LUIZ OTÁVIO GUIMARÃES ERVILHA

EFEITOS DE HERBICIDAS A BASE DE TRIAZINA SOBRE PARÂMETROS ENDÓCRINOS E REPRODUTIVOS DE MURINOS MACHOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

Orientadora: Mariana Machado Neves

VIÇOSA - MINAS GERAIS
2024

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
E73e
2024

Ervilha, Luiz Otávio Guimarães, 1997-
Efeitos de herbicidas a base de triazina sobre parâmetros endócrinos e reprodutivos de murinos machos / Luiz Otávio Guimarães Ervilha. – Viçosa, MG, 2024.
1 tese eletrônica (158 f.): il. (algumas color.).

Texto em português e inglês.
Orientador: Mariana Machado Neves.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Geral, 2024.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2024.642
Modo de acesso: World Wide Web.

1. Atrazina (Herbicida). 2. Eixo hipotalâmico-hipofisário-gonadal. 3. Infecundidade masculina. 4. Testosterona. 5. Espermatozoides. I. Neves, Mariana Machado, 1977-. II. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-Graduação em Biologia Celular e Estrutural. III. Título.

CDD 22. ed. 599.35

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

LUIZ OTÁVIO GUIMARÃES ERVILHA

EFEITOS DE HERBICIDAS A BASE DE TRIAZINA SOBRE PARÂMETROS ENDÓCRINOS E REPRODUTIVOS DE MURINOS MACHOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

APROVADA: 26 de agosto de 2024.

Assentimento:

Luiz Otávio Guimarães Ervilha
Autor

Mariana Machado Neves
Orientadora

Dedico à
minha mãe que sempre
me apoiou, me dando forças
para hoje estar aqui.

AGRADECIMENTOS

Primeiramente, agradeço a Deus por ter guiado meus caminhos e estar comigo nos momentos difíceis.

À minha mãe, Ana Rosa Guimarães Ervilha, cujo sobrenome faço questão de carregar. Agradeço pelo amor, suporte, força, dedicação, companheirismo, orações e por acreditar em mim, não medindo esforços para o meu melhor.

À minha família, por toda confiança, em especial à minha tia Maria Aparecida e minha prima Juliana. Aos meus entes falecidos, meu pai Luiz Carlos Ervilha e a minha avó Nilza.

Aos patinhos Izabela, Isabela, Mírian e Thainá. Nossas conversas, parcerias dentro e fora do laboratório, projetos, análises e almoços permitiram que estes e outros projetos saíssem do papel.

A Izabela, obrigado pela ajuda assídua nos cuidados do biotério. Foram meses de dedicação diária aos projetos que ali desenvolvemos. Agradeço, ainda, ao João Victor, Isabela, Luís Miguel e Thainá pelo auxilio durante o período experimental. As inúmeras caixas limpas, águas repostas e procedimentos realizados.

Ao Victor por acreditar em mim, me acompanhar nos momentos de estudos e tornar as coisas mais leves.

Aos meus amigos do Laboratório de Reprodução Animal e Toxicologia da UFV (LARAT), obrigado pelas dúvidas respondidas, conversas diárias e leveza no dia a dia.

Aos sempre presentes amigos de republica, de graduação e VRB. Sempre importantes para minha formação pessoal e profissional. Nossas conversas, cumplicidades e conselhos foram sempre de grande ajuda e merecem ser lembradas.

À Fernanda e Elizabeth, pelas conversas quase diárias, dúvidas respondidas e o entusiasmo pela implementação de técnicas em nossos trabalhos.

A Ana Cláudia, por me apresentar a pesquisa, me ensinar técnicas e possibilitar meu crescimento. Obrigado pelas oportunidades.

A minha sempre orientadora Profª Drª Mariana, por ter me dado a oportunidade de trabalho e confiança, me instruindo a pensar e me ensinando a discernir o melhor caminho. São nove anos de convívio e ensinamentos, que levarei para toda a vida.

Aos membros da banca, por dedicarem um tempo a avaliar o meu trabalho. Obrigado Fernanda Dias, Mônica Morais, Ana Souza e Leandro Licursi.

À Universidade Federal de Viçosa, minha querida UFV, e seus funcionários e professores, por todo apoio e suporte.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) pelo consentimento da bolsa.

Às agências de fomento FAPEMIG, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo auxílio financeiro que permite o funcionamento do laboratório e o desenvolvimento de nossas pesquisas.

EPÍGRAFE

O real não está na saída nem na chegada:
ele se dispõe para a gente é no meio da travessia.
Guimarães Rosa

RESUMO

GUIMARÃES-ERVILHA, Luiz Otávio, D.Sc., Universidade Federal de Viçosa, agosto de 2024. **Efeitos de herbicidas a base de triazina sobre parâmetros endócrinos e reprodutivos de murinos machos**. Orientadora: Mariana Machado Neves.

O uso de herbicidas é uma prática comum na agricultura moderna. Compostos à base de triazina, como a atrazina ou sua combinação com a mesotriona (Calaris®), destacam-se por sua eficácia no controle de ervas daninhas e custo relativamente baixo. Contudo, cada vez mais trabalhos demonstram que esses herbicidas podem atuar como desreguladores endócrinos, interferindo no eixo hipotálamo-hipófise-gônada (HPG) e causando distúrbios hormonais que afetam a saúde reprodutiva. Assim, este estudo investigou, primeiramente, os efeitos da atrazina sobre o eixo HPG de machos, a partir de uma revisão sistematizada e meta-analítica. Depois, avaliou-se a ação do Calaris®, uma nova tecnologia agrícola, sobre o sistema reprodutivo masculino e parâmetros de fertilidade em modelos experimentais, com foco nos mecanismos de toxicidade após exposição subcrônica. A revisão de literatura evidenciou a intensidade dos impactos da atrazina sobre as concentrações de hormônios importantes do eixo HPG em modelos murinos. Destacou-se reduções significativas nos níveis séricos do hormônio folículo estimulante, hormônio luteinizante e de testosterona, com aumento dos níveis séricos de estradiol após administração de altas concentrações de atrazina (> 100 mg Kg^{-1}), independente do tempo de exposição. Já no trabalho experimental, ratos Wistar machos foram expostos a duas concentrações de Calaris®, 2 e 20 mg Kg^{-1}, durante 70 dias por gavagem. Após o período experimental, os animais foram alocados para testes de fertilidade com fêmeas hígidas e, por fim, eutanasiados. Hipotálamo, testículos e epidídimos foram coletados e processados para avaliações histológicas, bioquímicas, enzimáticas, balanço mineral e análises de genotoxicidade. O soro foi recuperado para análises hormonais. Ainda, avaliamos a qualidade espermática e a viabilidade dos fetos. Os resultados foram comparados por ANOVA One-way seguido pelo teste de post hoc de Tukey. A exposição ao Calaris® resultou em estresse oxidativo e nitrosativo com desorganização do parênquima testicular, desbalanço mineral, danos no DNA de células testiculares, baixa quantidade e qualidade espermática e redução na fertilidade, mesmo na ausência de alterações nos níveis de testosterona. Os achados sugerem que a combinação de herbicidas pode ter efeitos adversos mais significativos do que os compostos isolados, considerando as baixas

concentrações de Calaris® utilizadas (2 e 20 mg Kg^{-1}), reforçando a necessidade de revisões nas práticas de uso desses produtos na agricultura.

