal	83.3	100	100	100	83.3	0-5 (1.5)	0-3 (1.2)	0-3 (1.5)	0-3 (0.7)	0-2 (0.5)
Subepithelial	100	100	100	100	83.3	0-7 (2.2) ^a	0-4 (1.7) ^{ab}	0-3 (1.7) ^{ab}	1-3 (1.8) ^{ab}	0-2 (0.6) ^b

Control (CTL), Glyphosate (GLY), Atrazine (ATZ), 2,4-dichlorophenoxyacetic acid (2,4-D), Mixture (MIX), Prostatic Intraepithelial Neoplasia (PIN), low-grade PIN (LGPIN), and high-grade PIN (HGPIN). Data are shown as mean. a,b Different letters indicate statistical differences among groups ($p \leq 0.05$).

Source: Extracted from Silva *et al.* (2024)

3.2 PERINATAL EXPOSURE TO BPA LEADS TO PRONOUNCED PROSTATIC MORPHOPHYSIOLOGICAL DISORDERS IN A RODENT MODEL OF INDUCED HYPERPLASIA

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O presente artigo segue as normas do periódico.

Perinatal exposure to BPA leads to pronounced prostatic morphophysiological disorders in a rodent model of induced hyperplasia

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Abstract

Bisphenol A (BPA) is a ubiquitous endocrine disruptor potentially harmful to male reproductive health. We aimed to investigate the impacts of perinatal exposure to a historically relevant and realistic dose of BPA on the ventral prostate under normal conditions and with prostatic hyperplasia in Mongolian gerbils (*Meriones unguiculatus*). Females were exposed to BPA (50 µg/kg/day) during gestation and lactation. The F1 male offspring were maintained until adulthood and subsequently treated with testosterone to induce prostatic hyperplasia. Morphological, molecular, and hormonal parameters were assessed on the ventral prostate. Testosterone-supplemented gerbils showed increased epithelium height and smooth muscle layer thickness. In the context of hyperplasia, perinatal exposure to BPA led to the onset of severe histopathologies (e.g., prostatic intraepithelial neoplasia, adenocarcinoma, and microacini), associated with increased cell proliferation. Perinatal BPA-exposed gerbils with prostatic hyperplasia showed increased pro-inflammatory markers (e.g., IL-6, COX-2, and F4/80), followed by a reduction in IL-10 protein levels. Regarding the steroid receptors, gerbils from this group presented a decrease in AR, followed by an increase in epithelial ERα expression. Molecularly, ERβ protein levels were higher in the prostate of perinatally exposed to BPA or testosterone-supplemented gerbils. Moreover, serum testosterone and estradiol levels increased after testosterone supplementation, whereas the T/E2 ratio increased in gerbils exposed to both treatments. Overall, the current study presents novel and comprehensive data on the life-long morphophysiological disorders caused by perinatal exposure to BPA on the ventral prostate of gerbils, highlighting the pronounced impacts observed in the context of hyperplasia.

Keywords: Endocrine-disrupting chemicals; inflammation; steroid receptors; testosterone; ventral male prostate.

Highlights

- Our novel study assessed the effects of BPA on the F1 gerbil prostate;
- Most prostatic disorders were found in gerbils with prostatic hyperplasia;
- BPA plus testosterone exhibited pro-inflammatory responses in the gerbil prostate;
- Perinatal BPA exposure affects long-term prostatic health.

1. Introduction

The increasing exposure to endocrine-disrupting chemicals (EDCs) represents a current environmental and public health concern, as the recurring contact with these substances is associated with reproductive dysfunctions across populations (Chen et al., 2022; Metcalfe et al., 2022). Scientific evidence indicates that these adverse effects are not only restricted to those primarily exposed, but also reflect on offspring developmental reprogramming through epigenetic mechanisms (Xin et al., 2015). In this context, the Developmental Origins of Health and Disease (DOHaD) hypothesis emphasizes that early life experiences permanently interfere with health status across the life course (Heindel et al., 2017). This scientific field highlights the potential multigenerational risk posed by daily exposure to EDCs, such as phthalates, plasticizers, and pesticides (Karlsson et al., 2021; Mahalingam et al., 2017; Rebuzzini et al., 2022).

Among the plasticizers, Bisphenol A (BPA) is a xenoestrogen and a major component of polycarbonate and epoxy resins, used in the production of plastics and

cosmetics (Figueiredo et al., 2020; Ishtiaq et al., 2024). BPA exposure occurs through inhalation, skin contact, and diet, causing adverse cellular effects, including DNA methylation (Santangeli et al., 2019), proteome changes in male germ cells (Rahman et al., 2021), deficiency in sperm motility (Mostari et al., 2022), and developmental disorders (Welch and Mulligan, 2022). Moreover, the mechanisms of BPA toxicity also include unintended binding to steroid receptors, such as estrogen receptor alpha (ER α) and beta (ER β), through interaction in the estrogen signaling pathways, which are important for regulating hormones in females and males (Liu et al., 2021).

The complex effects resulting from primary exposure to BPA have been well documented (Batista-Silva et al., 2025; Segovia-Mendoza et al., 2020; Song et al., 2021). Additionally, the multigenerational and transgenerational effects of BPA have been explored due to its ability to cross the placenta and disrupt key stages of fetal development, particularly during immune cell development and reproductive system maturation (Schönfelder et al., 2002). Furthermore, the fetal ability to metabolize and biotransform xenobiotics and toxic compounds remains underdeveloped at these stages (Xin et al., 2015). Perinatal exposure to BPA can result in recruitment and mobilization of inflammatory cells, damage to germ cells, and genotypic modifications, revealing greater susceptibility and cellular reprogramming during this period (Bonaldo et al., 2023; Jorgensen et al., 2016; Leonel et al., 2020).

The prostate stands out as a key hormone-responsive gland in males (Silva et al., 2024). Prostatic morphogenesis begins in the embryonic stage and continues until after sexual maturity is acquired. During this critical life stage, an accurate hormonal control is essential to maintain the gland's homeostasis (Lima et al., 2015). At this stage, exposure to EDCs may reprogram the prostate, making it more susceptible to proliferative insults such as benign prostatic hyperplasia (BPH) and cancer (Prins et

al., 2008; Scarano et al., 2019). BPH is a common condition that affects around 50% of men over the age of 50, driven in part by the androgenic insults that occur during aging (Guerra et al., 2024). Besides hormonal factors, the continuous exposure to EDCs, such as BPA, can impair the prostatic function in the long term, primarily due to their undesired binding to steroid hormones, which regulate the intrinsic development of the prostate (Lacouture et al., 2022; Sánchez et al., 2022). Despite advanced knowledge about the multigenerational effects caused by perinatal exposure to BPA, the long-term impact on the adult prostate remains poorly explored and requires further investigation. Additionally, the potential influence of perinatal exposure to BPA on adult hyperplastic prostate is still unknown.

Based on the above, the current study was designed to investigate the impacts of perinatal exposure to a historically relevant and realistic dose of BPA in the prostate of adult gerbils, under normal conditions and with prostatic hyperplasia. We postulated that BPA exposure during early development might reprogram the developing prostate, resulting in latent effects that become evident in adulthood. Using a rodent model with prostatic hyperplasia, we also hypothesized that these impacts may be amplified in testosterone-induced hyperplastic gerbils, resulting in more pronounced prostatic morphophysiological disorders. Particularly, our study focuses on the ventral prostate morphology, histopathology, immunohistochemical, and molecular expression of markers of cell proliferation and death, inflammation, steroid receptor expression, as well as hormone regulation in Mongolian gerbils.

2. Materials and Methods

2.1 Ethics approval statement

All experimental procedures involving the animals were conducted following the guidelines and recommendations of the National Council for Animal Control and Experimentation (CONCEA, Brazil) and approved by the Ethics Committee on the Use of Animals (CEUA, IBILCE, UNESP), protocols 235/2022 and 236/2022.

2.2. Animals and experimental treatment

2.2.1. BPA perinatal exposure protocol

Forty healthy female Mongolian gerbils (*Meriones unguiculatus*), 3 months of age, were mated with fertile males of the same age to form independent families (total of 40 pairs). After birth, the first litter was discarded to induce a second pregnancy in the females and to determine the first day of pregnancy. Discarding the first litter allows the exclusion of potential maternal stress caused by the initial pregnancy. Pregnant gerbils were subjected to the following experimental treatments: Bisphenol A (BPA) (n=20) – animals received a dose of 50 µg/kg/day of Bisphenol A (239.658, Sigma Aldrich, ≥ 99% purity) in 0.1 mL of corn oil (vehicle), via oral gavage, from the implantation day until the end of lactation (8th day of gestation and 21st day post-birth, respectively), totaling 39 days of exposure, following a protocol previously described (Ruiz et al., 2023); Control (C) (n=20) – animals received 0.1 mL of corn oil (vehicle), via oral gavage, during the same period. The chosen dose of BPA is within the range of concentrations reported in human biological samples (Casas et al., 2013; Lee et al., 2008). In addition, corresponds to a legacy regulatory level historically established by the Environmental Protection Agency (US EPA) and the European Food Safety Authority guidelines (EFSA) (EFSA, 2015; Vom Saal and Vandenberg, 2021). Although this dose exceeds the current safety thresholds, higher doses elicit measurable toxicological responses in a specific exposure period, such as the endocrine sensitization window.

At the end of lactation, one male offspring from each litter was separated and individually maintained in cages until adulthood (6 months of age). The remaining offspring were distributed to other studies.

2.2.2. *Postnatal treatments*

At 6 months of age, F1 adult males (N=40) were randomly distributed to the following experimental groups (n=10/group): Control (C) – animals perinatally treated with corn oil (vehicle); Bisphenol A (BPA) – animals perinatally exposed to BPA (50 µg/kg/day); Testosterone (T) – animals perinatally treated with corn oil (vehicle) that received, on alternate days and for 30 days, subcutaneous injections of testosterone cypionate (3 mg/kg, Deposteron, EMS, ≥ 99% purity) in 0.1 mL of corn oil; Bisphenol A plus Testosterone (BPA+T) – animals perinatally exposed to BPA (50 µg/kg/day) that received, on alternate days and for 30 days, subcutaneous injections of testosterone cypionate (3 mg/kg, Deposteron, EMS, ≥ 99% purity) in 0.1 mL of corn oil. The testosterone doses and pattern of administration were chosen based on a previous model for inducing hyperplasia in rodents of the same species (Castro et al., 2021).

To avoid any contamination, during all the experimentation, the pregnant and F1 gerbils were kept individually in BPA-free cages, on racks equipped with air exhaustion at a temperature of 22 ± 2 °C, controlled photoperiod (12L – 12D), and supplied *ad libitum* with water in BPA-free bottles (Alesco, Monte Mor, São Paulo, Brazil) and specific food (LABIMIX, Agromix – Brazil). The respective treatments were carried out at the Biotherium of the Department of Biological Sciences of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State University (UNESP).

2.3. Sampling

All gerbils were euthanized on the same day, at 7 months of age, after completing 30 days of postnatal treatment. The gerbils were weighed, euthanized by anesthetic overdose with Isoflurane 3%, and decapitated. For hormonal dosage, the blood was collected in specific tubes, centrifuged, and stored at -80°C. The prostatic complex, which comprises the anterior and dorsolateral lobes, was removed and weighed. Considering the anatomical position, the ventral prostate was identified, removed, weighed, and fixed in paraformaldehyde (4%, 24h) before being dehydrated in an increasing series of alcohol, processed, and embedded in paraffin in a Leica Semi Enclosed System (Histosec, Merck, Darmstad, Germany). The samples were sliced at 4 µm thickness for morphological, histopathological, and immunohistochemical evaluation (n = 6/group). To perform molecular assays, the ventral prostate was immediately removed, frozen, and stored at -80°C (n = 4/group). The adipose tissue samples (epididymal, visceral, and retroperitoneal fat) were also removed and weighed to obtain body fat parameters.

2.4. Morphological analysis

Morphological analyses included quantification of stromal fibers, as well as stereological and morphometric determination. To evaluate the stromal fibers, the histological sections were stained with picrosirius red (collagen fibers) and Gömöri's reticulin (reticular fibers). The histological sections of the ventral prostate were also stained with Hematoxylin-Eosin (HE) for general morphological, stereological, morphometric, and histopathological analyses. The collagen and reticular fibers, as well as the relative volumes of prostate tissue components (epithelium, muscular stroma, and non-muscular stroma), were calculated. For this purpose, 60 ventral

prostate fields per experimental group (15 fields/animal) were scanned at 400x magnification (BX61VS camera, Olympus) and analyzed by random sampling. The relative frequency was performed blind and quantified using the M130 multipoint test (Huttunen et al., 1981; Weibel, 1963), through Image Pro-Plus 6.0 software (Media Cybernetics, Inc., MD, USA). To perform morphometric analysis, the height of the epithelium and the smooth muscle layer (SML) thickness were determined in 450 measurements from each experimental group (75 measurements per animal) under light microscopy, using ImageJ software.

2.5. Histopathological study

Epithelial lesions, such as hyperplasia, atrophy, atypia (flat and papillary), prostatic intraepithelial neoplasia (PIN), low-grade PIN (LGPIN), high-grade PIN (HGPIN), total PIN, epithelial detachment, buds, microacini, microinvasive carcinoma, adenocarcinoma, and inflammatory foci (periductal, intraluminal, and subepithelial) were identified and quantified. The ventral prostate sections stained with HE were used to quantify the incidence and multiplicity of respective prostatic lesions in the experimental groups, by employing previously documented methodologies applied to gerbils (Gonçalves et al., 2013; Shappell et al., 2004). The α -smooth muscle actin (α -sma) technique was used to identify buds and microinvasive carcinomas. The incidence was expressed as the number of animals exhibiting each type of lesion (%), and the multiplicity was expressed by quantifying the number of lesions found in each histological section analyzed (average number of lesions/group). The entire histopathological study was carried out blindly in 3 different sections of a slide per animal (n=6/group), using the QuPath software (Version 0.4.2).

2.6. Immunohistochemistry procedures and analysis

Immunohistochemistry techniques were employed to evaluate biomarkers related to tissue integrity, cell proliferation and death, inflammatory profile, and steroid receptor expression. Representative histological sections of the ventral prostate were dewaxed, rehydrated, and submitted to antigen retrieval. The endogenous peroxide activity was blocked with 10% hydrogen peroxide in methanol for 20 min, followed by nonspecific protein blockage with 5% skimmed milk for 60 min. The sections were washed with buffer and incubated with their specific primary antibodies diluted in BSA 1% overnight at 4° C. BSA 1% was also used as a negative control. The slides were incubated with post-primary antibodies and polymer (Novolink™ polymer detection system 1, Leica Biosystems Newcastle Ltd.) for 60 min at 37°C or room temperature. Positive staining was detected with 3-30'-diaminobenzidine tetrahydrochloride (DAB, RE7270-CE, Leica Biosystems) and counterstained with hematoxylin. Epithelial and stromal immunostaining cells were analyzed to evaluate the α -sma and positive cells of phosphohistone-H3 (PHH3), activated caspase-3 (casp-3), interleukin-6 (IL-6), interleukin-10 (IL-10), cyclooxygenase-2 (COX-2), F4/80 macrophage, androgen receptor (AR), ER α , and ER β . The quantification and determination of positive cells were performed considering the total number of cells per compartment in one section per animal (n= 6/group), using the QuPath software (Version 0.4.2). The complete list of antibodies and protocols used in the immunohistochemical analyses is described in Table 1.

2.7. Immunoblotting assays

In order to quantify the protein levels, frozen ventral prostate samples were homogenized at 4 °C with an extraction buffer containing protease inhibitor (Protease Inhibitor Cocktail, P8340, Sigma Aldrich, St. Louis, MO, USA). The samples were submitted to electrophoresis in SDS page gel (12% polyacrylamide TRIS-glycine gels)

and transferred to nitrocellulose membrane (Amersham Protram, 10,600,003, GE Healthcare, Darmstadt, Germany). The membranes were incubated with 5% dry milk in TBSt for 60 min at room temperature and incubated with the following primary antibodies: IL-6 (anti-IL-6, rabbit monoclonal, 1:500, D5W4V, Cell Signaling), IL-10 (anti-IL-10, mouse monoclonal, sc-8438, 1:500, Santa Cruz Biotechnology), COX-2 (anti-COX-2, rabbit monoclonal, D5H5, 1:500, Cell Signaling), F4/80 (anti-F4/80, rabbit monoclonal, D2S9R, 1:500, Cell Signaling), AR (anti-AR, mouse monoclonal, sc-7305, 1:500, Santa Cruz Biotechnology), ER α (anti-ER α , mouse monoclonal, sc-8005, 1:500, Santa Cruz Biotechnology), ER β (anti-ER β , rabbit polyclonal, PA1-310B, 1:500, Invitrogen, Thermo Fisher), or β -actin (anti- β -actin, rabbit monoclonal, 13E5; 1:1000, Cell Signaling) overnight at 4 °C, and incubated with secondary antibodies (Antimouse IgG, HRP-linked Antibody, #7076, 1:2000, Cell Signaling or Antirabbit IgG, HRP-linked Antibody, #7074, 1:2000, Cell Signaling) for 2 h at room temperature. Subsequently, the membranes were incubated with Chemiluminescent Substrate (Cod. 34580, SuperSignal™ West Pico PLUS Luminol-Enhancer solution, ThermoFisher Scientific) and were revealed in an imaging system machine (ChemiDoc MP, BioRad, Hercules, CA, USA). The density of the bands was quantified using Image Lab software (BioRad Laboratories, Inc.) and was normalized using β -actin, as an endogenous protein.

2.8. Hormonal dosage

Serum total testosterone (T) and estradiol (E2) levels were quantified in duplicate in 6 animals/group, through enzyme immunoassay (EIA) commercial kits according to the supplier's protocol (USA Diagnostics; Cat No. USA 8005 and USA 9140).

2.9. Statistical analysis

Normal distribution of the data was checked by the Shapiro-Wilk test and submitted to one-way analysis of variance (ANOVA) followed by Tukey's test. Non-parametric data were subjected to the Kruskal–Wallis test followed by Dunn's test, using GraphPad Prism software (version 8.0, GraphPad Software Inc., San Diego, CA, USA). Results are expressed as mean $\pm$ standard error of the mean (SEM), considering a statistical significance level of 5% ($p \leq 0.05$).

3. Results

3.1. *Biometric data*

The biometric data included absolute and relative measures of body weight, body fat, prostatic complex, and the ventral prostate. However, these parameters did not show statistically significant differences among the experimental groups (Table 2).

3.2. *The prostate compartments were structurally remodeled by testosterone supplementation*

The ventral prostate of gerbils from the different experimental groups presented acini composed of simple cubic epithelium. The general morphology of the ventral prostate indicated the occurrence of acini embedded in a connective tissue composed of smooth muscle layer, blood vessels, reticular and collagen fibers that comprise the fibromuscular stroma, in addition to a luminal compartment. The picrosirius and Gömöri's reticulin techniques indicated that the percentage of collagen and reticular fibers remained unchanged among the experimental groups (Fig. 1). On the other hand, there were different alterations in the relative volume of the histological compartments of the prostate, as evidenced by the stereological analysis. An increase in the relative volume of epithelium was observed in the T compared to control ($p = 0.0238$) and BPA+T ($p = 0.0250$). There was an increase in the relative percentage of

muscular stroma in the T compared to all other groups ($p < 0.0001$). The volumetric percentage of the non-muscular stroma decreased in the BPA compared to the control ($p = 0.0001$) and BPA+T ($p = 0.0283$). The non-muscular stroma was also decreased in the T group compared to the control ($p = 0.0445$). The luminal compartment showed an increase in animals perinatally exposed to BPA compared to the T group ($p = 0.0379$). The relative volume of blood vessels did not differ between the experimental groups (Table 3). The morphometric analysis showed an increase in epithelium height and smooth muscle layer (SML) thickness in T compared to all other groups ($p < 0.0001$ and $p < 0.0010$) (Table 3).

3.3 BPA caused major prostate injuries in gerbils with prostatic hyperplasia

The ventral prostate of adult gerbils revealed an architecture consisting of acini surrounded by a simple cubic epithelium and a smooth muscular layer. The non-muscular stroma was rich in blood vessels and immune cells. The luminal compartment contained the prostatic fluid (Fig. 2 A-L). Meanwhile, alterations of different patterns were mainly identified in the prostate of gerbils perinatally exposed to BPA and with prostatic hyperplasia in adulthood. Analysis of the incidence and multiplicity of lesions showed an increase in hyperplasia foci in testosterone-supplemented animals compared to the control ($p = 0.0004$) and BPA ($p = 0.0010$). Proliferative disorders, such as low-grade prostatic intraepithelial neoplasia (LGPIN), were shown in gerbils from the BPA+T compared to those from the other experimental groups ($p = 0.0010$). High-grade prostatic intraepithelial neoplasia (HGPIN) was observed in BPA and BPA+T compared to control ($p = 0.0349$; $p = 0.0086$). Interestingly, total prostatic intraepithelial neoplasia (PIN total) was also observed in gerbils from the BPA ($p = 0.0186$) and BPA+T ($p < 0.0001$) groups compared to the control. The multiplicity of PIN total was also increased in the BPA+T group compared to T ($p = 0.0385$).

Alterations in epithelial dynamics were also seen in gerbils from the BPA+T group. Compared to control, there was an increase in atrophic epithelium in BPA+T ($p = 0.0056$). Gerbils from this group also presented an increase in epithelial detachment compared to all other groups ($p = 0.0056$). The number of microacini also increased in the BPA+T group compared to the T group ($p = 0.0284$). Moreover, malignant lesions such as adenocarcinoma were found in the prostate of gerbils from the BPA+T group when compared to the control ($p = 0.0162$). Finally, an increase in inflammatory foci was observed, including periductal inflammation in the BPA+T ($p = 0.0005$) and subepithelial inflammation in the T group ($p = 0.0057$), both compared to the control gerbils. Prostatic alterations such as planar atypia, papillary atypia, buds, microinvasive carcinoma, and intraluminal inflammation did not differ among the experimental groups (Fig. 2; Table 4).

3.4 BPA stimulated cell proliferation in gerbils with prostatic hyperplasia

Immunohistochemical analysis revealed an increase in epithelial expression of PHH3 in BPA+T compared to the control ($p = 0.0299$) and the T group ($p = 0.0232$) (Fig. 3A and B). The stromal expression of PHH3 and caspase-3 remained unchanged among the experimental groups (Fig. 3 C-F). However, the epithelial activated caspase-3/PHH3 ratio showed a decrease in the BPA+T compared to the control ($p = 0.0082$) (Fig. 3G).

3.5. BPA potentiates prostate inflammation in gerbils with prostatic hyperplasia

Here, we evaluated the incidence of different inflammatory markers and immune cells incidence on the adult gerbil prostate. IL-6, IL-10, COX-2 and F4/80-immunoreactive cells were determined through immunohistochemical and Western blotting techniques, and several alterations were reported. Immunoreactive cells for IL-

6, COX-2 and F4/80 were located in the stroma, while IL-10 was located in both the prostate epithelium and stroma (Fig. 4 A-U). An increase in IL-6-positive cells was observed in the prostate of gerbils from the T and BPA+T groups, compared to control ($p = 0.0348$; $p = 0.0114$) and BPA ($p = 0.0173$; $p = 0.0055$) (Fig. 4 A-E). COX-2 was present in the prostate of gerbils from all the experimental groups. However, there was an increase in the percentage of COX-2-positive cells in BPA+T when compared to the control ($p = 0.0027$) and T group ($p = 0.0122$) (Fig. 4 F-J). The macrophage infiltration, determined by the percentage of F4/80-positive cells, revealed an increase in BPA+T compared to all other groups ($p = 0.0009$) (Fig. 4 K-O). Although the percentage of IL-10-positive cells remained unchanged in the experimental treatments (Fig. 4 P-U), Western blotting analysis revealed a reduction in IL-10 protein concentration in BPA compared to control ($p = 0.0039$), and in the BPA+T group compared to control ($p = 0.0006$), and T ($p = 0.0137$) (Fig. 4X). Moreover, the protein concentration of COX-2 increased in animals exposed perinatally to BPA when compared to the other experimental groups ($p = 0.0068$) (Fig. 4Y). The relative density of F4/80 macrophages was not altered in the different experimental treatments (Fig. 4Z). The representative blots from Western blotting can be accessed in Supplementary Figure 1.

3.6. BPA and testosterone modulate the expression of steroid receptors on the ventral prostate

Immunoreactive cells as well as the protein expression of AR, ER α , and ER β were quantified, and several alterations were reported on the ventral prostate (Fig. 5). The epithelial expression of AR showed a decrease in BPA+T compared to the control ($p = 0.0423$) and BPA ($p = 0.0115$) (Fig. 5A and B). The stromal expression of AR also showed a decrease in BPA+T compared to BPA ($p = 0.0255$) (Fig. 5A and C). The ER α expression showed an increase in the epithelium from the BPA+T group

compared to the control ($p = 0.0011$) (Fig. 5D and E). Despite this, the stromal expression of ER α and ER β remained unaltered in the experimental groups (Fig. 5 F-I). Although the levels of AR and ER α proteins remained unchanged (Fig. 5 J-L), Western blotting analysis revealed increased ER β protein levels in the BPA ($p = 0.0413$) and T ($p = 0.0012$) groups compared to the control, as well as in the T group compared to the BPA+T ($p = 0.0014$) (Fig. 5J and M).

3.7. Testosterone and estradiol levels

As expected by the hormonal treatment, testosterone serum levels showed an increase in T and BPA+T compared to other groups ($p < 0.0001$) (Fig. 6A). Interestingly, E2 levels were also increased in T compared to the control ($p = 0.0099$) (Fig. 6B). The T/E2 ratio was also increased in the BPA+T compared to control ($p = 0.0211$) (Fig. 6C).

4. Discussion

To the best of our knowledge, this is the first study to assess the effects of perinatal exposure to BPA on the ventral prostate of adult male gerbils under both normal physiological conditions and testosterone-induced prostatic hyperplasia. To elucidate the impacts of a historically relevant and realistic dose of BPA exposure during a critical life stage and its long-term effects, we investigated morphological, molecular, and hormonal aspects. The data pointed to multiple morphophysiological outcomes on the ventral prostate, highlighting the gerbil as a relevant experimental model for studying long-term prostate disorders. The androgen intervention induced notable morphological alterations in the prostate, since the significant alterations were detected in testosterone-supplemented gerbils. Furthermore, gerbils perinatally exposed to BPA and supplemented with testosterone exhibited aggravated prostatic

outcomes, indicating that early-life BPA exposure leads to latent effects in the prostate, which are exacerbated in the context of hyperplasia in adulthood.

The biometric data remained unaltered, suggesting that treatments, individually or in combination, do not elicit overt systemic effects or measurable phenotypic modifications at the biometric level. Although there were no alterations in the percentage of collagen or reticular fibers, gerbils from the T group showed an increased volumetric percentage of epithelium and SML, as well as increased epithelium height and SML thickness. As expected, we also observed a higher incidence of hyperplastic foci in the T group, given the documented role of androgens in inducing prostatic hyperplasia (Biancardi et al., 2017; Pierorazio et al., 2010; Ramos et al., 2020). By the action of type 1 or type 2 5 α -reductase enzyme, testosterone is converted into dihydrotestosterone (DHT), a potent androgen that induces structural remodeling in the prostate, resulting in higher secretory activity and hypercellularity (Felisbino et al., 2019; Park et al., 2022). However, gerbils from the BPA+T group did not exhibit the same morphological alterations observed in the T group, revealing an antagonistic effect performed by BPA on the scenario of prostatic hyperplasia. It represents that perinatal exposure to BPA may reprogram the androgen responsiveness in the adult prostate, thereby counteracting androgen-induced structural disorders. Indeed, the antiandrogenic and estrogenic properties of the BPA are well documented in the literature (Cariati et al., 2019; Cimmino et al., 2020; Molina-Molina et al., 2013). Studies show that BPA impairs AR activation, transcriptional activity, and normal function (Pathak et al., 2024; Teng et al., 2013), which interrupts the binding of testosterone and might be a potential explanation for the structural integrity observed in the BPA+T group.

Alarming histological lesions were found on the ventral prostate of gerbils. In the context of severe injuries, we found an increased LGPIN, HGPIN, and total PIN in gerbils from the BPA and BPA+T groups. Changes in epithelial dynamics, such as atrophy, epithelial detachment, and microacini, were also seen in the BPA+T group. It appears to indicate a synergistic and potentiation of BPA when hyperplasia occurs, whereby perinatal exposure to BPA establishes a latent pro-proliferative and tumorigenic microenvironment that deteriorates in response to hyperplastic onset in adulthood (Wu et al., 2011). We also found areas affected by adenocarcinomas in the BPA+T group, suggesting an overall deterioration in histopathological features induced by BPA. In fact, the developmental exposure to BPA appears to play a pro-tumorigenic role, promoting the progression of carcinogenesis (Fatma Karaman et al., 2019; Prins et al., 2008; Taylor et al., 2020). In the current study, these latent morphological effects persist and remain evident upon the hyperplastic condition. Similarly, the perinatal exposure to BPA downregulated the expression of the *Mgmt* gene, involved in DNA repair and tumor suppression in adult Wistar rats (Sánchez et al., 2022). These findings support our histopathological data, wherein unrepaired prostate injuries became persistent and pronounced in adulthood, suggesting long-lasting epigenetic changes induced by BPA. The several prostate histopathologies observed in the BPA+T group are a consequence of the enhanced cell proliferation, as previously evidenced by increased epithelial PHH3 expression and reduced activated caspase-3/PHH3 ratio.

Perinatal BPA-exposed gerbils with prostatic hyperplasia induced by testosterone showed inflammatory insults, which can disrupt the normal functioning of the gland. BPA and testosterone have often been linked to the onset of inflammation in the prostate (Bernardo et al., 2015; Brandt et al., 2014; Castro et al., 2021; Facina et al., 2021). The increased expression of pro-inflammatory markers such as IL-6,

COX-2, and F4/80 indicates active inflammatory process, tumor angiogenesis, and macrophage infiltration, respectively (Castro et al., 2021; Song et al., 2017). IL-6 is regulated by oncogenes and tumor suppressors, and both COX-2 and F4/80 are known to be upregulated in prostate cancer and BPH (Culig, 2014; Kim et al., 2011; Wang et al., 2012). The divergence observed in COX-2 expression, as assessed by immunohistochemistry and Western blotting, likely reflects the intrinsic differences between the techniques. Immunohistochemistry allows the detection of the spatial distribution and relative abundance of the protein within specific cellular and tissue compartments, whereas Western blotting provides quantitative information on the overall protein content. Nevertheless, in the general biological context, gerbils perinatally exposed to BPA and presenting prostatic hyperplasia exhibited increased COX-2 levels, reinforcing the activation of a latent inflammatory status associated with the BPA exposure. Interestingly, we also observed a reduction in IL-10 protein levels in BPA and BPA+T gerbils. IL-10 is a key anti-inflammatory cytokine that plays a critical role in modulating immune responses by suppressing pro-inflammatory responses (Freitas et al., 2020; Zhang et al., 2024). This finding indicates that BPA induces a state of reprogramming process that, in the long term, establishes an unrepaired and persistent inflammatory microenvironment in the prostate, a condition even more pronounced in gerbils with prostatic hyperplasia. Considering the physiological role of the prostate in male fertility, persistent inflammatory insults can impair the normal functioning of the gland in addition to favoring the appearance of neoplasias, such as those reported in our histopathological data (Motrich et al., 2018).

Regarding the expression of steroid receptors, we observed a reduction in AR, followed by an increased ER α expression in BPA+T. These results agree with our morphological findings, since the decrease in AR may be related to a drop in androgen

sensitivity, as an attempt at involution of the gland and restoration of homeostasis (Antoniassi et al., 2017; Cordeiro et al., 2008). ER α is typically modulated under endocrine disruption. The increased response observed in gerbils perinatally exposed to BPA and presenting prostatic hyperplasia suggests the involvement of estrogen-related proliferation pathways, since ER α stimulates epithelial cell proliferation in the prostate (Sanches et al., 2017; Silva et al., 2024). On the other hand, the ER β protein levels were individually higher in perinatal BPA-exposed or testosterone-supplemented gerbils, whereas reduced in the combined treatment, which might be interpreted as an adaptive response and protective mechanism to mitigate the proliferative disorders since this receptor has antiproliferative and anti-inflammatory properties (Habib et al., 2015; Hong et al., 2022; Ren et al., 2021).

Testosterone and estradiol levels were increased in gerbils with prostatic hyperplasia. The excess exogenous testosterone possibly enhanced aromatase activity, which converts androgens into estrogens, explaining the elevated E2 levels in the T group (Allkanjari and Vitalone, 2015). Conversely, the increase in the T/E2 ratio in the BPA+T group suggests poor responsiveness and impaired estrogen signaling performed by BPA at physiological levels, leading to hormonal imbalance. Further studies should focus on identifying the precise molecular targets of developmental BPA exposure and its long-term consequences in the context of other reproductive pathologies, while also evaluating the translational relevance to human health.

5. Conclusion

In summary, the current study demonstrates that perinatal exposure to BPA leads to a long-lasting disruption in prostate health, which becomes more pronounced under hyperplastic conditions in adulthood. The observed alterations, which include structural remodeling, tissue injury, cell proliferation, inflammation, dysregulation of

steroid receptor expression, and hormonal imbalance, indicate that exposure to BPA in a crucial life stage reprograms the prostate to further pathological disorders. We emphasize the critical vulnerability of developmental windows to BPA exposure, also highlighting the long-term prostatic consequences, particularly in a hormonally challenged context.

Data availability

Data will be made available on request.

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Declaration of conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Stella Bicalho-Silva: Writing – original draft, Visualization, Methodology, Conceptualization. **Vitor Grigio:** Formal analysis, Methodology. **Thalles F. R. Ruiz:** Formal analysis, Methodology. **Marília F. Calmon:** Methodology. **Paula Rahal:** Resources. **Sebastião R. Taboga:** Funding acquisition, Resources, Visualization. **Fernanda C. A. Santos:** Writing – review & editing, Visualization. **Patrícia S. L. Vilamaior:** Data curation, Project administration, Writing – review & editing, Visualization.