Palavras-chave: Atrazina. Eixo hipotálamo-hipófise-gônada. Fertilidade masculina. Testosterona. Espermatozoides.

ABSTRACT

GUIMARÃES-ERVILHA, Luiz Otávio, D.Sc., Universidade Federal de Viçosa, August, 2024. **Effects of triazine-based herbicides on hormonal and reproductive parameters in male murine models**. Adviser: Mariana Machado Neves.

The use of herbicides is a common practice in modern agriculture. Triazine-based compounds, such as atrazine or its combination with mesotrione (Calaris®), stand out for their effectiveness in weed control and relatively low cost. However, more and more studies demonstrate that these herbicides can act as endocrine disruptors, interfering with the hypothalamic-pituitary-gonadal (HPG) axis and causing hormonal disorders that affect reproductive health. Therefore, this study first investigated the effects of atrazine on the HPG axis of males, based on a systematic and meta-analytical review. Then, the action of Calaris®, a new agricultural technology, on the male reproductive system and fertility parameters in experimental models was evaluated, focusing on the mechanisms of toxicity after subchronic exposure. The literature review demonstrated the intensity of the impacts of atrazine on the concentrations of important hormones of the HPG axis in murine models. Significant reductions in serum levels of follicle-stimulating hormone, luteinizing hormone, and testosterone were noted, with increased serum levels of estradiol after administration of high concentrations of atrazine (> 100 mg Kg^{-1}), regardless of the exposure time. In the experimental work, male Wistar rats were exposed to two concentrations of Calaris®, 2 and 20 mg Kg^{-1}, for 70 days by gavage. After the experimental period, the animals were allocated to fertility tests with healthy females and, finally, euthanized. The hypothalamus, testes, and epididymis were collected and processed for histological, biochemical, and enzymatic evaluations, mineral balance, and genotoxicity analyses. The serum was recovered for hormonal analyses. Furthermore, we evaluated sperm quality and fetal viability. The results were compared by One-way ANOVA followed by Tukey's post hoc test. Exposure to Calaris® resulted in oxidative and nitrosative stress with disorganization of the testicular parenchyma, mineral imbalance, DNA damage in testicular cells, low sperm quantity and quality, and reduced fertility, even in the absence of changes in testosterone levels. The findings suggest that the combination of herbicides may have more significant adverse effects than the isolated compounds, considering the low concentrations of Calaris® used (2 and 20 mg Kg^{-1}), reinforcing the need for reviews in the practices of using these products in agriculture.

Keywords: Atrazine. Hypothalamic-pituitary-gonad axis. Male fertility. Testosterone. Sperm.

SUMÁRIO

CAPÍTULO 1

1. Justificativa

Os avanços tecnológicos e a globalização das práticas agrícolas contribuíram para a melhoria e o aumento significativo na produção de alimentos (Hailu, 2023). Este é um passo crucial em uma realidade onde a demanda por alimentos está aumentando. O uso de agroquímicos, especialmente herbicidas, é um método eficaz para minimizar perdas de plantações devido a ervas daninhas (Parven et al., 2024). No entanto, o uso contínuo e muitas vezes indiscriminado desses compostos resulta em eventos adversos para o meio ambiente. Em particular, os efeitos em organismos não-alvo, como humanos e animais, tornam-se uma preocupação.

A atrazina é um herbicida da classe das triazinas. É amplamente utilizado em várias culturas porque é altamente eficiente e relativamente barato. No entanto, apesar de sua popularidade e benefícios nas práticas agrícolas, o uso do herbicida também tem sido associado a uma série de resultados negativos para a saúde animal e humana, principalmente devido às suas capacidades de desregulação endócrina. Estudos laboratoriais e dados epidemiológicos demonstram irrefutavelmente que a exposição à atrazina pode prejudicar a função do eixo hipotálamo-hipófise-gonadal (HPG) (Wirbisky e Freeman, 2015; Chang et al., 2022; Das et al., 2023). O eixo HPG é responsável por regular e manter a integridade dos órgãos reprodutivos, como os testículos e o epidídimo nos machos. Alterações no eixo HPG prejudicam o equilíbrio hormonal, causando uma série de problemas, especialmente no que diz respeito à fertilidade e à saúde reprodutiva geral (Acevedo-Rodriguez et al., 2018). Dado o uso generalizado de atrazina e compostos relacionados, é necessário buscar por uma compreensão mais profunda de seus efeitos biológicos em organismos não-alvo. Investigar seu potencial como desregulador

endócrino é crucial para identificar os riscos reais que esses compostos representam. Resíduos em alimentos, água e solo representam uma fonte significativa de exposição para a população humana.

A combinação de herbicidas, como a atrazina com o herbicida mesotriona, uma prática comum no contexto agrícola, levanta preocupações adicionais (Tudi et al., 2021; Araujo et al., 2023). Esses produtos são aplicados para melhorar o controle de ervas daninhas, mas as interações químicas entre eles podem potencializar os efeitos adversos na saúde reprodutiva. É necessário investigações não apenas do impacto individual de cada composto, mas também dos efeitos combinados. Propomos investigar os efeitos da atrazina, sozinha e em combinação com mesotriona, no sistema reprodutor masculino usando modelos bem estabelecidos. Avaliaremos os mecanismos pelos quais esses compostos interferem no equilíbrio hormonal e comprometem a função reprodutiva, com atenção especial à função testicular e à fertilidade.

Os resultados apresentados aqui podem contribuir para a reavaliação dos padrões de segurança relacionados ao uso de herbicidas na agricultura. Por exemplo, não há regulamentação adequada sobre os níveis permitidos de mesotriona na água potável. Uma compreensão mais profunda dos efeitos desses compostos é essencial para o desenvolvimento de estratégias de mitigação de riscos, promovendo uma agricultura mais sustentável e segura.