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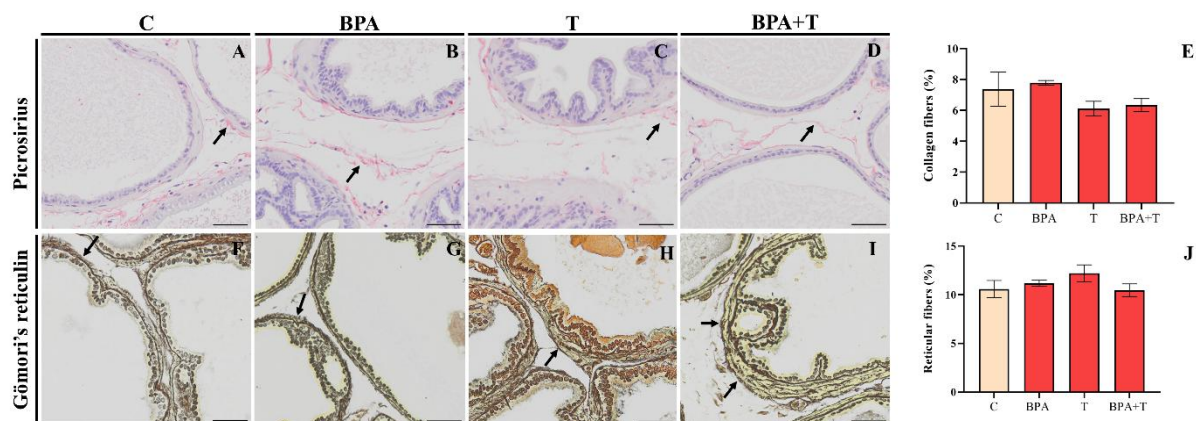
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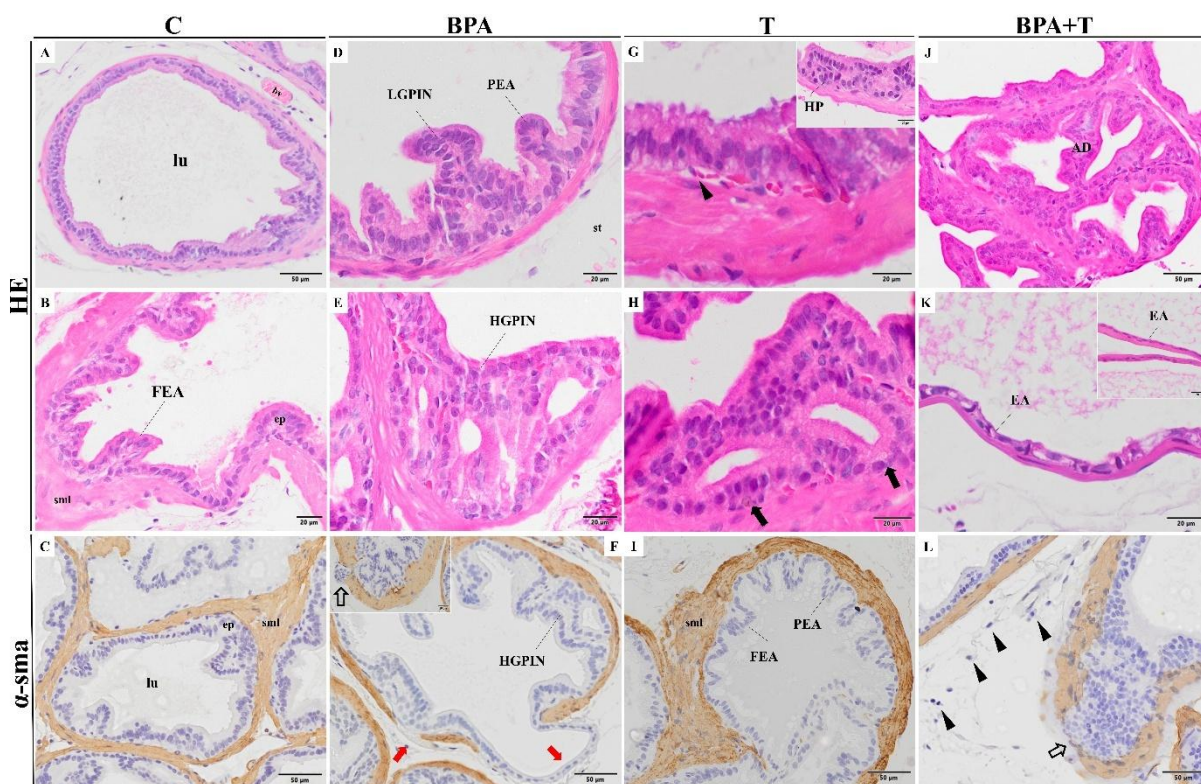
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Figure 1



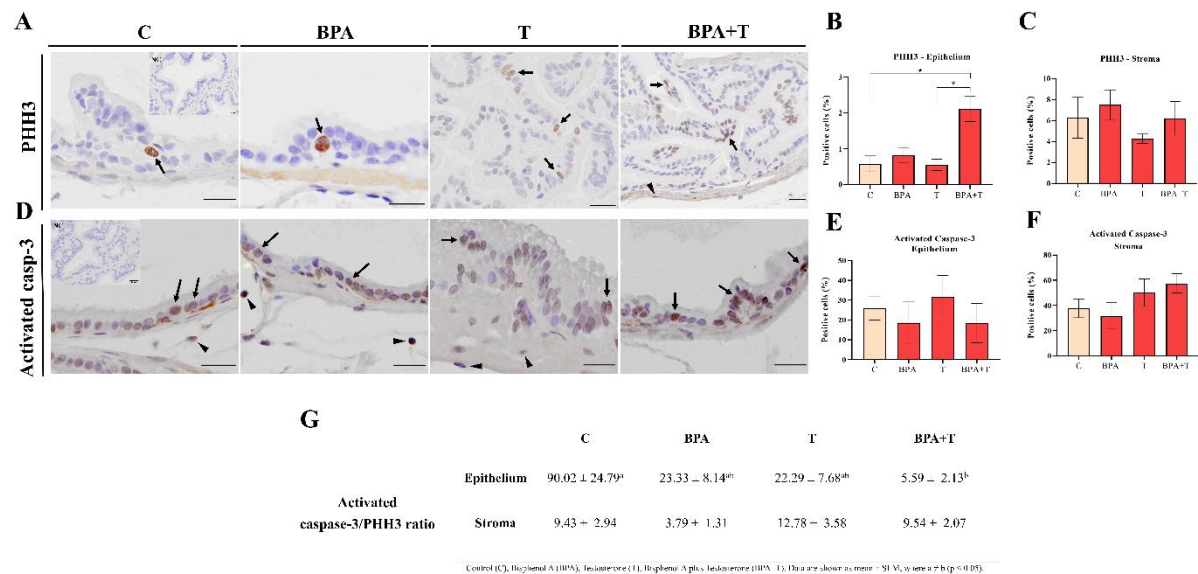
Source: Extracted from Bicalho-Silva *et al.* (2025)

Figure 2



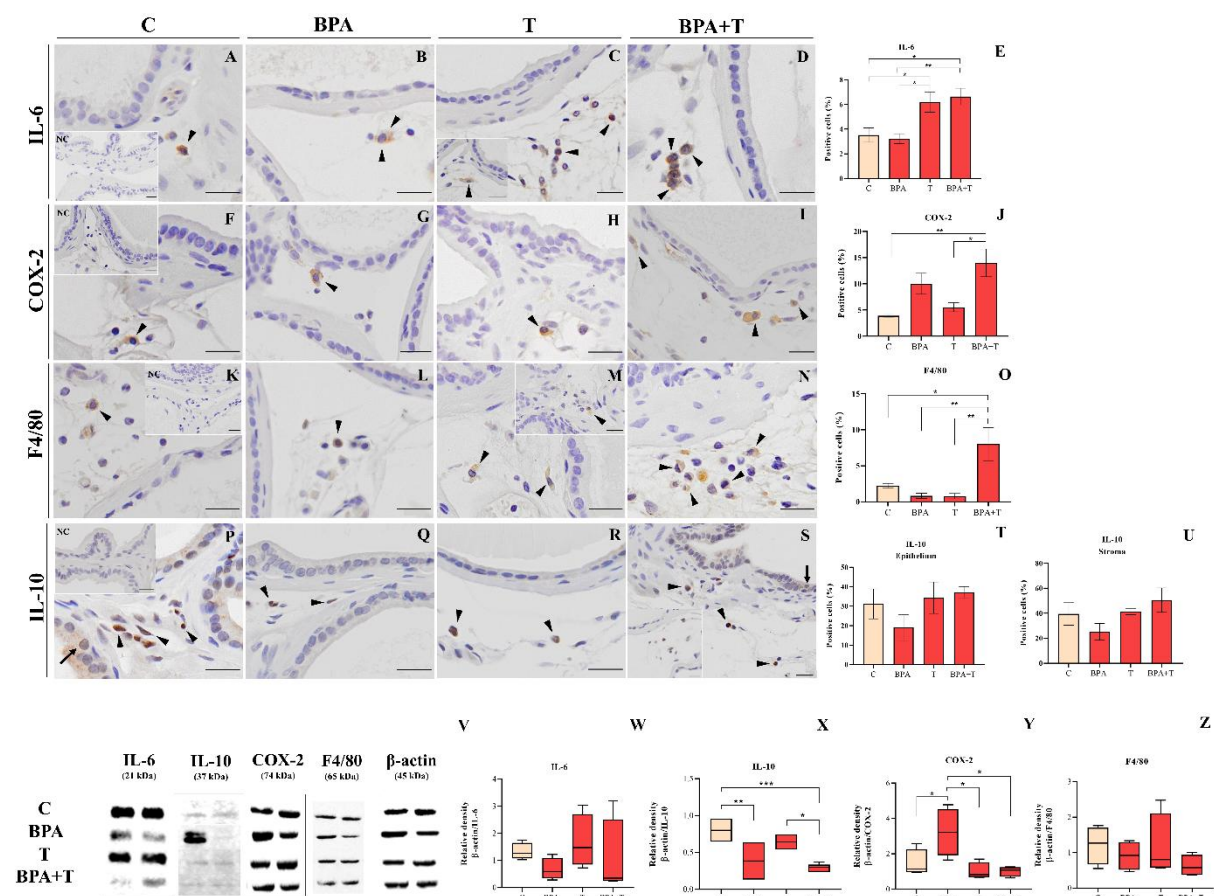
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Figure 3



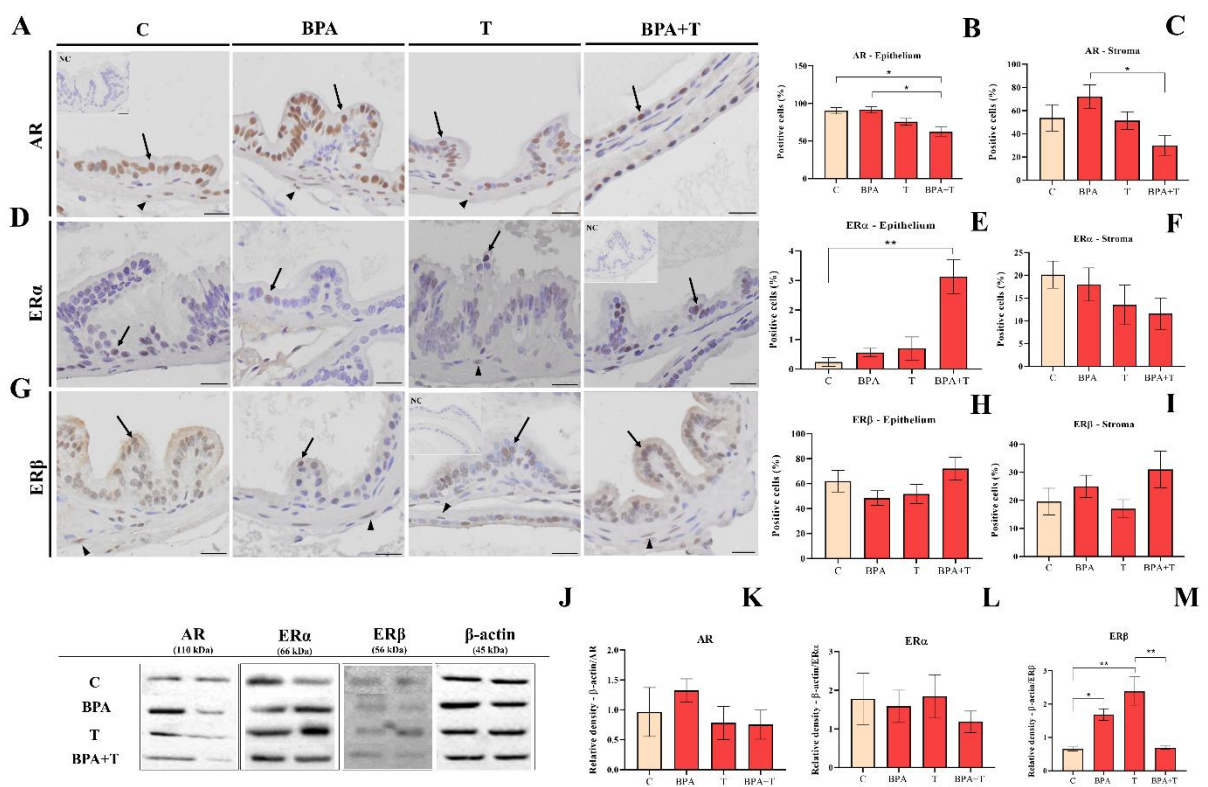
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Figure 4



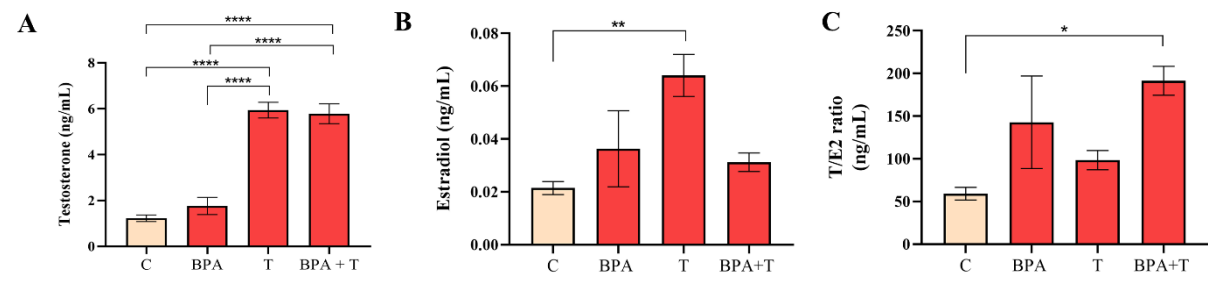
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Figure 5



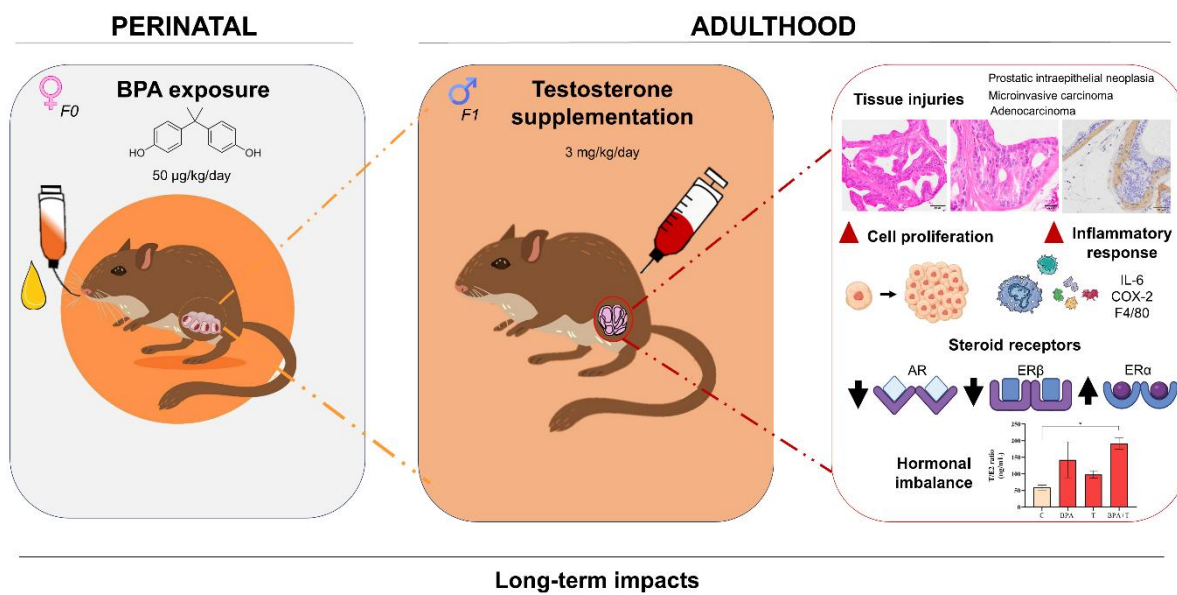
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Figure 6



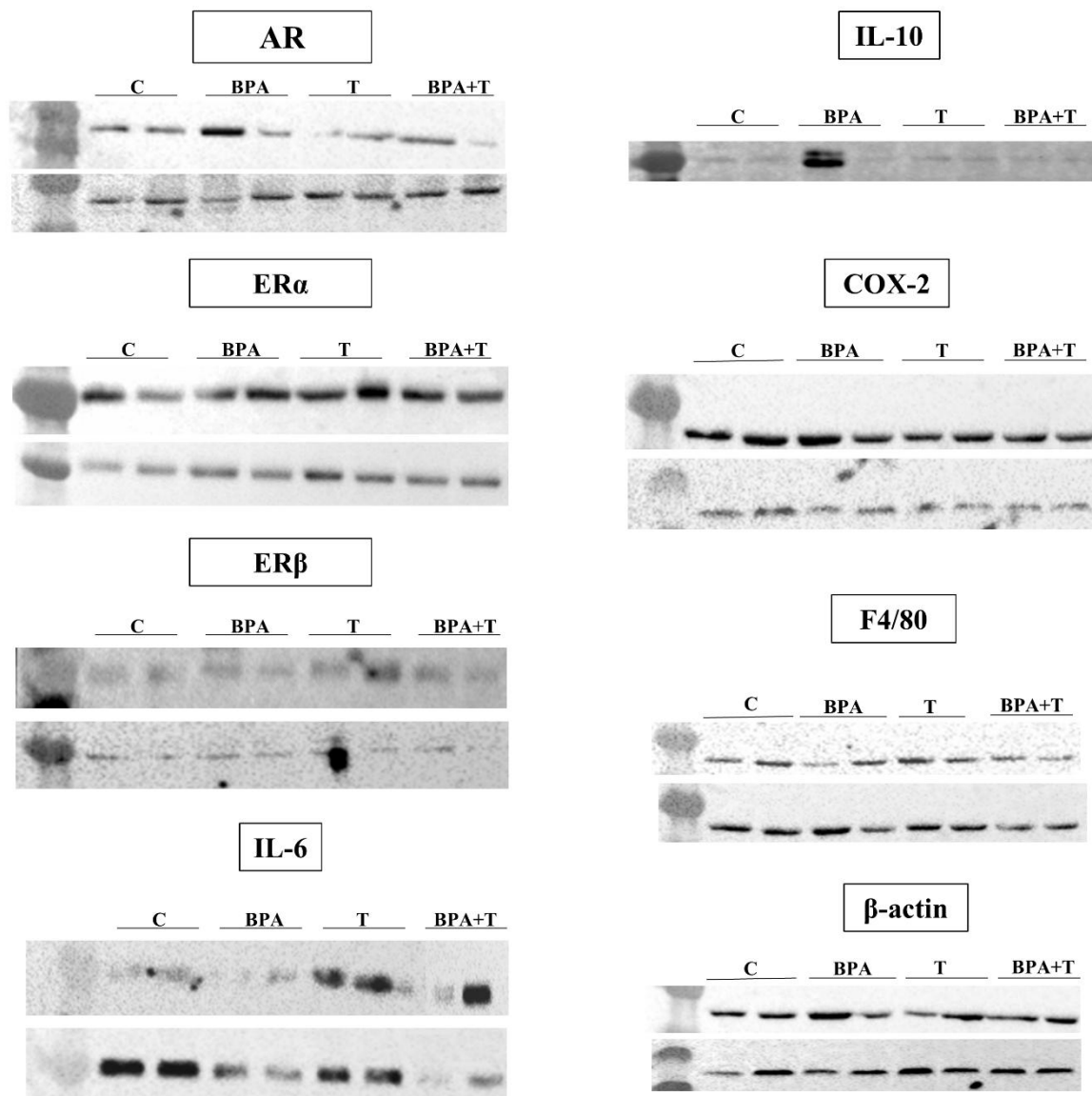
Source: Extracted from Bicalho-Silva *et al.* (2025)

Figure 7



Source: Extracted from Bicalho-Silva *et al.* (2025)

Supplementary Figure 1



Source: Extracted from Bicalho-Silva *et al.* (2025)

Table 1: Description of antibodies and protocols used in the immunohistochemical analysis.

	Antigen retrieval	Washing buffer	Primary antibody reference	Primary antibody dilution
α -sma	10 mM sodium citrate buffer pH 6.0 (90° C, 50 min)	TBS	α -sma, mouse monoclonal, 1A4, Santa Cruz Biotechnology (sc-32251)	1:100 in BSA 1% overnight at 4° C
PHH3	10 mM sodium citrate buffer pH 6.0 (99° C, 60 min)	PBS	PHH3, rabbit polyclonal, Ser10, Cell Signaling (9701)	1:75 in BSA 1% overnight at 4° C
Casp-3	10 mM sodium citrate buffer pH 6.0 (90° C, 30 min)	PBS	Activated caspase-3, rabbit polyclonal, Novus (NB100-56113)	1:75 in BSA 1% overnight at 4° C
IL-6	TRIS-EDTA buffer pH 9.0 (98° C, 30 min)	TBS + tween	IL-6, rabbit monoclonal, Santa Cruz Biotechnology (D5W4V)	1:100 in BSA 1% overnight at 4° C
IL-10	TRIS-EDTA buffer pH 9.0 (98° C, 30 min)	TBS + tween	IL-10, mouse monoclonal, Santa Cruz Biotechnology (sc-8438)	1:100 in BSA 1% overnight at 4° C
COX-2	TRIS-EDTA buffer pH 9.0 (98° C, 30 min)	TBS + tween	Cox-2, rabbit monoclonal, Cell Signaling (D5H5)	1:100 in BSA 1% overnight at 4° C
F4/80	TRIS-EDTA buffer pH 9.0 (98° C, 30 min)	TBS + tween	F4/80, rabbit monoclonal, Cell Signaling (D2S9R)	1:100 in BSA 1% overnight at 4° C
AR	10 mM sodium citrate buffer pH 6.0	PBS	anti-AR, mouse monoclonal, Santa Cruz Biotechnology (sc-7305)	1:50 in BSA 1% overnight at 4° C
ER α	TRIS-EDTA buffer pH 9.0 (98° C, 45 min)	TBS + tween	anti-ER α , mouse monoclonal, Santa Cruz Biotechnology (sc-8005)	1:75 in BSA 1% overnight at 4° C
ER β	TRIS-EDTA buffer pH 9.0 (98° C, 45 min)	TBS + tween	anti-ER β , rabbit polyclonal, Invitrogen, Thermo Fisher (PA1-310B)	1:75 in BSA 1% overnight at 4° C

Source: Extracted from Bicalho-Silva *et al.* (2025)

Table 2: Biometric parameters analyzed in Mongolian gerbils perinatally exposed to BPA and supplemented by testosterone in adulthood (n=10/group).

	C	BPA	T	BPA+T
Body weight (g)	77.60 ± 1.85	75.80 ± 1.98	76.70 ± 1.97	76.89 ± 2.49
Absolute weight of body fat (g)	1.74 ± 0.25	1.76 ± 0.20	1.89 ± 0.23	2.64 ± 0.68
Relative weight of body fat (g)	2.21 ± 0.02	2.32 ± 0.02	2.44 ± 0.02	2.55 ± 0.02
Absolute weight of prostatic complex (g)	0.27 ± 0.00	0.29 ± 0.01	0.33 ± 0.02	0.31 ± 0.01
Relative weight of prostatic complex (g, x10 ³)	3.49 ± 0.07	3.94 ± 0.02	4.40 ± 0.02	3.97 ± 0.01
Absolute weight of ventral prostate (g)	0.026 ± 0.00	0.025 ± 0.00	0.031 ± 0.00	0.026 ± 0.00
Relative weight of ventral prostate (g, x10 ⁴)	3.34 ± 0.14	3.63 ± 0.51	4.11 ± 0.28	3.32 ± 0.23

Control (C), BPA (BPA), Testosterone (T), BPA+T (BPA plus Testosterone). Data are shown as mean ± SEM ($p \leq 0.05$).

Source: Extracted from Bicalho-Silva *et al.* (2025)

Table 3: Stereological and morphometric parameters analyzed in gerbils perinatally exposed to BPA and supplemented by testosterone in adulthood (n=6/group).

	C	BPA	T	BPA+T
<i>Stereology (%)</i>				
Epithelium	18.09 ± 1.73 ^a	19.07 ± 1.62 ^{ab}	24.07 ± 1.99 ^b	16.81 ± 1.13 ^a
Muscular stroma	8.26 ± 0.63 ^a	8.22 ± 0.52 ^a	12.83 ± 1.29 ^b	8.57 ± 0.44 ^a
Non-muscular stroma	22.62 ± 2.44 ^a	10.02 ± 0.80 ^{bc}	15.91 ± 1.71 ^b	17.23 ± 1.35 ^{abd}
Lumen	49.70 ± 2.77 ^{ab}	56.83 ± 2.47 ^a	45.09 ± 3.73 ^b	55.74 ± 2.31 ^{ab}
Blood vessel	1.30 ± 0.25	1.81 ± 0.28	1.78 ± 0.11	1.65 ± 0.24
<i>Morphometry (μm)</i>				
Epithelium height	18.84 ± 0.84 ^a	16.21 ± 1.96 ^a	27.51 ± 1.97 ^b	17.96 ± 1.49 ^a
SML thickness	11.45 ± 1.24 ^a	10.82 ± 0.72 ^a	16.72 ± 1.85 ^b	11.39 ± 0.55 ^a

Control (C), BPA (BPA), Testosterone (T), BPA+T (BPA plus Testosterone), Smooth Muscle Layer (SML). Data are shown as mean ± SEM, where a ≠ b ≠ c ≠ d ($p \leq 0.05$).

Source: Extracted from Bicalho-Silva *et al.* (2025)

Table 4: Incidence and multiplicity of lesions quantified on the prostate sections of Mongolian gerbils perinatally exposed to BPA and supplemented by testosterone in adulthood (n=6/group)

	Incidence (%)				Multiplicity			
	C	BPA	T	BPA+T	C	BPA	T	BPA+T
Hyperplasia	100	100	100	100	0-8 (5.0) ^a	2-11 (6.1) ^a	9-41 (20.3) ^b	2-26 (14.1) ^{ab}
<i>PIN</i>								
LGPIN	100	100	100	100	0-8 (4.6) ^a	3-19 (9.6) ^a	3-20 (9.4) ^a	5-47 (17.7) ^b
HGPIN	100	100	100	100	1-8 (2.1) ^a	6-28 (10.6) ^b	3-33 (9.2) ^{ab}	3-24 (12.0) ^b
PIN total	100	100	100	100	0-8 (6.8) ^a	3-28 (20.7) ^b	3-33 (18.2) ^{ab}	3-47 (30.6) ^{bc}
<i>Epithelial atypia</i>								
Flat	100	83.3	100	100	0-8 (3.1)	0-27 (4.2)	1-10 (3.7)	0-7 (3.5)
Papillary	100	100	100	100	7-63 (33.4)	6-60 (37.1)	6-57 (29.5)	4-82 (38.7)
Epithelial atrophy	16.6	83.3	83.3	100	0-11 (0.0) ^a	0-8 (2.7) ^{ab}	0-9 (3.3) ^{ab}	0-13 (6.2) ^b
Epithelial detachment	0	0	0	83.3	0-0 (0.0) ^a	0-0 (0.0) ^a	0-0 (0.0) ^a	0-16 (0.7) ^b
Buds	100	100	100	100	1-25 (12.6)	2-28 (12.1)	1-30 (14.7)	2-32 (15.4)
Microacini	100	100	100	100	1-20 (11.2) ^{ab}	0-22 (8.2) ^{ab}	1-16 (5.4) ^a	3-29 (16.4) ^b
Microinvasive carcinoma	50	83.3	83.3	100	0-2 (0.5)	0-3 (1.1)	0-3 (1.1)	0-5 (1.5)
Adenocarcinoma	16.6	83.3	83.3	100	0-3 (0.0) ^a	0-8 (1.7) ^{ab}	0-3 (1.0) ^{ab}	0-7 (2.6) ^b
<i>Inflammation</i>								
Periductal	33.3	83.3	0	100	0-6 (0.0) ^a	0-5 (1.3) ^{ab}	0-4 (1.9) ^{ab}	0-13 (7.3) ^b
Intraluminal	0	0	16.6	16.6	0-0 (0.0)	0-0 (0.0)	0-1 (0.0)	0-5 (0.0)
Subepithelial	0	83.3	83.3	50	0-0 (0.0) ^a	0-2 (0.8) ^{ab}	0-7 (1.8) ^b	0-3 (0.7) ^{ab}

Control (C), Bisphenol A (BPA), Testosterone (T), Bisphenol A plus Testosterone (BPA+T). Prostatic Intraepithelial Neoplasia (PIN), low-grade PIN (LGPIN), high-grade PIN (HGPIN). Data are shown as mean $\pm$ SEM, where $a \neq b \neq c$ ($p \leq 0.05$).

Source: Extracted from Bicalho-Silva *et al.* (2025)

3.3 BETA-CARYOPHYLLENE MITIGATES METABOLIC DYSFUNCTION IN THE TESTES OF GERBILS PERINATALLY EXPOSED TO BPA

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β -caryophyllene mitigates metabolic dysfunction in the testes of gerbils perinatally exposed to BPA

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Abstract

Bisphenol A (BPA) is a persistent environmental pollutant harmful to male reproductive health. Although linked to testicular disorders, the underlying mechanisms and potential therapeutic agents to mitigate their adverse effects need to be fully explored. We investigated the impacts of perinatal BPA exposure and the therapeutic potential of β -caryophyllene (BCP) in the testes of adult Mongolian gerbils (*Meriones unguiculatus*). Pregnant females were exposed to a historically relevant and realistic dose of BPA (50 μ g/kg/day) during gestation and lactation. The F1 male offspring were maintained until adulthood and received a 30-day treatment with BCP (50mg/kg/day). We assessed the biometric, hormonal, sperm motility, oxidative markers, and metabolic testicular parameters. BPA-exposed gerbils showed decreased progressive sperm motility, as well as increased non-progressive sperm movement. ^1H -NMR metabolomics revealed testicular metabolic reprogramming, including deficits in central energy metabolism (e.g., acetate), nucleoside biosynthesis (adenosine, IMP), vitamin metabolism (pyridoxine, taurine), and phospholipid integrity (ethanolamine, O-phosphoethanolamine). Pathway analysis revealed disturbances in pyruvate metabolism/gluconeogenesis, purine metabolism, and glycerophospholipid metabolism, confirming altered testicular homeostasis. Notably, the BCP consumption rescues sperm motility and mitigates BPA-induced metabolic dysfunction by restoring testicular contents of acetate, adenosine, inosine, IMP, ethanolamine, and O-phosphoethanolamine. Perinatal exposure to BPA programs latent testicular dysfunction through the establishment of a toxic metabolic memory that manifests in adulthood. However, the 30-day BCP intervention mitigated BPA-induced testicular metabolic dysfunctions. This study provides novel descriptive insights into BPA-

induced testicular toxicity and highlights BCP as a promising therapeutic agent to counteract the long-term reproductive consequences of early-life exposures.

Keywords: Male infertility; Phytocannabinoid; Sperm motility; Testicular metabolome.

Highlights

- Perinatal exposure to BPA causes impaired sperm progressive motility in adulthood;
- Testicular dysfunction in BPA-exposed gerbils is associated to metabolic shifts;
- Perinatal exposure to BPA reprograms testicular energy and phospholipid metabolism;
- BCP treatment rescues sperm motility and testicular metabolic health in adulthood.

Abbreviations

¹H-NMR, proton nuclear magnetic resonance; 4-HNE, 4-hydroxynonenal; BCP, β -caryophyllene; BPA, Bisphenol A; BTB, blood-testis barrier; CB2-R, cannabinoid receptor type 2; CEUA, ethics committee on the use of animals; CONCEA, national council for animal control and experimentation; DNP, 2,4-dinitrophenol; DOHaD, developmental origins of health and disease; ECS, endocannabinoid system; EDCs, endocrine-disrupting chemicals; EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; FRAP, ferric reducing antioxidant power assay; GSI, gonadosomatic index; HTF, human tubal fluid; IMP, inosine monophosphate; PLS-DA, partial least squares discriminant analysis; Nitrotyrosine, NT; s-PLSDA, sparse partial least squares discriminant analysis.

1. Introduction

There is a documented global decline in male fertility concomitant with the ubiquitous presence of environmental pollutants, which represents one of the most pressing public health challenges of the 21st century [1]. Due to their low antioxidant capacity, high concentration of polyunsaturated fatty acids, and sensitive germinative epithelium, the testes are the main target of environmental toxicants and endocrine-disrupting chemicals (EDCs) [2–4]. Among the myriad synthetic compounds that act as toxicants, EDCs have garnered significant attention for their ability to interfere with hormonal signaling pathways critical for reproductive health [5].

Among the most prevalent EDCs, Bisphenol A (BPA) is used in consumer products, from food containers to thermal paper [6]. BPA is widely produced and released into the environment, with reported concentrations of 251 ng/mL in wastewater, 384.8 ng/mL in food samples, 937.49 ng/g in biological fluids, 208.55 ng/mL in personal care products, and up to 154.820 µg/g in other materials [7]. This widespread use has resulted in pervasive human exposure, with detectable BPA levels identified in the urine of over 90% of individuals in developed countries [8], and evidence of its presence throughout both prenatal and postnatal stages of life [9,10].

The Developmental Origins of Health and Disease (DOHaD) hypothesis offers a conceptual framework for understanding the latent effects of EDCs. It proposes that transient environmental insults during critical windows of developmental plasticity, such as the perinatal stage, can program physiological alterations that predispose individuals to disease later in life [11]. The developing male reproductive system is particularly vulnerable during this stage, as key processes including testicular organization, germ cell proliferation, and endocrine programming are being established. This susceptibility is further heightened by the capacity of BPA to easily cross the blood–testis barrier (BTB) and accumulate in

maternal tissues and fluids, thereby directly disrupting the testicular microenvironment and interfering with developmental programming [12–15].

Evidence from rodent models demonstrates that maternal exposure to low doses of BPA disrupts the crucial process of male reproductive development, including spermatogenesis, sexual differentiation and maturation, and testicular function, ultimately resulting in reduced fertility in the offspring [16–18]. In this context, perinatal exposure to BPA constitutes a particularly insidious threat, capable of inducing a persistent "toxic memory" that compromises reproductive efficiency well into adulthood, long after the initial chemical has been eliminated. Despite growing advances in these outcomes, the underlying metabolic mechanisms through which perinatal BPA exposure mediates testicular toxicity remain poorly explored.

The development of plant-derived compounds with relevant pharmacological properties has proved to be an efficient alternative to counteract the testicular injuries caused by short and long-term exposure to environmental toxicants [19,20]. β -caryophyllene (BCP) is a phytocannabinoid found in several plant species that acts as a natural antioxidant and anti-inflammatory compound [21]. BCP is a potent and selective agonist for the cannabinoid receptor type 2 (CB2-R), a key component of the endocannabinoid system (ECS) that emerged as a master regulator of inflammation, cellular stress, and homeostasis, with its components expressed throughout the male reproductive tract [22]. Recently, BCP showed protective and therapeutic effects against cardiac and testicular oxidative stress, in addition to alleviating the damage to sperm quality and quantity in the mouse testis [23–25]. The treatment with BCP also proved to be successful in controlling proliferative and inflammatory disorders in rodent prostate cells [26]. Thus, it appears as a potential candidate to ameliorate testicular insults caused by exposure to environmental pollutants. Here, we test the hypothesis that the enduring testicular dysfunction programmed by

perinatal BPA exposure is the manifestation of a self-perpetuating state of metabolic collapse. Given that the legacy of early-life BPA may be related to impaired testicular metabolic functioning, this study aims to investigate BPA's early-exposure capacity to induce a measurable and persistent metabolic phenotype. We further hypothesize that the adult testis, following perinatal BPA exposure, may remain functionally compromised. To address this challenge, we also explore potential therapeutic reversal by BCP, a natural bicyclic sesquiterpene abundant in the essential oils of plants such as black pepper, cloves, and cannabis [26]. If latent BPA toxicity is indeed connected to a state of chronic metabolic dysfunction, then a molecule like BCP, which specifically engages the body's primary stress-resolution pathways, has the potential to mitigate the deleterious effects. Overall, using Mongolian gerbil as a relevant rodent model of perinatal BPA exposure, we aim to (1) characterize the long-term functional, oxidative, and metabolic consequences in the adult testes; (2) test the hypothesis that this latent toxicity manifests as a persistent metabolic dysregulation; and (3) determine if this pathological state, programmed in early life, can be reversed or mitigated by a 30-day intervention in adulthood with dietary BCP treatment. The testes of Mongolian gerbils are an effective model for reproductive studies because this species has high spermatogenic efficiency compared to other mammalian species that have already been investigated [27]. In addition, it has proven to be a useful experimental model in scientific research, as it has similarities with human reproductive biology [28].