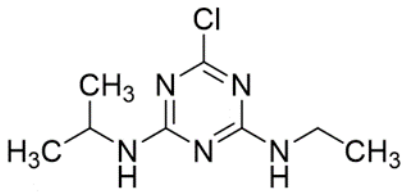
2. Revisão de Literatura

2.1 Herbicidas a base de triazina

Agroquímicos são facilitadores para a produção de alimentos em larga escala pela agricultura. A aplicação de defensivos agrícolas é uma tecnologia simples e eficaz

para controlar as perdas de plantações e aumentar a produção. Basicamente, esses compostos químicos previnem a perda de colheitas por pragas, doenças e ervas daninhas, tornando os alimentos mais acessíveis e baratos, bem como permitem que a produção aconteça em áreas com condições climáticas adversas (Gianessi, 2013). No entanto, a utilização imprudente destes compostos apresenta riscos à saúde pública e animal, uma vez que os efeitos dos herbicidas não se limitam apenas aos organismos-alvo, podendo afetar adversamente outros organismos no ambiente (Gore et al. 2015; Ikeji et al., 2023). Dentre os defensivos agrícolas temos os herbicidas, voltados para o combate de ervas daninhas que podem interferir negativamente no crescimento e desenvolvimento das culturas de valor econômico (Tudi et al., 2021).

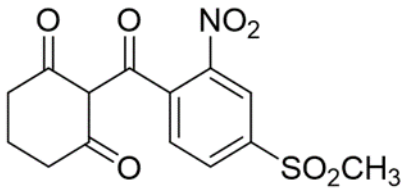
Os químicos triazinas, como a atrazina, são herbicidas agrícolas amplamente utilizados em culturas de milho, sorgo, cana-de-açúcar, algodão e abacaxi (Ikeji et al., 2023). A atrazina (6-cloro-N_2-etil-N_4-isopropil-1,3,5-triazina-2,4-diamina) apresenta amplo espectro, sendo o segundo herbicida mais utilizado no mundo (Kumar et al., 2013; Solomon et al., 2013; Fig. 1). Os átomos de nitrogênio no anel aromático da atrazina a tornam tóxica e duradoura no ambiente (Boopathy, 2017). Quando a atrazina entra no solo e no sedimento, ela se combina com diferentes componentes do ambiente por meio de adsorção e depois se acumula no ecossistema (Gao et al., 2018). Devido ao seu uso generalizado, a atrazina e seus metabólitos derivados, como a deetilatrazina, contaminam a água e o solo, principalmente em campos agrícolas, onde são altamente persistentes na água (águas subterrâneas e superficiais) e resistentes à degradação, perdurando por vários anos no ambiente (He et al., 2019). Por isso, sua existência no solo e nos sedimentos tem recebido grande preocupação por parte da comunidade científica, devido aos seus potenciais riscos ecológicos, carcinogênicos e como desregulador endócrino (Stradtman e Freeman., 2021).



2-cloro-4-etilamino-6-isopropilamino-1,3,5-triazina
(atrazina)

Figura 1: Molécula de atrazina.

Com crescente demanda por produção de alimentos, o desenvolvimento de novos defensivos e a atualização de produtos já conhecidos ganha destaque, visando aumentar a eficácia dos produtos. Neste contexto, a combinação de métodos já utilizados torna-se uma alternativa frente à produção de alimentos (Qu et al., 2021). Dentre as novas combinações de químicos vendidos comercialmente temos o Calaris®, que compreende uma mistura de atrazina com o composto mesotriona. A mesotriona é um herbicida seletivo utilizado no controle de plantas daninhas de folhas largas em culturas de milho, sorgo, algodão e batata. A substância ativa da mesotriona é a 2-(4-metoxi-6-metil-1,3,5-triazin-2-il)-5-[[(metilsulfinil)carbamoil]amino]benzoico, que age inibindo a enzima 4-hidroxifenilpiruvato dioxigenase (HPPD), responsável pela síntese de pigmentos fotossintéticos nas plantas daninhas (Fig. 2). A mesotriona é moderadamente solúvel em água, o que a torna capaz de contaminar águas superficiais e subterrâneas. Consequentemente, o herbicida e seus resíduos já foram detectados como poluentes em ecossistemas aquáticos (Wang et al., 2018). Desta forma, com a aplicação generalizada de mesotriona, sua capacidade de retenção no solo e a possibilidade de entrar nas águas superficiais por escoamento superficial também foi potencializada (Barchanska et al., 2011). A combinação atrazina/mesotriona apresenta potencial comercial atraente, sendo mais eficaz no combate a ervas daninhas que os compostos utilizados de forma individual.



2-(4-mesil-2-nitrobenzoil)cicloexano-1,3-diona
(mesotriona)

Figura 2: Molécula de mesotriona.

Como esses compostos foram desenvolvidos para atuar em tecidos vegetais, organismos não-alvos, como representantes da fauna, podem apresentar reações adversas quando entram em contato com esses agentes. No entanto, os efeitos toxicológicos nestes indivíduos ainda são pouco conhecidos. Recentemente, Araujo et al. (2021) registraram efeitos nocivos na abelha sem ferrão *Partamona helleri* frente a exposição aguda ao Calaris®, acarretando mudanças comportamentais e alterações na morfofisiologia do aparelho digestivo. Em mamíferos, sabe-se que a atrazina pode causar efeitos negativos em diversos órgãos, inclusive aqueles direta ou indiretamente relacionados com o eixo hipotalâmico-hipofisário-gonadal. Sabe-se que a exposição crônica ao herbicida pode levar a disfunções hormonais, afetando a liberação de gonadotrofinas pelo hipotálamo e, consequentemente, perturbando a função testicular (Wirbisky e Freeman, 2015). A interferência da atrazina na sinalização endócrina pode inibir a esteroidogênese e a espermatogênese, além de promover alterações histológicas em testículos e epidídimos. Dentre as patologias epididimárias temos presença de vacúolos no epitélio, inflamações intersticiais e a presença de células germinativas anormais no lúmen do ducto (Abarikwu et al., 2021). Esses efeitos adversos são preocupantes, pois sinalizam um potencial efeito sobre a fertilidade masculina. Além disso, mecanismos moleculares advindos dessas alterações parecem envolver a desregulação de vias críticas de sinalização hormonal e a geração de estresse oxidativo (Abarikwu et al., 2023). Partindo do pressuposto que o

Calaris® é uma mistura de atrazina com mesotriona, espera-se uma interferência desse produto em parâmetros reprodutivos masculinos de mamíferos.