2. Materials and Methods

2.1. Chemicals

Bisphenol A (239.658, ≥99% purity) and β-caryophyllene (W225207, ≥95% purity) were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Ethics Statement

All experimental procedures involving animals agreed with the guidelines of the National Council for Animal Control and Experimentation (CONCEA, Brazil) and were approved by the recommendations of the Ethics Committee on the Use of Animals (CEUA, IBILCE, UNESP), under protocol numbers 235/2022 and 236/2022. The experimental procedures involving animals were also performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.3. Animals and study design

2.3.1 Perinatal exposure to BPA

Twenty-four adult (3 months of age) female Mongolian gerbils (*Meriones unguiculatus*) were mated with fertile males to form independent families (n=6 pairs/group; total of 24 pairs). After birth, the first litter was discarded to induce a second pregnancy and determine the first day of pregnancy, following a protocol previously described [29]. Pregnant gerbils were randomly separated and assigned to the following experimental groups: Bisphenol A (BPA) (n=18) - animals received a dose of 50 µg/kg/day of BPA diluted in 0.1 mL of corn oil, via oral gavage, from the implantation day until the end of lactation (8th day of gestation - 21st day post-birth), a total of 39 days of exposure; Control (C) (n=6) – females that received 0.1 mL of corn oil (vehicle), via oral gavage, during the same period. The dose of BPA was chosen considering the levels reported in pregnant women and children [30,31]. In addition, corresponds to a historically relevant level established by the Environmental Protection Agency (EPA) and the European Food Safety Authority guidelines [32,33]. In 2023, based on toxicological evidence, EFSA recommended a reduction of this

threshold to 0.2 ng/kg bw/day [34]. Despite this, higher doses allow measurable toxicological responses in a specific exposure period, such as the perinatal stage.

At the end of the exposure period, one male offspring from each litter was separated and maintained until 6 months of age, in order to compose the experimental groups. In this rodent species, the age of 6 months represents a well-established period corresponding to adulthood. Any surplus animals were assigned to other studies.

2.3.2 Postnatal treatments

At 6 months of age, the F1 adult male gerbils (n=24) were randomly assigned to the different experimental groups (n=6/group): Control (C) – gerbils perinatally treated with corn oil (vehicle); Bisphenol A (BPA) – gerbils perinatally exposed to BPA (50 µg/kg/day); Bisphenol A plus β-caryophyllene (BPA+BCP) – gerbils perinatally exposed to BPA (50 µg/kg/day) that received 50 mg/kg/day of β-caryophyllene, diluted in 0.1 mL of corn oil, via oral gavage, for 30 days; Bisphenol A plus vehicle (BPA+V) - gerbils perinatally exposed to BPA (50 µg/kg/day) and that received 0.1 mL of corn oil (vehicle), via oral gavage, for 30 days. The BPA+V group was included to control for variables and thereby isolate the specific effects attributable to BCP. The selected BCP dose was determined considering its well-documented safety and therapeutic potential, as evidenced by previous studies demonstrating its ability to control cell proliferation and inflammation in the prostate, along with its antioxidant and anti-neuroinflammatory properties in preclinical models [26,35]. The schematic representation of the experimental design is shown in Figure 1.

Animal handling occurred at the Biotherium of the Department of Biological Sciences of the Institute of Biosciences, Humanities and Exact Sciences of the São Paulo State University (UNESP, Brazil). To avoid any contamination, all the animals used in this study were kept in BPA-free cages, on racks equipped with air exhaustion, controlled temperature (22 ± 2 °C) and photoperiod (12h light – 12h dark), supplied with water in BPA-free bottles

(Alesco, Monte Mor, SP, Brazil) and food *ad libitum* (LABIMIX – Ratos e Camundongos; Agromix Indústria e Comércio de Alimentos).

2.4. Sample collection

At the end of experimentation, at 7 months of age, the gerbils were weighed, euthanized by anesthetic deepening (Isoflurane 3%), and decapitated. Blood samples were collected to measure the blood glucose. The blood was also collected, centrifuged, and stored at -80°C for hormonal dosage. The testes were removed, weighed, flash-frozen, and lyophilized for ¹H-NMR analysis. The gonadosomatic index (GSI%) was obtained by the following formula: $GSI = GW/BW \times 100$, where GW = gonad weight and BW = body weight [20]. The body fat was removed for calculating the adiposity index (%), estimated by the formula [(sum of epididymal, retroperitoneal, and visceral fat/body weight) $\times$ 100] [36].

2.5. Sperm motility

The sperm motility determination was performed immediately after euthanasia. Sperm was collected through perforations in the epididymis cauda, in 1 mL of modified HTF medium (Human Tubal Fluid, Irvine Scientific, CA, USA), at 34 °C. Briefly, 10 μ L of sperm solution was added to a Makler counting chamber (Sefi-Medical Instruments, Israel), and the evaluation of sperm motility was assessed by visual estimation at 200x magnification (n=6/group, 100 spermatozoa/animal) under a phase-contrast microscope (Olympus BX60, Olympus Corporation, Japan). Spermatozoa were classified as motile with progressive movement, motile without progressive movement, and immotile [37].

2.6. Blood glucose

Blood samples (n=6/group) were collected immediately after the decapitation from blood taken from the cervical region, using a glucose test strip coupled to a digital glucometer (Accu-Chek Active®, Roche, Mannheim, Germany).

2.7. Protein extraction and quantification

Total protein was extracted from 50 mg of frozen testicular tissue samples using the Triton-X buffer (Triton-X 100, HEPES 10 mM, EGTA 0.5 mM), supplemented with a lysis buffer (100 mM PMSF, 1% protease inhibitor cocktail, and 100 mM sodium orthovanadate). A total of 50 μ L of this lysis buffer was added to the tissue homogenate, and the resulting solution was agitated for 10 min at room temperature. The protein concentration was quantified using a commercial kit, Pierce™ BCA Protein Assay Kit, following the manufacturer's instructions. Decrescent concentrations of BSA were used as a standard curve. The absorbances were measured at 560 nm using a multiplate reader (MultiSkan Go, Thermo Fisher Scientific, Vantaa, Finland). Total protein values were used for oxidative markers analyses and the FRAP assay.

2.8. Oxidative markers and FRAP assay

To determine the oxidative damage to protein and lipids, we used 15 μ g of extracted protein to measure the levels of nitrotyrosine (NT), 4-hydroxynonenal (4-HNE), and 2,4-dinitrophenol (DNP) by the Slot-blot technique [38]. In brief, the PVDF membranes were incubated overnight at 4°C with primary antibodies (NT, 4-HNE, and DNP) and then with secondary antibodies for 90 min at room temperature. The membranes were washed with filtered PBS, incubated with ECL (NZYTech, Lisboa, Portugal), and read in BioRad ChemiDoc MP (BioRad, Hemel Hempstead, UK). For total protein loading, the Ponceau Staining (NZYTech, Lisboa, Portugal) was used. Bands density was quantified by Image Laboratory software (BioRad, Hemel Hempstead, UK). The complete list of antibodies used in the oxidative status analyses is described in Supplementary Table 1.

The Ferric Reducing Antioxidant Power Assay (FRAP) analysis was performed according to Benzie and Strain 1996 [39]. In brief, 6 μ L of protein extract was added, in duplicate, to the working FRAP solution. The absorbance was read at 0, 40, and 120 minutes

after incubation, at 593 nm, using a microplate reader (MultiSkan Go, Thermo Fisher Scientific, Vantaa, Finland). The ascorbic acid was used as a standard curve. Results were expressed as nmol/ μ g protein.

2.9. *Untargeted testicular metabolome and spectral analysis*

Testicular tissue extracts were prepared using a combined extraction of polar and non-polar metabolites. The testicular polar extracts were dissolved in deuterium water (D_2O). ^1H -NMR spectra were recorded using a 500 MHz Bruker Avance III HD spectrometer equipped with a 5 mm TXI probe (Bruker Corporation, Billerica, MA, USA), working at 298 K. Solvent-suppressed 1D- ^1H -NMR spectra were acquired using a zgpr pulse sequence, with a sweep width of 7 kHz, relaxation delay of 7 s, pulse angle of 30° , acquisition time of 2.3 s, and 64 scans. An amount of 225 μ L of a mixture containing 10 mM of sodium fumarate with 180 μ L of testicular tissue extracts was transferred to an NMR tube and analyzed. The untargeted metabolites identification and characterization were performed by Chenomx profiler software (NMR suite, version 8.3), in accordance with the Metabolomics Standards Initiative guidelines for metabolite identification [40]. Spectra were processed by applying a line broadening of 0.2 Hz before Fourier transformation and manually phased and baseline-corrected. Sodium fumarate (6.50 ppm) was used as a standard reference for chemical shifts and metabolite quantification, using the Bruker TopSpin software 4.5.0. Results are expressed as metabolite content (μ mol/mg of tissue). The information about the levels of the characterized metabolites was summarized in Supplementary Table 2. Metabolomic data were analyzed through the MetaboAnalyst 6.0 program (available on: <https://www.metaboanalyst.ca/>) (accessed on 23 July 2025), where partial least squares discriminant analysis (PLS-DA), sparse partial least squares discriminant analysis (s-PLSDA), heatmaps, and pathways analysis were performed.

2.10. *Hormonal dosage*

Total testosterone and estradiol levels were measured in duplicate using an aliquot of the serum of gerbils (n=6/group), through specific commercial kits following the supplier's protocol (USA Diagnostics, Belo Horizonte, MG, Brazil).

2.11. Statistical analysis

The data were submitted to a unifactorial one-way analysis of variance (ANOVA), followed by a Fisher's LSD test. Non-parametric data were subjected to the Kruskal–Wallis test followed by Dunn's test. The results are expressed as mean $\pm$ standard deviation in the table and as mean $\pm$ minimum to maximum values in the graphs, considering $p < 0.05$. All statistical analyses were carried out using GraphPad Prism (version 8.0, GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Oral BCP intervention increased the blood glucose levels in gerbils

To determine the long-term systemic impact of perinatal BPA exposure and the possible mitigating effect of subsequent adult treatment with BCP, we first assessed key biometric and hormonal parameters. Analysis of adult male gerbils revealed no significant alterations in body weight, adiposity index, absolute testes weight, or the gonadosomatic index (GSI) across the different experimental groups. Furthermore, circulating serum levels of total testosterone and estradiol also remained statistically unaltered among the groups. In contrast to the stable hormonal profile, we observed a significant difference in metabolic status. Blood glucose levels were significantly elevated in the BPA+BCP group compared to the control ($p=0.0311$), although remaining in normal fasting levels. There were no statistically significant differences in body weight, absolute or relative testis weight, GSI, or the adiposity index among the experimental groups (Table 1).

3.2. Perinatal exposure to BPA compromises sperm motility, while BCP treatment reverses this effect in adult gerbils

To elucidate the long-term consequences of early-life BPA exposure and to assess the potential therapeutic efficacy of BCP, we characterized the reproductive phenotype of adult male gerbils subjected to both treatments. At the functional level, perinatally BPA-exposed gerbils exhibited a significant impairment in sperm quality compared to age-matched controls. Perinatal exposure to BPA reduced the rate of spermatozoa with progressive movement compared to the control ($p < 0.0001$). Gerbils from BPA+BCP and BPA+V groups also showed reduced sperm with progressive movement compared to control ($p < 0.0001$; $p < 0.0001$). Furthermore, perinatal exposure to BPA led to an increase in the rate of spermatozoa with non-progressive movement compared to control gerbils ($p = 0.0002$). This deterioration in a key fertility parameter occurred in the absence of any overt systemic toxicity. On the other hand, the 30-day BCP consumption led to a recovery of sperm with progressive movement ($p = 0.0416$), followed by a reduction in sperm with non-progressive movement ($p = 0.0100$) compared to perinatally BPA-exposed gerbils. The percentage of immotile sperm did not differ among the experimental groups (Fig. 2).

3.3. BPA exposure did not promote alterations in oxidative markers, although BCP intervention increased NT levels in adult gerbils

Regarding the oxidative markers, the testes of adult gerbils from the BPA+BCP group showed an increased NT level (a marker of protein damage by reactive nitrogen species) compared to control ($p = 0.0152$), BPA ($p = 0.0333$), and BPA+V ($p = 0.0019$) (Fig. 3A). However, the levels of 4-HNE (an end-product of lipid peroxidation) or DNP derivatives (that indicate protein carbonylation) did not differ among the experimental groups (Fig. 3B and C). This absence in oxidative damage was coupled with non-statistically significant

alterations in the total antioxidant capacity in the testicular tissue of gerbils from all groups, as measured by the FRAP assay (Fig. 3D). Despite this, we hypothesized that BPA-associated latent toxicity could manifest as a testicular metabolic dysregulation and that BCP could play a mitigating role in this scenario. To test this hypothesis, we employed a metabolomics approach.

3.4. Untargeted testicular metabolomics identifies BPA-induced metabolic alterations mitigated by 30-day BCP intervention

To define the molecular fingerprint of BPA-induced metabolic alterations and the potential therapeutic role of BCP, we performed untargeted ^1H NMR metabolomics on polar extracts of the testicular tissue. Multivariate statistical analysis revealed a significant alteration of the global testicular metabolome in gerbils subjected to experimental treatments. Supervised Partial Least Squares Discriminant Analysis (PLS-DA) shows distinct metabolic profiles among experimental groups (Fig. 4A-F). The PLS-DA also demonstrated a clear and robust separation between the metabolic profile of adult testis perinatally exposed to BPA (Fig. 4A), which presents a distinct pattern of global testicular metabolome to those treated with BCP (Fig. 4D). The s-PLS-DA model (Fig. 4G-L) confirmed this separation, underscoring the statistical power of the metabolic shift induced by BCP treatment (Fig. 4J). The heatmap represents the variation in metabolite levels and their relationships between samples and the testicular metabolites in different experimental treatments (Fig. 5).

To a better understanding, the metabolites were grouped into different metabolic classes: central energy metabolism (acetate, lactate, succinate, creatine phosphate); amino acids (alanine, glutamate, valine); nucleosides (adenosine, inosine monophosphate (IMP), inosine, uridine, guanosine); metabolism of vitamins and derivatives (pyridoxine, taurine, glycolate); and phospholipids metabolism (ethanolamine, O-phosphoethanolamine, choline,

O-phosphocholine). The complete list of identified metabolites is shown in Supplementary Table 2.

Perinatal BPA exposure resulted in a decrease in testicular acetate content compared to gerbils from the control group ($p=0.0020$). On the other hand, the acetate levels were restored after 30 days of treatment with BCP ($p=0.0147$) (Fig. 6A). The 30-day BCP treatment also increased lactate ($p=0.0498$) and creatine phosphate ($p=0.0158$) levels compared to the BPA group (Fig. 6B and C). Additionally, succinate content was higher in the BPA+V than in the BPA group (Fig. 6D). We found a similar pattern in the metabolites associated with amino acid metabolism. The testicular content of alanine and glutamate was higher in gerbils exposed to the BPA+BCP group when compared to those perinatally exposed to BPA ($p=0.0257$; $p=0.0219$) (Fig. 7A and B). However, the testicular content of valine did not differ among the experimental groups (Fig. 7C). Regarding the testicular content of metabolites related to nucleoside metabolism, there was a decrease in adenosine ($p=0.0246$), IMP ($p=0.0111$), and inosine ($p=0.0334$) in perinatally BPA-exposed gerbils when compared to the control. The content of these metabolites was also reduced in BPA-exposed gerbils compared to the BPA+BCP group ($p=0.0068$; $p=0.0191$; $p=0.0007$) (Fig. 8 A-C). No differences were detected in the testicular content of uridine or guanosine in adult gerbils after experimental treatments (Fig. 8D and E). In addition, we found alterations in metabolites associated with vitamin metabolism. Particularly, the perinatal exposure to BPA promoted a decrease in taurine ($p=0.0039$), pyridoxine ($p=0.0031$), and glycolate ($p=0.0195$) contents compared to control (Fig. 9). Pyridoxine and glycolate contents were also reduced in perinatally BPA-exposed gerbils in comparison with the BPA+BCP group ($p=0.0466$; $p=0.0177$) (Fig. 9B and C). Reduced levels of glycolate were also observed in the BPA group, compared to the BPA+V ($p=0.0236$) (Fig. 9C). Finally, regarding metabolites related to phospholipid metabolism, all treatments caused a significant decrease in ethanolamine

testicular content in adult gerbils compared with control ($p=0.0193$) (Fig. 10A). On the other hand, perinatally BPA-exposed gerbils showed a reduction in O-phosphoethanolamine testicular content compared to the control ($p=0.0425$) and BPA+BCP groups ($p=0.0307$) (Fig. 10B). No significant differences were detected in the testicular content of choline and O-phosphocoline (Fig. 10C and D). Figure 11 represents a pathway analysis in the testes of Mongolian gerbils subjected to different experimental treatments. The plots map the metabolic pathways based on their statistical significance (y-axis: $-\log_{10}(p)$) and the magnitude of their impact (x-axis: Pathway Impact). The perinatal exposure to BPA resulted in significant metabolic disturbances. The most prominently affected pathways, showing the highest statistical significance, were those related to pyruvate metabolism and glycolysis or gluconeogenesis. Other pathways that were also significantly impacted include alanine, aspartate, and glutamate metabolism, selenocompound metabolism, purine metabolism, and glycerophospholipid metabolism (Fig. 11A). Compared to the control, BCP and vehicle intervention showed main alterations in vitamin B6 metabolism and glycerolipid metabolism, respectively (Fig. 11B and C). On the other hand, Fig. 11D indicates that BCP treatment, when compared with non-treated gerbils, most significantly impacts testicular taurine and hypotaurine metabolism. Other affected pathways include alanine, aspartate, and glutamate metabolism, arginine biosynthesis, purine metabolism, and nitrogen metabolism, which clearly differ from the vehicle data (Fig. 11E and F).

4. Discussion

This study showed, for the first time, a novel description of the latent metabolic toxicity caused by BPA and the efficient role of BCP in mitigating testicular metabolic disorders. We demonstrate that perinatal exposure to a historically and realistic dose of BPA programs an early-life state of testicular dysfunction that later manifests in adulthood. Our central finding is that this "toxic memory" culminates in a metabolic alteration in the adult testis. This effect

is characterized by a series of mechanisms, including chronic deficits in central energy metabolism and failure of biosynthetic pathways essential for maintaining cellular homeostasis. This metabolically compromised state is incompatible with the high energetic and synthetic demands of regular sperm physiology, leading to the observed decline in sperm motility. Here, we provide a descriptive exploration illustrating how transient developmental exposure to BPA can generate early-life insults with latent effects that later emerge in adulthood, ultimately leading to reproductive impairment in adult animals. On the other hand, we present BCP as a safe and effective natural compound to counteract the BPA-induced testicular metabolic dysfunction.

Xenoestrogens like BPA interfere with endocrine signaling, producing undesired responses within the testis, such as hormonal reprogramming of testicular development and epigenetic changes, which result in damage to sperm production and quality [41–43]. In our study, we showed that exposure to BPA during a critical stage of development led to reduced progressive sperm motility in adult gerbils, a key parameter directly associated with male infertility. Supporting this evidence, a recent study reported that fetal exposure to BPA caused a reduction in CATSPER2 mRNA expression in Wistar rats, an essential gene governing sperm motility and fertilization capacity [44]. Additionally, parental exposure to BPA has been shown to induce alterations in sperm quality parameters across several experimental models [45–47]. Moreover, in a study published in 2020, transgenerational exposure to a low dose of BPA (1 µg/g diet) resulted in adverse effects on sperm function and impaired male reproductive capacity persisting up to the F2 generation [48]. These transgenerational outcomes have been attributed to BPA-induced DNA and histone damage, as well as aberrant methylation patterns, leading to heritable alterations in gene expression across the genome [45]. Thus, BPA appears to leave an epigenetic legacy that

transcends primary exposure, perpetuating reproductive dysfunction across multiple generations.

Testicular metabolome outcomes revealed the molecular signature of BPA's toxic memory, suggesting the establishment of a self-perpetuating vicious cycle of metabolic dysfunction that explains its persistence long after the initial chemical exposure has ceased. Our testicular metabolomic profiling suggests that perinatal BPA exposure induces latent metabolic reprogramming in the adult testes, even in the absence of significant alterations in estradiol and testosterone or testicular oxidative stress-related markers. Despite this, changes in other hormones cannot be ruled out. The significant alterations in pathways such as pyruvate metabolism, glycolysis/gluconeogenesis, purine metabolism, and glycerophospholipid metabolism highlight a disruption of cellular energy homeostasis and structural integrity [49,50]. The observed decrease in acetate, a crucial substrate for lipid synthesis and energy production [51,52], alongside reductions in adenosine and IMP, vital for nucleotide synthesis and energy currency [53], points to a compromised capacity for both energy generation and cellular repair. Furthermore, the depletion of pyridoxine and taurine, essential cofactors and osmolytes with antioxidant roles [54,55], and the significant reduction in ethanolamine, a key component of phospholipids, collectively indicate a severe impairment in testicular metabolic homeostasis and membrane integrity [56]. Taken together, the untargeted metabolomic findings indicate a dysregulation of multiple testicular metabolic pathways, essential for energy production, structural integrity, and antioxidant defense, which may result in impaired spermatogenesis and contribute to male infertility. Our findings corroborate studies that point to multiple biochemical disturbances associated with BPA exposure, including alterations in essential metabolic pathways and intermediates of glycolysis, the Krebs cycle, β oxidation of long-chain fatty acids, and nucleoside metabolism [57].

Importantly, the current study indicates that the latent testicular dysfunction induced by perinatal exposure to BPA is not entirely irreversible. The 30-day therapeutic intervention with BCP in adulthood mitigated the adverse testicular effects, rescuing sperm motility and restoring the metabolic phenotype toward an intermediate state between non-exposed and perinatally exposed gerbils. Here, the data pointed to a restoration of testicular acetate levels, suggesting an improvement in energy substrate availability. The normalization of testicular contents of adenosine, IMP, and inosine levels further indicates a recovery in purine metabolism, which is crucial for ATP synthesis and nucleic acid repair [58]. The increase in testicular alanine and glutamate levels, key amino acids involved in energy metabolism, further supports a broad metabolic rebalancing towards homeostasis [59,60]. Interestingly, BCP treatment also improved testicular O-phosphoethanolamine levels, suggesting a positive impact on phospholipid synthesis and preserving membrane integrity [56]. Notably, elevated blood glucose levels were observed in BCP-treated gerbils. This finding suggests that BCP may have induced a systemic metabolic adjustment, increasing circulating glucose as part of an adaptive energy-mobilizing response aimed at supporting testicular recovery, leading to a transient systemic alteration. Likewise, the 30-day BCP intervention also resulted in increased testicular nitrotyrosine levels. Although this seems unexpected, it can represent an adaptive response, such as a transient increase during cellular repair and remodeling, or a modulation of nitric oxide signaling that is not solely indicative of oxidative damage [61].

Nevertheless, BCP-treated gerbils exhibited a restoration of testicular pyridoxine levels, which had been previously reduced by perinatal exposure to BPA. This finding underscores the antioxidant role of BCP, since pyridoxine is a key vitamin involved in sperm maturation, as well as in the improvement of sperm parameters and seminal quality [62]. These findings may be linked with the capacity of BCP in restoring normal sperm

progression. Notably, the metabolic pathway analysis further highlights BCP's ability to modulate key testicular metabolic networks, including pyridoxine and glycolate metabolism, which are critical for osmoregulation and antioxidant defense in the testis [63–65]. In general, we attribute the therapeutic effects of BCP to its features, which include potent antioxidant and anti-inflammatory properties [66–68]. BCP has a known agonistic activity on the endocannabinoid system, which is present and functionally active in the testes, in both somatic and germ cells [69–71]. Moreover, our findings agree with previous scientific reports, showing the functional and protective effects of BCP in restoring the reproductive health of males exposed to different environmental pollutants [24,68].

Despite the robust findings, this study has some limitations that should be discussed. The Mongolian gerbil serves as a valuable and reliable model for reproductive studies, but the translational potential of these specific outcomes to human physiology requires further investigation, given species-specific differences. Furthermore, we focused on a single EDC that does not fully capture the complexity of real-world environmental exposures, which often involve synergistic or antagonistic effects from mixtures of contaminants. Finally, future studies should also aim to fully elucidate the precise molecular targets and signaling pathways through which BCP exerts its beneficial metabolic effects in the testes, and explore the long-term efficacy and safety of such interventions in a systemic broader context.

5. Conclusion

In summary, this study provides compelling evidence that perinatal exposure to BPA induces a latent testicular metabolic dysfunction that becomes evident in adulthood, ultimately compromising sperm motility. This “toxic memory” is characterized by a testis-specific metabolic collapse rather than an upregulation of oxidative stress–related markers, unveiling a novel mechanism underlying BPA-induced reproductive toxicity. We demonstrate that BCP, a naturally occurring compound, mitigates these long-term testicular

impairments by restoring key metabolic pathways, thereby underscoring its therapeutic potential. Collectively, these findings highlight the critical vulnerability of the perinatal stage and the urgent need for interventions to counteract the adverse effects of environmental EDCs. Future investigations should explore the translational relevance of these results to human reproductive health, aiming to better understand and mitigate the latent impacts of EDC exposure, which represent a paramount concern for safeguarding the reproductive health of future generations.

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Data availability

Data will be made available on request.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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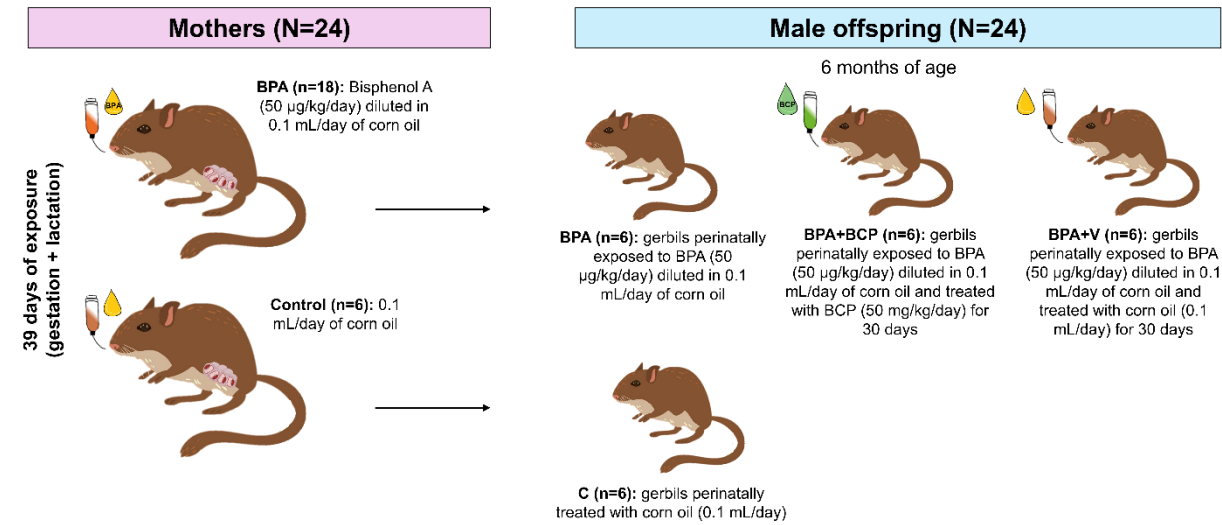
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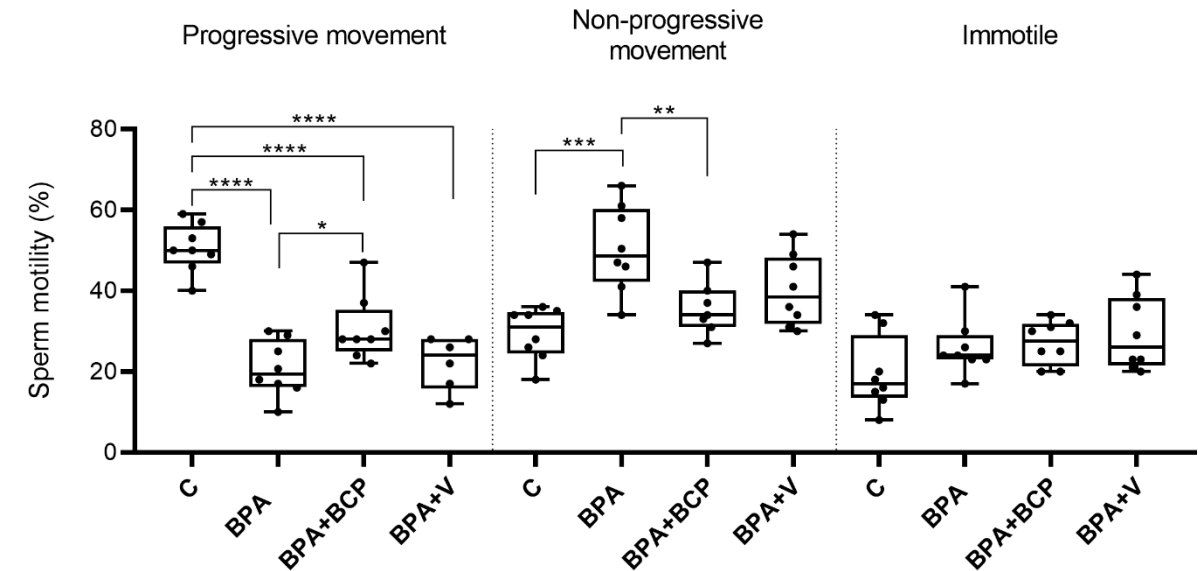
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Figure 1



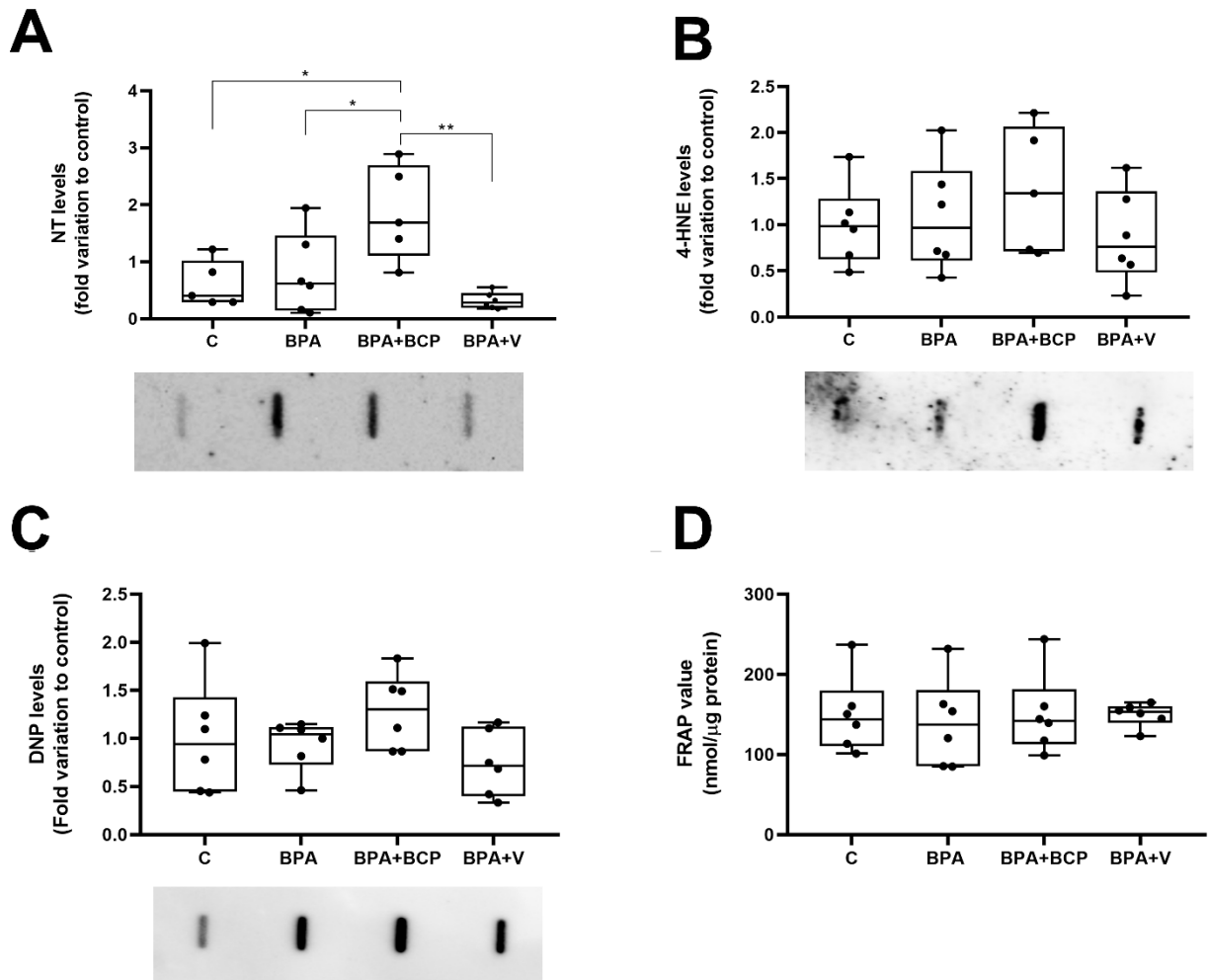
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Figure 2



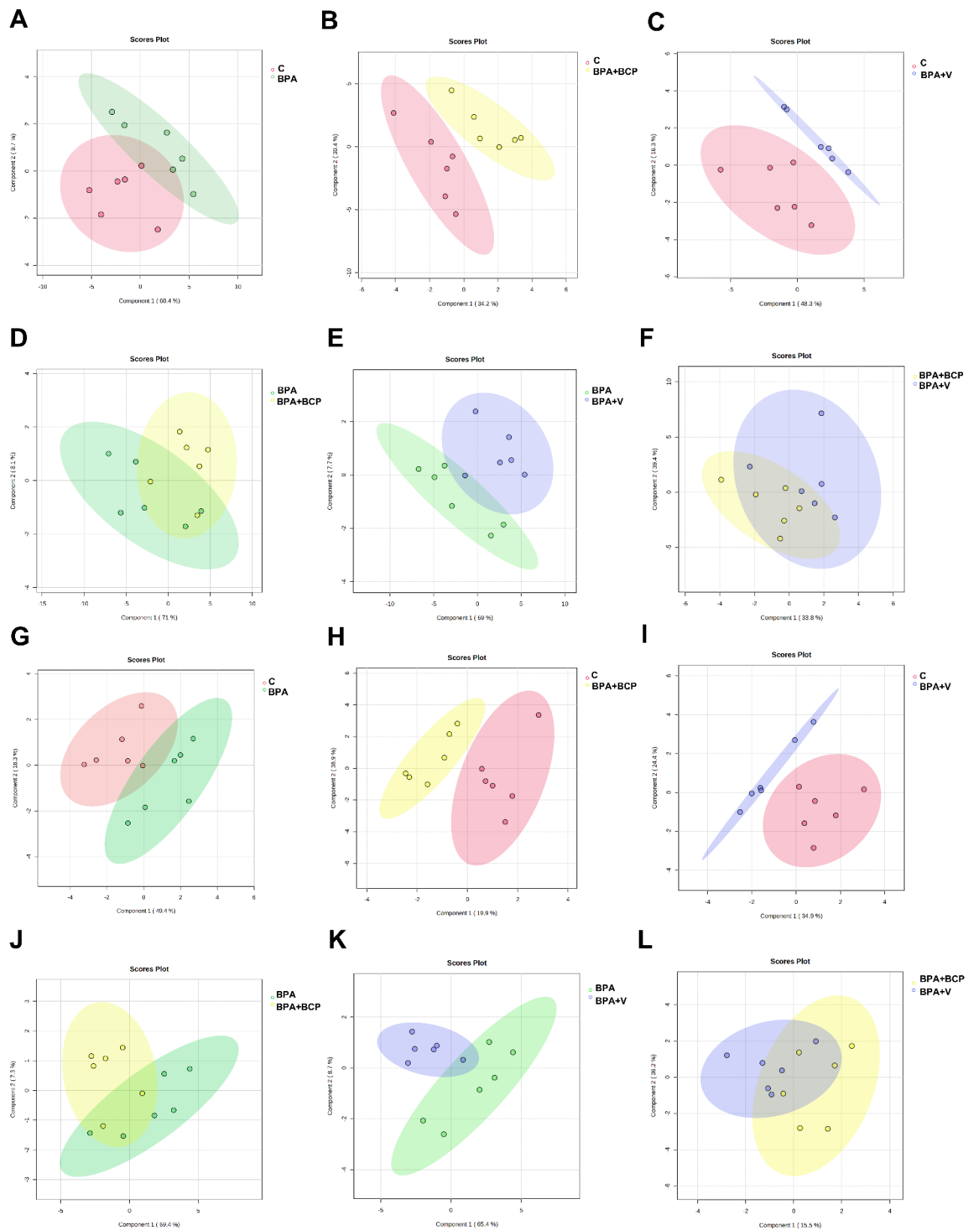
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Figure 3