2.2 Órgãos reprodutores masculinos

2.2.1 Histofisiologia dos testículos

Os testículos são gônadas masculinas pares localizadas no escroto, fora da cavidade abdominal, possuindo uma estrutura complexa e adequada para exercer funções endócrinas e exócrinas. Os testículos são envoltos pela túnica albugínea, uma cápsula de tecido conjuntivo denso, da qual partem septos de tecido conjuntivo (Setchell e Breed, 2005; França et al., 2016). O parênquima do órgão é ocupado por centenas de estruturas concêntricas, denominadas de túbulos seminíferos, que são intercaladas com uma região intersticial (Fig. 3). Com comprimento considerável, mais de 500 metros nos homens (Cameron, 2007), os túbulos seminíferos são altamente convolutos dentro do testículo e correspondem a, aproximadamente, 90% do volume testicular. Os túbulos seminíferos são formados pela túnica própria, composta de lâmina basal, lâmina própria de tecido conjuntivo e camada de células contráteis distribuídas concentricamente denominadas de células mioides. Essas células tem elementos contráteis em seu citoplasma que favorecem a movimentação do conteúdo luminal por movimentos peristálticos (Zhou et al., 2019). Além disso, o túbulo seminífero apresenta o epitélio seminífero/germinativo e o lúmen tubular (Li et al., 2024). No epitélio seminífero ocorre a produção de espermatozoides, pelo processo denominado espermatogênese, refletindo a função exócrina do órgão. Entre os constituintes do epitélio seminífero estão as células somáticas, denominadas de Sertoli, e as células da linhagem germinativa (Fig. 3). As células de Sertoli formam a estrutura dos túbulos seminíferos, com sua base sobre a membrana basal da túnica própria e seu ápice orientado em direção ao lúmen do túbulo seminífero. As células de Sertoli realizam

diversas funções, como nutrição, sustentação e produção de fatores de crescimento necessários para manter um ambiente adequado à atividade das células germinativas ligadas à espermatogênese (Zhao et al., 2014; França et al., 2016). Já o interstício é formado por tecido conjuntivo frouxo, no qual encontramos vasos sanguíneos, fibroblastos, vasos linfáticos, células do sistema de defesa e células de Leydig, também chamadas de células intersticiais (Fig. 3). É no interstício que ocorre a produção do andrógeno, uma função endócrina do órgão. A produção de hormônios, sendo o principal a testosterona, é realizada pelas células de Leydig, cujo processo é denominado esteroidogênese (Zhou et al., 2019). As extremidades dos túbulos seminíferos são contínuas aos túbulos retos, os quais se comunicam com os ductos eferentes que fornecem conexão para o transporte de espermatozoides até o ducto epididimário. O testículo é um dos órgãos dos mamíferos que é altamente sensível aos efeitos disruptivos endócrinos de tóxicos, a ação de xenobióticos tende a desorganizar o epitélio seminífero, comprometendo a função do órgão (Luccio-Camelo e Prins, 2011). Análises histológicas e histomorfométricas podem indicar o comprometimento da função testicular.

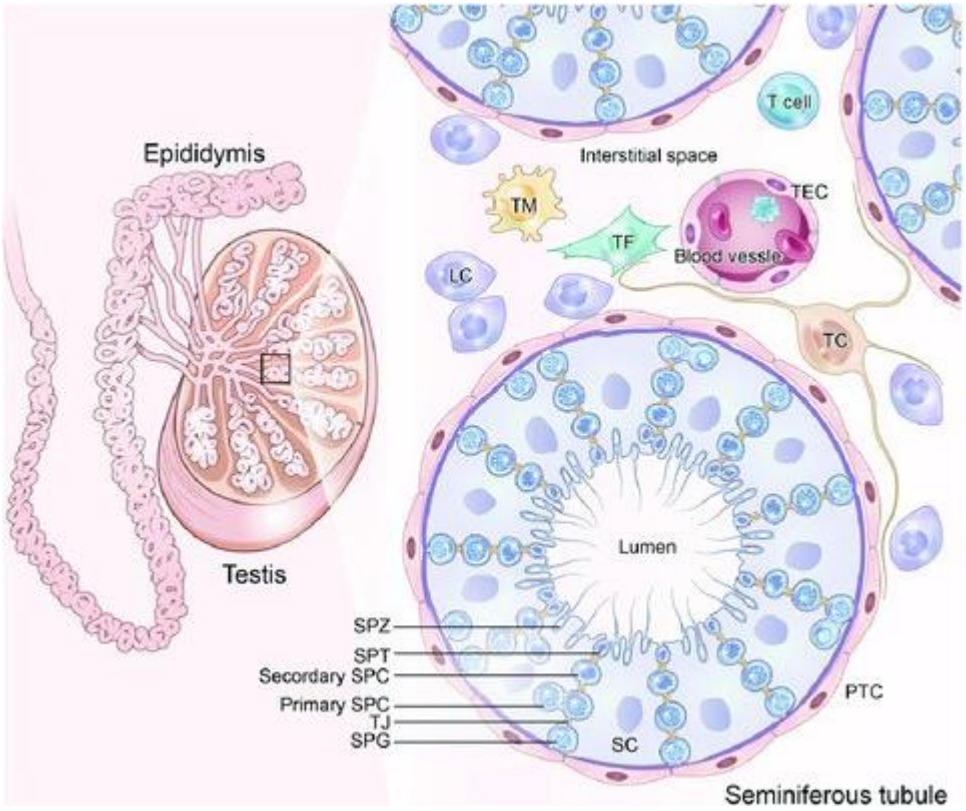


Figura 3: Organização dos testículos. Corte transversal diagramático do testículo mostrando a localização do túbulo seminífero e do espaço intersticial (esquerda). Nos túbulos seminíferos (direita), células germinativas em diferentes estágios de maturação estão amparadas pelas células de Sertoli (SCs). Espermatozoides são apresentações na região do lúmen do túbulo seminífero. O túbulo seminífero é cercado por células mioides peritubulares (PTCs). No espaço intersticial, vasos sanguíneos, células de Leydig (LCs), telócitos (TCs), fibroblastos testiculares (TFs), macrófagos testiculares (TMs) e algumas outras células imunes estão presentes. SPC: espermatócito; SPG: espermatogônia; SPT: espermátide; SPZ: espermatozoides; TEC: célula endotelial testicular; TJ: junção Sertoli-Sertoli (Ma et al., 2023).

Os espermatozoides são células haploides, pequenas e móveis, com a função de fecundar o ovócito. Espermatócitos, espermátides e, consequentemente, os espermatozoides aparecem pela primeira vez na puberdade, a partir da proliferação das células espermatogoniais, marcando o início da ocorrência de um ciclo espermatogênico completo. Sua produção espermática nos túbulos seminíferos exige uma proteção contra o reconhecimento pelo sistema imunológico. Para isso, o epitélio seminífero conta com a formação da barreira hemato-testicular ou barreira Sertoli-Sertoli, gerando um ambiente imunologicamente privilegiado (Mruk e Cheng, 2015; Figueiredo et al., 2021; Luaces et

al., 2023). Para isso, células de Sertoli vizinhas formam zônulas de oclusão entre elas que impedem a passagem de macromoléculas ao longo do epitélio, criando uma diferença entre a composição molecular das porções basal e adluminal (Gerber et al., 2016). A porção basal do epitélio é formada por espermatogônias, o tipo celular mais imaturo, seguida de espermatócitos em leptóteno e espermatócitos em zigóteno, enquanto a porção adluminal é composta por espermatócitos em paquíteno, espermatócitos secundários e espermátides, o tipo celular mais maduro e diferenciado, sendo liberadas no lúmen originando os espermatozoides presentes no lúmen tubular (Mruk e Cheng, 2015; Gerber et al., 2016). A avaliação da morfologia e da motilidade espermática podem ser bons indicativos da qualidade na produção do gameta. A alteração desses parâmetros após exposição a tóxicos pode indicar efeitos negativos na reprodução masculina.

2.2.2 Histofisiologia dos epidídimos

Os epidídimos são órgãos pares anexos aos testículos, localizados na superfície posterior dos testículos. O órgão desempenha um papel fundamental na maturação, armazenamento e transporte dos espermatozoides, além de reabsorver fluidos de forma a promover a concentração do gameta (Robaire e Hinton, 2015; James et al., 2020). O epidídimo é normalmente dividido em três regiões, cabeça (caput), corpo (corpus) e cauda (Fig. 4). Espécies de roedores ainda apresentam uma região anterior à cabeça, conhecida como segmento inicial. Do mediastino testicular saem os dúctulos eferentes, que transportam os espermatozoides dos testículos até o segmento inicial do epidídimo (Robaire e Hinton, 2015). Ao contrário do testículo formado por centenas de túbulos seminíferos, o epidídimo é formado por um único, longo e convoluto ducto que se conecta ao ducto deferente. Basicamente, o epidídimo é formado por um compartimento ductal e outro interductal (Castro et al., 2017). O compartimento ductal é composto pelo ducto

epididimário em si, com seu epitélio pseudoestratificado cilíndrico, composto por células principais, basais e claras, apresentando em algumas regiões, células apicais e estreitas (Robaire e Hinton, 2015; Zhao et al., 2020). O outro compartimento é formado por um tecido conjuntivo com vasos sanguíneos e linfáticos e células de defesa (Kempinas and Klinefelter, 2014). O epitélio epididimário é revestido por uma membrana basal espessa e cercada por uma camada muscular definida.

Os tipos celulares do epitélio presentes nas regiões da cabeça, corpo e cauda do epidídimo são as células principais, basais e claras (Fig. 4). As células principais constituem aproximadamente 80% das células epiteliais do órgão e apresentam microvilosidades longas na porção apical das células, chamadas de estereocílios, que aumentam a superfície para absorção e secreção de fluidos. Estas células são fundamentais para a proteção e manutenção de um ambiente luminal adequado para o desenvolvimento dos espermatozoides, formando junções estreitas umas com as outras para produzir a barreira hematoepididimária (Mita et al., 2011; Lin e Troyer, 2014; Robaire e Hinton, 2015; Cyr et al., 2018). As células basais, como o próprio nome sugere, estão localizadas na base do epitélio. Atuam como células-tronco do epitélio epididimário e contribuem para a renovação e reparo do epitélio (Robaire e Hinton, 2015). As células claras estão presentes na região da cauda e têm uma aparência clara ao microscópio devido à sua baixa densidade de organelas citoplasmáticas. São responsáveis pelo descarte de gotículas citoplasmáticas eliminadas pelos espermatozoides e contêm H^+ATPase que acidificam o fluido luminal, um processo importante para a manutenção do ambiente ácido necessário para a preservação da viabilidade dos espermatozoides durante seu armazenamento (Breton et al., 2019).

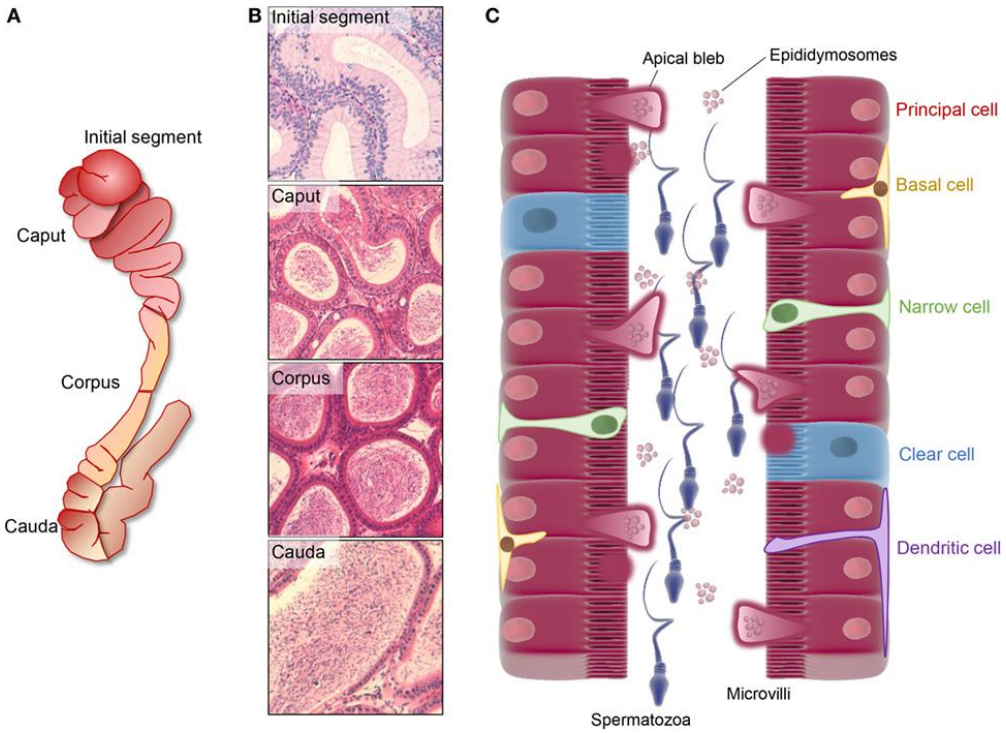


Figura 4: Estrutura e distribuição esquemática dos principais tipos de células no epidídimo. O epidídimo, do camundongo, é geralmente dividido em quatro segmentos: o segmento inicial, a cabeça, o corpo e a cauda do epidídimo. As células principais dominam o epitélio epididimário ao longo de todo o comprimento do epidídimo e são particularmente ativas em termos de biossíntese e secreção de proteínas nos segmentos epididimários proximais. Além das células principais, as células claras são distribuídas esporadicamente por todo o epitélio dos segmentos caput, corpus e cauda na maioria das espécies estudadas. Um conjunto de tipos adicionais de células foi descrito no epidídimo, incluindo células basais, células apicais, células halo, células estreitas (encontradas apenas no segmento inicial e na zona intermediária) e células imunológicas (Zhou et al., 2018).