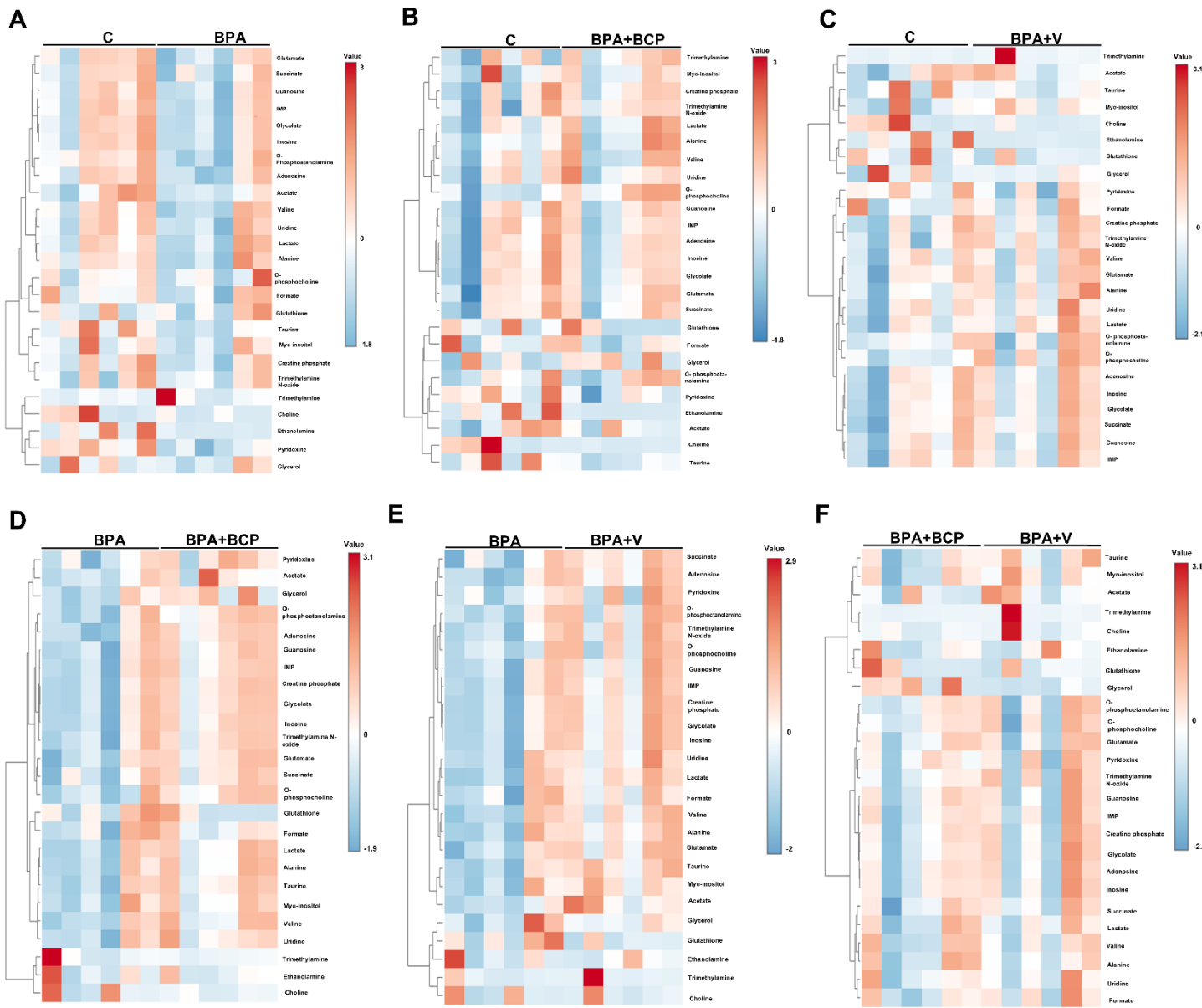
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Figure 4



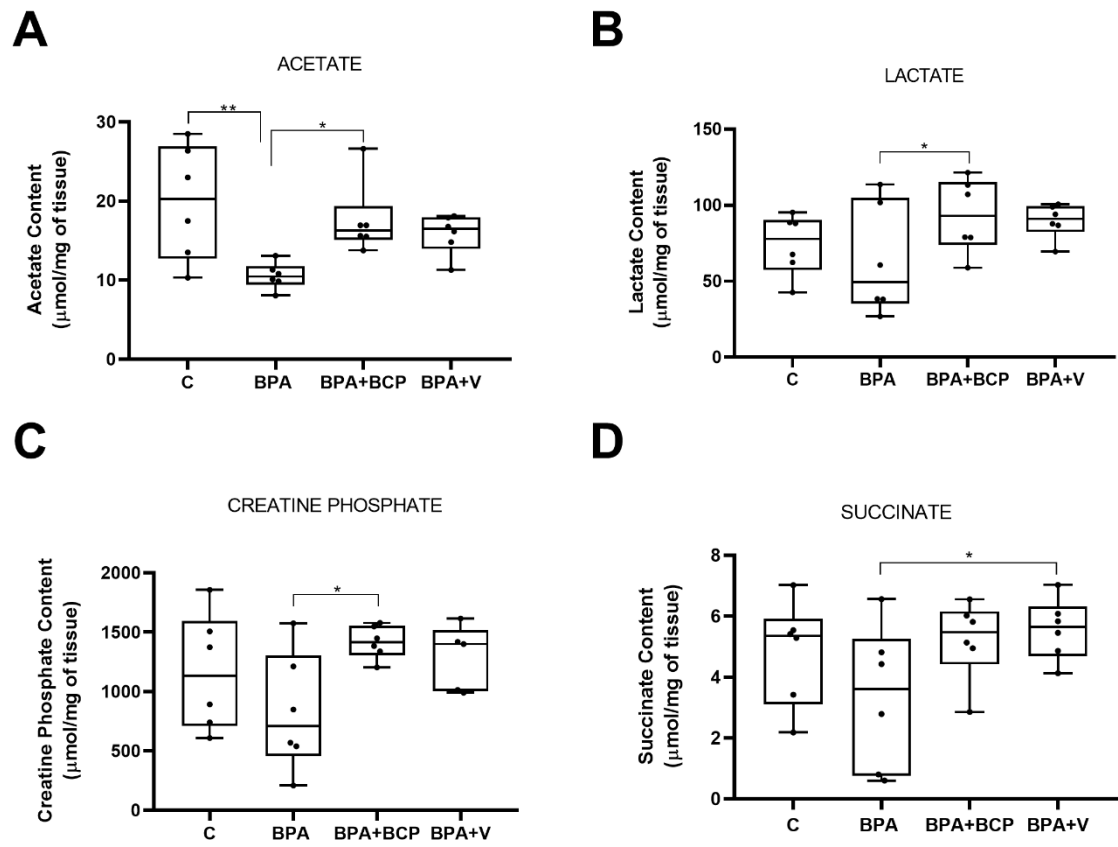
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Figure 5



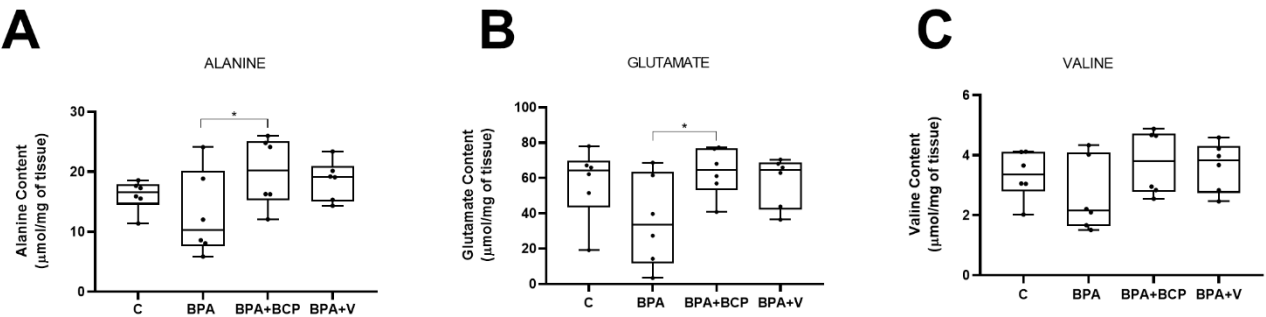
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Figure 6



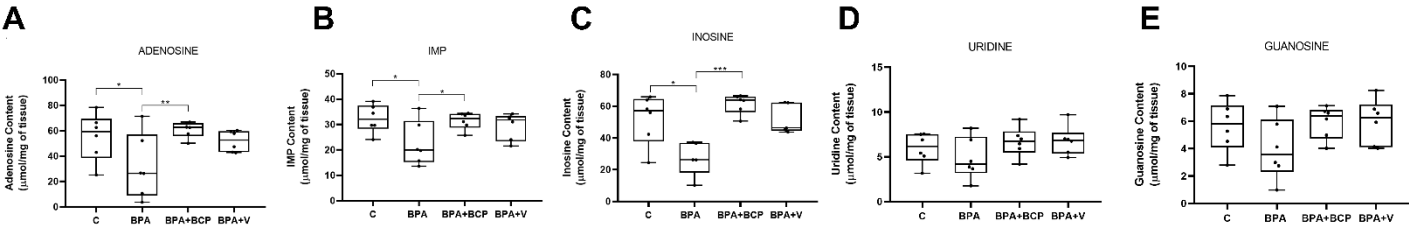
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Figure 7



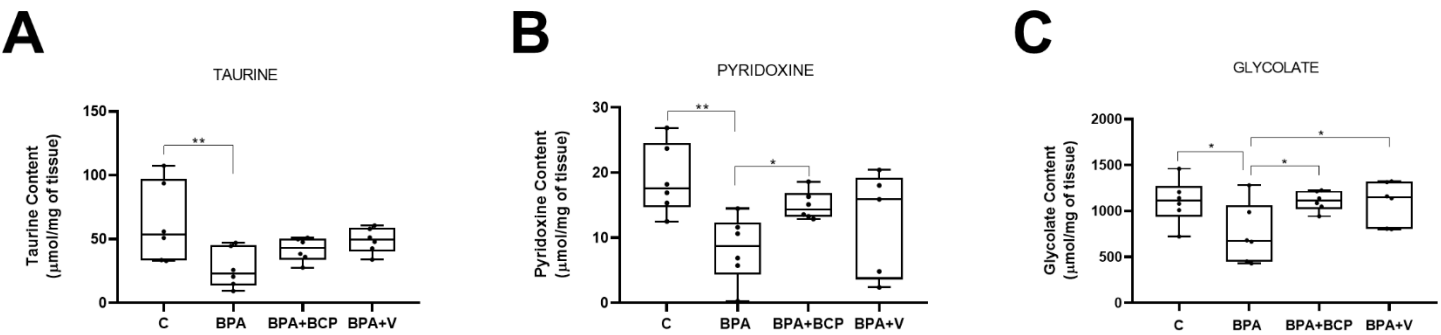
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Figure 8



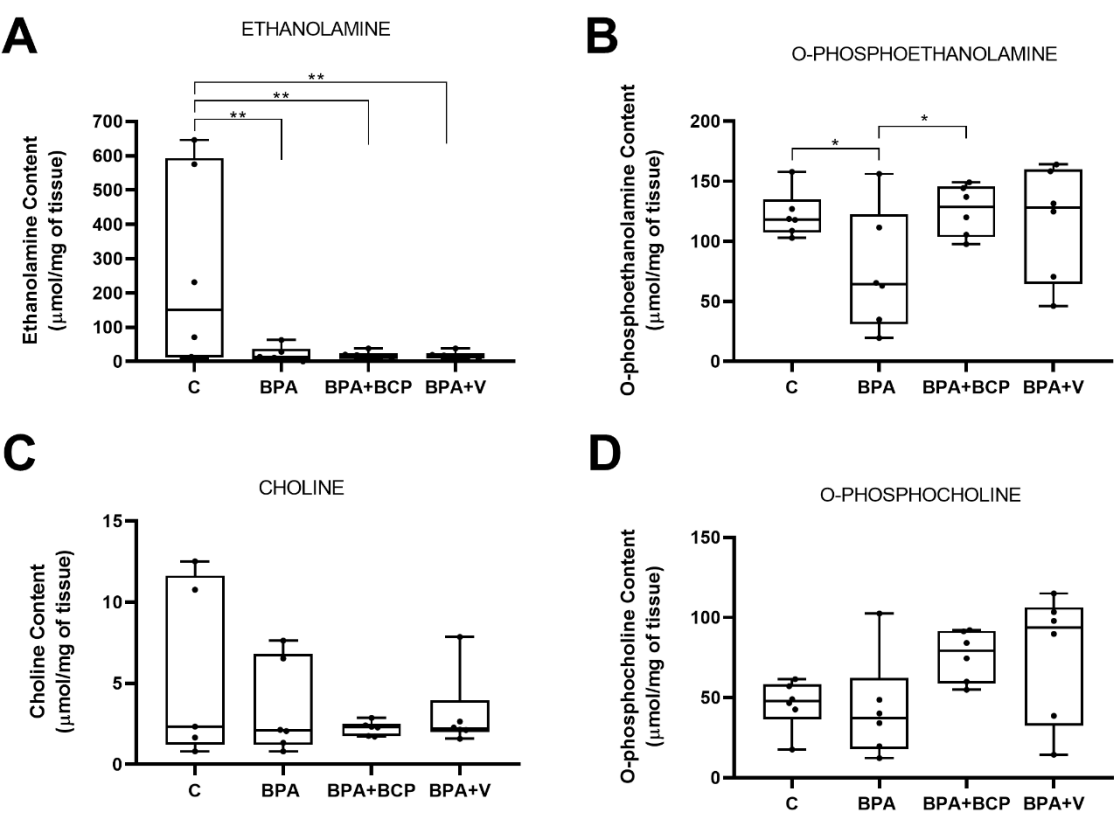
Source: Extracted from Bicalho-Silva *et al.* (2026)

Figure 9



Source: Extracted from Bicalho-Silva *et al.* (2026)

Figure 10



Source: Extracted from Bicalho-Silva *et al.* (2026)

Figure 11

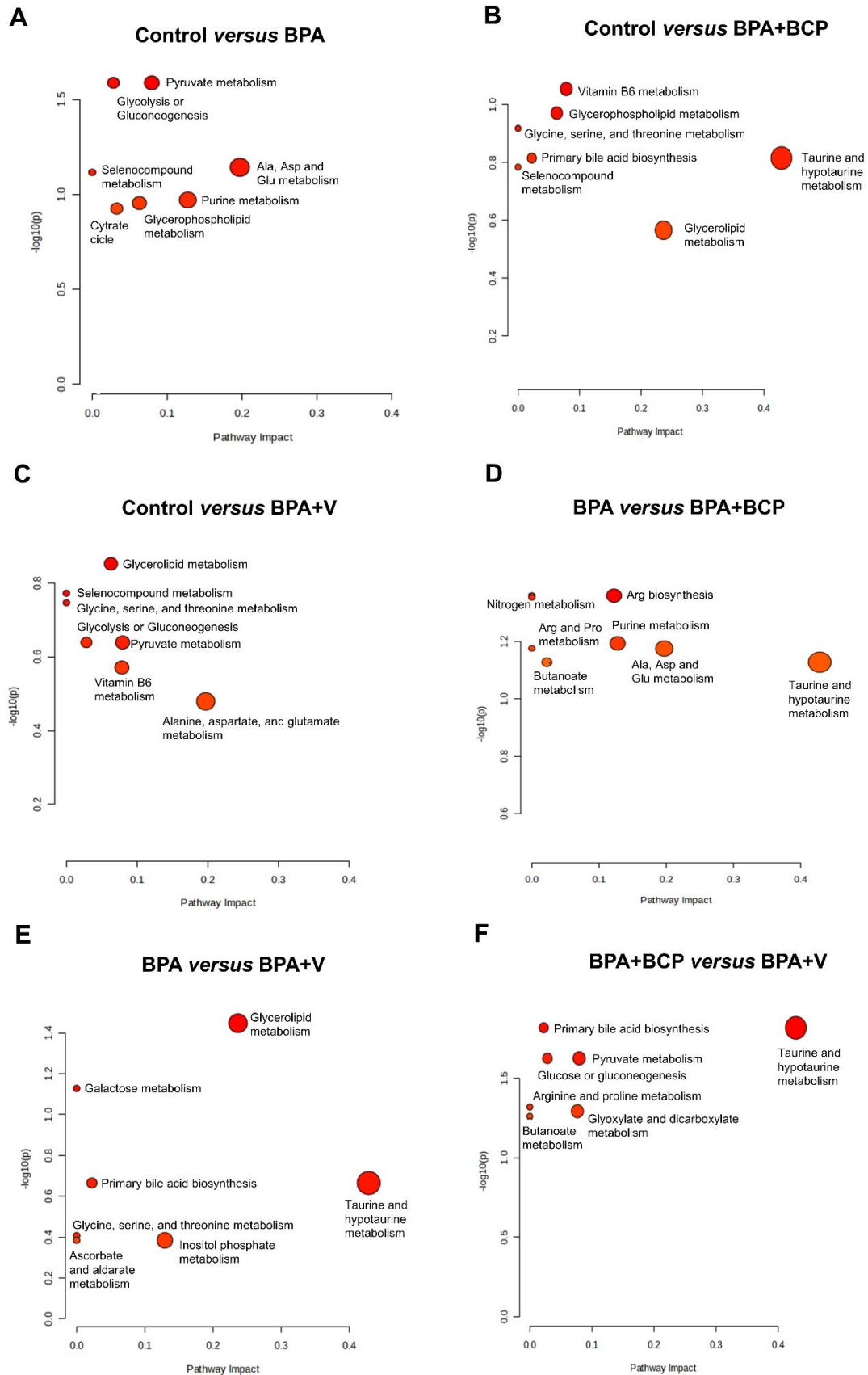
Source: Extracted from Bicalho-Silva *et al.* (2026)

Table 1: Biometric and hormonal data analyzed in Mongolian gerbils after perinatal exposure to BPA and BCP treatment in adulthood.