Uma das principais funções do epidídimo é promover a maturação dos espermatozoides, um processo que envolve modificações morfológicas, bioquímicas e funcionais. Durante a passagem pelo epidídimo, os espermatozoides adquirem motilidade progressiva e capacidade de fertilização (Cornwall, 2009; James et al., 2020). Esse processo de maturação depende de interações complexas dos espermatozoides com substâncias sintetizadas e secretadas pelo epitélio do epidídimo de forma organizada e dependente da região. Expressão gênica diferencial nas células principais dependente da região epididimária, hormônios e íons, contribuem para um perfil proteico luminal distinto dentro de cada região (Robaire e Hinton, 2015). Tudo isso resulta em modificações na membrana plasmática dos espermatozoides e ativam caminhos de

sinalização intracelular essenciais para a fertilização (Cornwall, 2009). A cauda do epidídimo atua como principal local de armazenamento dos espermatozoides. Aqui, os espermatozoides podem permanecer viáveis por várias semanas antes de serem ejaculados. Além disso, o epidídimo íntegro protege os espermatozoides contra substâncias nocivas, como xenobióticos e espécies reativas de oxigênio.

A quiescência metabólica dos espermatozoides estocados na cauda é mantida por um ambiente luminal com baixo pH, contribuindo para a preservação da viabilidade celular (Robaire e Hinton, 2015). A movimentação dos espermatozoides é facilitada por contrações peristálticas suaves da musculatura lisa que envolve o ducto epididimário, que apresenta maior espessura na região da cauda (Junqueira e Carneiro, 2013). Durante a ejaculação, a contração dessa musculatura lisa é fundamental para a liberação dos espermatozoides da cauda para o ducto deferente. A função do epidídimo é altamente dependente da regulação hormonal. A testosterona, produzida pelas células de Leydig nos testículos, é convertida em diidrotestosterona (DHT) nas células epiteliais do epidídimo, onde tem efeitos críticos para a manutenção da função do órgão (Robaire e Hinton, 2015). Através da ligação aos receptores de andrógeno nas células epiteliais, a DHT influencia a transcrição de genes essenciais para a secreção de proteínas, a absorção de íons e a manutenção do pH no fluido luminal. O estradiol também desempenha um papel na modulação da função epididimária, influenciando a expressão gênica e a secreção de proteínas epididimárias (Hess et al., 2011).

2.3 Controle endócrino envolvido na reprodução masculina

O eixo hipotálamo-hipófise-gonadal (HPG) é parte crucial na regulação da função reprodutiva em mamíferos. O eixo é formado por uma complexa interação endócrina entre o hipotálamo, a hipófise e os testículos. Assim, a produção adequada de

espermatozoides e hormônios sexuais, como a testosterona, está garantida. A compreensão detalhada do eixo HPG é fundamental para o entendimento da fisiologia testicular.

O hipotálamo é uma pequena região localizada na base do cérebro, abaixo do tálamo e acima da hipófise. Histologicamente, a região é composta por diferentes tipos de neurônios, incluindo neurônios neuroendócrinos que secretam hormônios diretamente na circulação sanguínea e células gliais (Junqueira e Carneiro, 2013). Embora seja uma região compacta, o hipotálamo é importante no controle hormonal e na resposta ao estresse (Vázquez-Cuevas et al., 2017). Encabeçando o eixo HPG, o hipotálamo desempenha um papel central na sua regulação. Entre os neurônios hipotalâmicos, os mais importantes para o eixo HPG são os neurônios do hormônio liberador de gonadotrofinas (neurônios GnRH), que secretam o hormônio liberador de gonadotrofinas (GnRH) de maneira pulsátil. A secreção pulsátil de GnRH é crucial, pois regula a liberação de hormônios gonadotróficos pela hipófise anterior (Wang et al., 2024).

Os neurônios GnRH não atuam de forma isolada, ao contrário, são modulados por diversos sinais neuroendócrinos e metabólicos, que ajustam a frequência e amplitude dos pulsos de GnRH, influenciando diretamente a função reprodutiva. Por exemplo, os neurônios da kisspeptina codificam o peptídeo kisspeptina, um componente crucial para a regulação da liberação de GnRH. O peptídeo se liga a receptores específicos (KISS1R) localizados na superfície dos neurônios GnRH. Essa ligação ativa vias de sinalização intracelulares que aumentam a liberação de GnRH (Starrett e Moenter, 2023). Neurotransmissores também afetam a atividade dos neurônios GnRH. O glutamato (excitatório) e o ácido gama-aminobutírico (GABA, inibitório) são exemplos de moléculas capazes de influenciar a atividade dos neurônios GnRH, ajustando a frequência de pulsos de GnRH. Ainda, há ação de outro grupo de neurônios sobre a ação de GnRH.

Os neurônios KNDy secretam os neuropeptídios kisspeptina, neurocinina B (estimulatória) e dinorfina (inibitória), que interferem na liberação pulsátil de GnRH (Odetayo et al., 2023; Wang et al., 2024). A liberação pulsátil do GnRH é importante na sua ação na hipófise. Alterações na frequência e amplitude de liberação do GnRH vão impactar a liberação de LH e FSH. Alterações no hipotálamo podem indicar problemas graves, não apenas para o sistema endócrino.