	C	BPA	BPA+BCP	BPA+V
Adiposity index (%)	2.2 ± 0.8	2.4 ± 1.1	2.4 ± 1.3	2.1 ± 0.7
Body weight (g)	77.6 ± 5.8	75.6 ± 5.9	77.0 ± 6.4	75.0 ± 9.8
Right testis weight (g)	0.64 ± 0.04	0.64 ± 0.10	0.66 ± 0.04	0.62 ± 0.09
Left testis weight (g)	0.68 ± 0.01	0.59 ± 0.12	0.65 ± 0.04	0.65 ± 0.09
Absolute testes weight (g)	1.31 ± 0.08	1.25 ± 0.22	1.32 ± 0.08	1.26 ± 0.19
GSI (%)	1.6 ± 0.0	1.6 ± 0.2	1.7 ± 0.1	1.6 ± 0.1
Blood glucose levels (mg/dL)	72.6 ± 4.9 ^a	76.3 ± 7.8 ^{ab}	85.1 ± 4.7 ^b	83.5 ± 9.7 ^{ab}
Total testosterone serum levels (ng/mL)	1.2 ± 0.3	1.7 ± 0.9	1.5 ± 0.5	1.4 ± 0.8
Estradiol serum levels (pg/mL)	21.4 ± 6.0	36.3 ± 35.2	48.6 ± 20.3	36.7 ± 13.1

Control (C), Bisphenol A (BPA), Bisphenol A plus β -caryophyllene (BPA+BCP), Bisphenol A plus vehicle (BPA+V), Gonadosomatic index (GSI). Data are presented as mean followed by standard deviation, where a $\neq$ b ($p \leq 0.05$).

Source: Extracted from Bicalho-Silva *et al.* (2026)

Supplementary Table 1: Description of primary and secondary antibodies used in this research.

Antibody	Source	Dilution	Vendor	Cat. No
DNP	Rabbit	1:5000	Sigma–Aldrich, St. Louis, MO, USA	D9656
Nitro-tyrosine	Mouse	1:5000	Cell Signaling Technology, Leiden, Netherlands	9691
4-HNE	Goat	1:5000	Merck Millipore, Temecula, USA	AB5605
Mouse	Goat	1:5000	Sigma–Aldrich, St. Louis, MO, USA	A3562
Rabbit	Goat	1:5000	Sigma–Aldrich, St. Louis, MO, USA	A3687
Goat	Rabbit	1:5000	Sigma–Aldrich, St. Louis, MO, USA	A4187

DNP: 2,4-dinitrophenol; 4-HNE: 4-hydroxynonenal.

Source: Extracted from Bicalho-Silva *et al.* (2026)

Supplementary Table 2: Assignment of resonances in the NMR spectra of testis polar extracts.

	Compound	H multiplicity	Chemical shift, δ (ppm)
1	Acetate	singlet	1.90
2	Adenosine	singlet	8.33
3	Alanine	doublet	1.47
4	Choline	singlet	3.19
5	Creatine phosphate	singlet	3.03
6	Ethanolamine	multiplet	3.13
7	Formate	singlet	8.44
8	Glutamate	multiplet	2.32
9	Glutathione	multiplet	4.56
10	Glycerol	double doublet	3.64
11	Glycolate	singlet	3.92
12	Guanosine	singlet	7.99
13	IMP	singlet	8.22
14	Inosine	doublet	6.09
15	lactate	doublet	1.32
16	<i>Myo</i> -inositol	triplet	4.05
17	O-phosphocholine	multiplet	4.16
18	O-phosphoethanolamine	multiplet	3.97
19	Pyridoxine	singlet	7.66
20	Succinate	singlet	2.39
21	Taurine	triplet	3.41

	Compound	H multiplicity	Chemical shift, δ (ppm)
22	Trimethylamine	singlet	2.88
23	Trimethylamine N-oxide	singlet	3.25
24	Uridine	doublet	7.86
25	Valine	doublet	0.98

Source: Extracted from Bicalho-Silva *et al.* (2026)

4. CONCLUSÃO

A presente tese reúne quatro estudos que investigaram os impactos da exposição a diferentes DEs na próstata e nos testículos de uma espécie de roedor de singular interesse científico. Os DEs estudados incluem metais potencialmente tóxicos, formulações comerciais de herbicidas e suas misturas e BPA. Particularmente, os terceiro e quarto estudos investigaram os efeitos da exposição perinatal ao BPA, considerando estágios cruciais do desenvolvimento, o intrauterino e a lactação. No quarto estudo, avaliamos também o possível papel protetivo do BCP frente às desordens metabólicas testiculares provocadas pelo BPA.

Coletivamente, os resultados alcançados destacam os diferentes mecanismos de ação atribuídos a cada desregulador endócrino avaliado. Os metais pesados não essenciais e potencialmente tóxicos causam efeitos adversos na próstata de murinos por prejudicar a homeostase da glândula por meio de desequilíbrio hormonal e ligações indesejadas aos receptores esteroides. O desequilíbrio hormonal é evidenciado, principalmente, por alterações nos níveis de testosterona, que levam a distúrbios no desenvolvimento da próstata, especialmente no contexto de exposições precoces. Além disso, desencadeiam a produção excessiva de espécies reativas de oxigênio (EROs) em desfavor da defesa antioxidante celular. O estabelecimento do estado pró-oxidativo tecidual inclui aumento da peroxidação lipídica e da oxidação de proteínas, que podem desencadear insultos inflamatórios e alterações estruturais na próstata de modelos murinos.

A exposição prolongada (90 dias) às formulações comerciais de glifosato, atrazina e 2,4-D e sua mistura causou diferentes insultos na próstata ventral, demonstrando sinergismo entre os compostos. Entretanto, alguns achados apontam para antagonismos entre exposição individual e combinada, entre eles, o que mostra diferenças importantes na toxicodinâmica e toxicocinética dos herbicidas quando combinados. O estudo conclui que mesmo em concentrações ambientalmente relevantes, os herbicidas podem causar alterações histológicas e hormonais na próstata de gerbilos machos, incluindo desequilíbrios hormonais, alterações estruturais e fisiológicas. A longo prazo, esses insultos podem comprometer a saúde prostática e a função reprodutiva.

A exposição durante a gestação e lactação ao BPA provocou efeitos prostáticos latentes que se tornaram tardiamente evidentes, sobretudo em gerbilos com hiperplasia prostática. A exposição a uma dose histórica, anteriormente

considerada segura e realista, de BPA durante um estágio crítico do desenvolvimento reprogramou a próstata adulta e a tornou suscetível a desordens que incluem histopatologias, aumento de importantes marcadores pró-inflamatórios, alterações em receptores esteroides e desbalanço hormonal. Esses achados foram potencializados no contexto hormonal alterado, caracterizado pela hiperplasia prostática. O estudo fornece evidências de que a exposição perinatal ao BPA causa impactos tardios na próstata que são, ainda, pronunciados na próstata hiperplásica, ressaltando os prejuízos em indivíduos predispostos ou que já desenvolveram essa condição patológica.

O quarto estudo conclui que a exposição perinatal ao BPA causa uma latente disfunção metabólica testicular, uma vez que reprograma os testículos em desenvolvimento e os torna propensos a disfunções metabólicas que se tornam tardiamente evidentes. Essas disfunções são caracterizadas por déficits em metabólitos cruciais, associados a vias bioquímicas essenciais para o estabelecimento e manutenção da homeostase testicular. Além disso, o estudo mostrou o potencial do BPA em reduzir a motilidade espermática, um parâmetro-chave para a avaliação da fertilidade masculina. Apesar dos potenciais efeitos adversos provocados pela exposição perinatal ao BPA, a intervenção com o BCP por 30 dias levou ao restabelecimento da motilidade espermática, bem como restaurou o conteúdo de metabólitos testiculares. Os dados deste estudo apontam para a necessidade de investigar compostos com potenciais efeitos terapêuticos, capazes de mitigar os efeitos adversos decorrentes da exposição a DEs e a outros poluentes ambientais. Além disso, o estudo destaca a importância da tradução dos achados em roedores para a saúde reprodutiva humana, dadas as diferenças particulares entre as espécies, a fim de resguardar e preservar a saúde reprodutiva das próximas gerações. Ademais, os terceiros e quartos estudos destacam e corroboram a hipótese postulada pela DOHaD, que determina que alterações em fases particulares do desenvolvimento podem fisiologicamente reprogramar e predispor o indivíduo a distúrbios tardios.

Embora distintos, os estudos apresentam uma conclusão comum acerca dos impactos reprodutivos causados pela exposição constante a DEs e da urgente necessidade de intervenções públicas para uma melhor qualidade de vida humana e ambiental, bem como da necessidade de se criar estratégias para mitigar e conter os danos causados pelos DEs ao meio ambiente.

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APÊNDICE I

CERTIFICADOS DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS



Instituto de Biociências, Letras e Ciências Exatas
Rua Cristovão Colombo, 2265 - Jardim Nazareth - CEP 15054-000 São José do Rio Preto - SP - Brasil
Tel: (17) 3221-2480 / 3221-2563

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação do risco de exposição a herbicidas e suas misturas: impactos sobre os órgãos reprodutivos de machos", registrada com o nº. 244/2023 - CEUA, sob a responsabilidade do PATRICIA SIMONE SIMONE LEITE VILAMAIOR, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou de ensino), encontra-se de acordo com os Preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada ad referendum pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), do IBILCE/UNESP, em 24/04/2023.

Finalidade: Pesquisa Científica

Vigência da Autorização: 27/03/2023 - 27/03/2026

ESPÉCIES/LINHAGENS/RAÇAS	Qtde. Animais	Idade	Origem
Descrição			
Meriones unguiculatus (Gerbilos da Mongólia)	50 machos, 0 fêmeas, 0 sem distinção	Idade De 6 a 9 meses	Biotério de Manutenção e Experimentação de Roedores - IBILCE

São José do Rio Preto, 25/04/2023

Luiz Henrique Florindo - Vice-presidente da CEUA

OBSERVAÇÕES

- 1) O Relatório Final deverá ser encaminhado em Formulário próprio à CEUA no prazo de até 30 (trinta) dias após o término da pesquisa.
- 2) Qualquer alteração na pesquisa deverá ser encaminhada à CEUA para apreciação.



Instituto de Biociências, Letras e Ciências Exatas
Rua Cristóvão Colombo, 2265 - Jardim Nazareth - CEP 15054-000 São José do Rio Preto - SP - Brasil
Tel: (17) 3221-2480 / 3221-2563

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que a proposta intitulada "Efeitos do β -Carbopreno na próstata de gerbilos após exposição gestacional e lactacional ao Bisfenol A e suplementados pela Testosterona na fase adulta", registrada com o n.º 236/2022 - CEUA, sob a responsabilidade do PATRÍCIA SIMONE LEITE VILAMAIOR, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou de ensino), encontra-se de acordo com os Preceitos da Lei n.º 11.794, de 08 de outubro de 2008, do Decreto n.º 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada ad referendum pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), do IBILCE/UNESP, em 29/06/2022.

Finalidade: Pesquisa Científica

Vigência da Autorização: 27/06/2022 - 31/08/2025

ESPÉCIES/LINHAGENS/RAÇAS

Descrição	Qtd. Animais	Idade	Origem
Meriones unguiculatus (Gerbilos da Mongólia)	90 machos, 0 fêmeas, 0 sem distinção	Idade De 180 a 240 dias	Biotério de Manutenção e Experimentação de Roedores

São José do Rio Preto, 27/02/2023



Luiz Henrique Florentino - Vice-presidente da CEUA

OBSERVAÇÕES

- 1) O Relatório Final deverá ser encaminhado em Formulário próprio à CEUA no prazo de até 30 (trinta) dias após o término da pesquisa.
- 2) Qualquer alteração na pesquisa deverá ser encaminhada à CEUA para apreciação.

APÊNDICE II

OUTRAS PRODUÇÕES DA MESMA TEMÁTICA DESENVOLVIDAS PELO GRUPO DE PESQUISA DURANTE O DOUTORADO

1. Effects of androgenic modulation on the morphophysiology of the adrenal cortex of male gerbils

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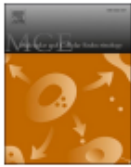
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Effects of androgenic modulation on the morphophysiology of the adrenal cortex of male gerbils

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ARTICLE INFO

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Castration
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Testosterone

ABSTRACT

The study aimed to investigate the repercussions of androgen modulation on the adrenal cortex of male gerbils, focusing on the morphophysiology, proliferation, and cell death, as well as the expression of hormone receptors and steroidogenic enzymes. Mongolian gerbils (*Meriones unguiculatus*) were divided into three experimental groups: Control (C), Testosterone (T), animals received injections of testosterone cypionate and Castrated (Ct), animals underwent orchietomy. The results showed that castration increased the zona fasciculata and promoted cell hypertrophy in all zones. Testosterone supplementation increased cell proliferation and cell death. Androgen modulation promoted an increase in AR, *Era*, and ER β . Castration promoted an increase in the CYP19, while decreasing 17 β HSD enzymes. Testosterone supplementation, on the other hand, reduced CYP17 and increased CYP19 and 3 β HSD enzymes. By analyzing the effects of androgen supplementation and deprivation, it can be concluded that testosterone is responsible for tissue remodeling in the cortex, regulating the rate of cell proliferation and death, as well as cell hypertrophy. Testosterone also modulate steroid hormone receptors and steroidogenic enzymes, consequently affecting the regulation, hormone synthesis and homeostasis of this endocrine gland.

2. Coconut oil affects aging-related changes in Mongolian gerbil liver morphophysiology

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RESEARCH PAPER

Coconut oil affects aging-related changes in Mongolian gerbil liver morphophysiology

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Abstract

Aging causes changes in liver morphophysiology, altering hepatocyte morphology and organ function. Due to its antioxidant and anti-inflammatory properties, coconut oil has been used as a therapeutic agent in diets, in an attempt to attenuate alterations in the liver naturally caused by aging. Herein, we evaluated the effects of coconut oil consumption during aging on Mongolian gerbil liver morphophysiology. The animals were divided into three experimental groups: the gerbils in the Adult Control Group (AC) were euthanized at 3 months of age, the gerbils in the Old Control Group (OC) at 15 months of age, and the gerbils in the Coconut Oil Group (CO) received 0.1 ml/day of coconut oil for 12 months and were euthanized at 15 months of age. Prolonged consumption of coconut oil during aging prevented the animals and the liver from gaining mass. However, the other results showed that coconut oil intensified the morphophysiological alterations of aging, promoting an increase in the hepatocyte cytoplasm and nuclei. In addition, an increase in blood vessels, reticular fibers, lipid droplets, and lipofuscin granules were observed in the CO group. Finally, the results also demonstrated that coconut oil promotes an increase in lipid peroxidation, indicated by an increase in MDA levels. We therefore conclude that coconut oil has the potential to intensify the morphophysiological alterations that occur in the liver during aging.

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Keywords: Angiogenesis; Antioxidants; Lipid peroxidation; Natural compounds; Phytotherapy.

3. Dehydroepiandrosterone sulphate (DHEAS) supplementation impacts adrenal cortex morphophysiology of aged female gerbils

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Dehydroepiandrosterone sulphate (DHEAS) supplementation impacts adrenal cortex morphophysiology of aged female gerbils

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ABSTRACT

During the process of aging, it is common for women to take dehydroepiandrosterone sulphate (DHEAS) supplements to prevent adrenopause. However, the potential effects of this supplementation on the adrenal cortex have not yet been fully elucidated. Therefore, the present study aimed to analyze the effects of DHEAS supplementation on the adrenal cortex of female Mongolian gerbils during the aging process. The experiment was conducted by dividing the aged female gerbils (18 months of age) into two groups (n = 5). The control group received no treatment, while the experimental group received 60 mg/kg of DHEAS for 5 weeks. The adrenal glands of both groups were then subjected to morphological, hormonal and immunohistochemistry analyses. The results showed that DHEAS supplementation led to a significant increase in the accumulation of lipofuscin granules in the adrenal cells. Furthermore, decreases in ERα and ERβ and the enzymes CYP17 and 17βHSD, and an increase in the 5α-reductase enzyme in the adrenal cortex were also observed. The results suggest that DHEAS supplementation has a negative feedback effect on the adrenal cortex, affecting its morphophysiology and, consequently, the gland's functionality. In addition, DHEAS supplementation does not reverse all aspects of the effects of aging on adrenal gland homeostasis.

4. Estrogen Therapies Enhance Mammary Carcinogenesis in Aging Gerbil Females Under Endocrine Disruption

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