A hipófise, também conhecida como glândula pituitária, é uma pequena glândula endócrina localizada na base do cérebro. Por seu tamanho reduzido, seu manuseio torna-se desafiador. Ela desempenha um papel crucial na regulação de várias funções fisiológicas através da secreção de hormônios que controlam outras glândulas endócrinas e diversos processos corporais (Junqueira e Carneiro, 2013). A hipófise é dividida em duas partes principais, cada uma com funções e características distintas. A hipófise anterior é a porção maior da glândula, composta por células glandulares que produzem e secretam hormônios. A hipófise anterior responde ao GnRH secretando dois hormônios gonadotróficos principais, o hormônio luteinizante (LH) e o hormônio folículo-estimulante (FSH). Pulsos mais frequentes de GnRH tendem a favorecer a secreção de LH, sendo crucial para a regulação da produção de testosterona nos homens. Pulsos menos frequentes de GnRH tendem a estimular a liberação de FSH. A frequência e a amplitude dos pulsos de GnRH podem variar com o estado nutricional, estresse, e outros fatores. Juntos, FSH e LH desempenham papeis complementares na regulação das funções testiculares (Junqueira e Carneiro, 2013).

O FSH desempenha um papel crucial na regulação da função das células de Sertoli. As células de Sertoli fornecem suporte estrutural e nutricional para as células germinativas em desenvolvimento, desempenhando funções essenciais na espermatogênese (Ruthig e Lamb, 2022). As células de Sertoli possuem receptores

específicos para o FSH na sua membrana. A ligação do FSH aos seus receptores ativa a enzima adenilato ciclase, que converte ATP (adenosina trifosfato) em cAMP (Monofosfato cíclico de adenosina). O cAMP atua como um segundo mensageiro, propagando o sinal dentro da célula de Sertoli. O aumento nos níveis de cAMP ativa a proteína quinase A (PKA), que fosforila várias proteínas alvo dentro da célula. A PKA também pode ativar fatores de transcrição que regulam a expressão de genes específicos necessários para as diferentes funções das células de Sertoli (Li et al., 2024). Entre alguns dos papeis do FSH, estão o estímulo das células de Sertoli a produzirem proteínas, como a proteína ligante de andrógeno (ABP), que se liga à testosterona e concentra esse hormônio no túbulo seminífero, essencial para a maturação das células germinativas, a secretar fatores de crescimento e citocinas que promovem a proliferação e diferenciação das células germinativas (Ruthig e Lamb, 2022). Ainda, o FSH promove a formação e manutenção das junções de oclusão entre as células de Sertoli, que formam a barreira hemato-testicular, essencial para proteger as células germinativas do sistema imunológico e criar um microambiente adequado para a espermatogênese (Mruk e Cheng, 2015). O FSH estimula a capacidade das células de Sertoli de fagocitar restos citoplasmáticos e células germinativas degeneradas, mantendo a saúde e a eficiência do epitélio seminífero. Além de regular a apoptose das células germinativas, garantindo que um número adequado de espermatogônias entre no processo de espermatogênese e que células defeituosas sejam eliminadas (Ruthig e Lamb, 2022; Li et al., 2024).

O LH estimula a produção de testosterona pelas células de Leydig nos testículos (Smith e Walker, 2014; Wang et al., 2024). As células de Leydig apresentam retículo endoplasmático desenvolvido, grande número de mitocôndrias e presença de vesículas lipídicas (Zirkin e Papadopoulos, 2018). São células responsáveis pela síntese do principal hormônio masculino, a testosterona (Bhattacharya e Dey, 2023). Este andrógeno

é importante na manutenção e produção adequada, em quantidade e qualidade, dos espermatozoides e também influencia fenótipos masculinos secundários, como pelos e massa corporal. Na membrana plasmática das células de Leydig há receptores específicos para a ligação de LH (LHR). Estes receptores são proteínas transmembrana que pertencem à família dos receptores acoplados à proteína G (GPCRs). A ligação do LH ao LHR é altamente específica e ocorre com alta afinidade (Wang et al., 2017; Zirkin e Papadopoulos, 2018). Com o estímulo do LH, o colesterol armazenado como gotas lipídicas no citoplasma de Leydig, é transportado para a membrana mitocondrial externa e translocado para a membrana mitocondrial interna (Zirkin e Papadopoulos, 2018; Monageng et al., 2023). O processo de translocação entre as membranas mitocondriais é realizado pela proteína StAR (proteína reguladora aguda esteroidogênica), um importante passo que garante o sucesso na produção do andrógeno. A enzima P450scc (enzima de clivagem de cadeia lateral de colesterol), também chamada de CYP11a1, converte o colesterol em pregnenolona. A qual pode seguir dois caminhos para a produção de testosterona. No retículo endoplasmático liso, a pregnenolona é transformada em (i) progesterona pela ação da enzima 3β-hidroxiesteróide desidrogenase (HSD) ou em (ii) 17OH-Pregnenolona pela enzima P450c17. A 17OH-Pregnenolona é convertida em desidroepiandrosterona pela enzima 17,20 liase, que é convertida em androstenediona pela 3β HSD. Já a progesterona é convertida pela enzima P450c17 em 17OH-Progesterona e pela enzima 17,20 liase é convertida em androstenediona. A androstenediona, nos dois caminhos, é convertida em testosterona pela enzima 17β HSD (Monageng et al., 2023). A testosterona pode ser convertida em estrógeno ou metabolizada para sua forma ativa de DHT, atuando nas células do epitélio seminífero, por intermédio de Sertoli, no processo de espermatogênese (Zirkin e Papadopoulos, 2018; Monageng et al., 2023). O estradiol é um dos principais estrógenos e, apesar de estar

presente em níveis muito baixos nos homens, é importante para a modulação da libido. Ainda, tem papel crucial no feedback negativo no eixo HPG, regulando a secreção das gonadotrofinas e, consequentemente, a produção de testosterona.

É importante ressaltar que o eixo HPG é regulado por mecanismos de feedback, podendo receber influências negativas, diminuindo a síntese e/ou liberação do hormônio, ou positivas. No entanto, no contexto masculino, o feedback positivo não desempenha um papel significativo na regulação do eixo HPG. A testosterona, produzida pelas células de Leydig, exerce feedback negativo sobre o hipotálamo e a hipófise, inibindo a secreção de GnRH e, consequentemente, de LH e FSH (Corradi et al., 2016). Além disso, a inibina, uma proteína produzida pelas células de Sertoli, exerce feedback negativo específico sobre a secreção de FSH pela hipófise (Corradi et al., 2016). O estradiol, produzido a partir de andrógenos, exerce efeitos negativos no hipotálamo e hipófise (Guercio et al., 2020).

2.4 Metodologias de estudos em toxicologia

Além de considerarmos parâmetros morfológicos básicos, como aspectos histológicos dos órgãos importantes para o sistema reprodutor masculino, em especial testículo e epidídimo, diferentes metodologias podem ser empregadas para o estudo da toxicidade de xenobióticos em parâmetros reprodutivos masculinos. Modificações na morfologia do parênquima desses órgãos podem estar associadas a disfunções endócrinas, além de parâmetros bioquímicos e moleculares defeituosos. Muitas vezes, agentes tóxicos interagem com organelas altamente ativas, como as mitocôndrias, ativando vias relacionadas ao estresse oxidativo e inflamação. Associados ao desbalanço hormonal, essas alterações moleculares podem resultar em defeitos na formação do espermatozoide, com motilidade e morfologia prejudicadas. Para avaliar a extensão desses danos, além de

análises espermáticas podemos considerar análises do sistema antioxidante e marcadores de estresse oxidativo, bem como análises que avaliem defeitos genéticos e, em última instância, parâmetros de fertilidade.

2.4.1 Atividade de antioxidantes enzimáticos e não-enzimáticos

O estresse oxidativo é um fenômeno patológico resultante do desequilíbrio entre a produção de espécies reativas de oxigênio (ERO)/radicais livres e a capacidade dos sistemas antioxidantes endógenos de neutralizá-las (Pizzino et al., 2017). Essas moléculas reativas levam à oxidação de biomoléculas, incluindo lipídios, proteínas e DNA, resultando em disfunção celular e, em último caso, morte celular. A compreensão dos mecanismos subjacentes ao estresse oxidativo e das respostas celulares associadas é parte crucial para a investigação da ação de poluentes ambientais e suas interações celulares. Considerando a ação desse poluente, o processo de detoxificação, que elimina toxinas e substâncias nocivas, pode ajudar a reduzir a carga dessas substâncias toxicas no organismo (Sies, 2017). Todavia, o processo gera EROs como subproduto, que devem ser neutralizadas pelas enzimas antioxidantes. Uma falha e/ou a sobrecarga desse processo resulta em estresse oxidativo.

As células possuem um aparato de enzimas antioxidantes que trabalham em conjunto para neutralizar EROs e minimizar os danos oxidativos. Entre as principais enzimas antioxidantes estão a superóxido dismutase (SOD), catalase (CAT) e glutationa s-transferase (GST). A SOD é a primeira linha de defesa contra as EROs. A enzima catalisa a dismutação do radical superóxidos (O_2^-) em oxigênio molecular e peróxido de hidrogênio (H_2O_2) (Wang et al., 2018). O processo de formação do radical O_2^- ocorre naturalmente durante o processo de respiração celular nas mitocôndrias. Em condições fisiológicas normais, a maior parte do oxigênio utilizado na produção de ATP é reduzido

a água e uma pequena quantidade em O_2^-. Os poluentes ambientais podem interferir diretamente na homeostase celular/mitocondrial, danificando a estrutura da cadeia transportadora e gerando maior quantidade de O_2^-. Com o aparato enzimático ainda em funcionamento, a SOD neutraliza o radical O_2^- formado (Wang et al., 2018). Todavia, os poluentes também podem interagir com a estrutura enzimática ou sobrecarrega-la, dificultado esse processo de neutralização. A SOD existe em diferentes isoformas, cada qual localizada em compartimentos celulares distintos e com sítios catalíticos dependentes de diferentes elementos. Cu/Zn-SOD está localizada no citoplasma e é dependente de cobre e zinco. Já a Mn-SOD está presente na matriz mitocondrial e depende do elemento manganês (Blackney et al., 2014). A enzima alterna entre os estados oxidados e reduzidos do metal no seu centro ativo para facilitar a dismutação do O_2^-. O processo ocorre, então, de forma cíclica. Essa capacidade de alternas rapidamente entre estados oxidados e reduzidos é essencial para a atividade enzimática da SOD. Alterações na concentração de seus cofatores (Cu, Zn e Mn) podem reduzir a eficiência da enzima.

A CAT é uma enzima tetramérica com grupos heme (Fe^{3+}), local ativo da enzima, que pertence a segunda linha de defesa do sistema antioxidante. A função primária da catalase é catalisar a decomposição do H_2O_2 em água (H_2O) e O_2, protegendo a célula dos efeitos tóxicos do H_2O_2. A CAT é uma das enzimas mais eficientes, decompondo milhões de moléculas de H_2O_2 por segundo, e é especialmente importante em tecidos com alta atividade metabólica, onde a produção de H_2O_2 é elevada (Nandi et al., 2019).

A GST é uma enzima multifuncional envolvida na detoxificação de xenobióticos e produtos da peroxidação lipídica através da conjugação com glutationa (GSH). As GSTs facilitam a ligação de GSH a substratos eletrofílicos, tornando-os mais solúveis e facilmente excretáveis. Além disso, as GSTs desempenham um papel importante na neutralização de hidroperóxidos lipídicos, contribuindo para a manutenção da integridade

das membranas celulares e na proteção contra danos oxidativos. O processo de conjugação com a GSH, catalisado pela GST, torna os substratos mais solúveis e mais facilmente excretados (Allocati et al., 2018). Um dos exemplos de compostos neutralizados pela GST, está o H_2O_2, cuja conjugação com a GSH resulta em H_2O.

Uma alternativa para avaliar a capacidade antioxidante total da amostra é a análise de FRAP (*Ferric Reducing Ability of Plasma*). A técnica baseia-se na capacidade das substâncias presentes na amostra de reduzir o íon férrico (Fe^{3+}) a íon ferroso (Fe^{2+}) em condições ácidas. Fe^{3+} pode oxidar alguns antioxidantes em meio ácido. O Fe^{2+} resultante forma um complexo colorido com o tripiridiltriazina (TPTZ), que é quantificado por espectrofotometria (Demirci-Çekiç et al., 2022).

2.4.2 Marcadores de estresse oxidativo

Para avaliar o impacto do estresse oxidativo, diversos biomarcadores são utilizados, cada um refletindo diferentes aspectos do dano oxidativo e da resposta antioxidante. O malondialdeído (MDA) é um dos principais produtos finais da peroxidação lipídica, um processo no qual os lipídios das membranas celulares são oxidados, resultando na formação de aldeídos reativos. As EROs são exemplos de moléculas que interagem com fosfolipídeos, subtraindo átomos de hidrogênio, gerando um radical lipídico (L^-). O L^- pode reagir com o O_2 formando um radical peroxilipídico (LOO^-) que interage com outro ácido graxo gerando hidroperóxido lipídico (LOOHs), extremamente instável e que se decompõem em várias espécies reativas, incluindo o MDA. O MDA reage com grupos amina em proteínas e ácidos nucleicos, formando compostos estáveis que são indicativos de dano oxidativo. A quantificação de MDA, geralmente por métodos como a reação com ácido tiobarbitúrico (TBARS), é amplamente utilizada como um indicador de danos oxidativos em lipídios (Cordiano et al., 2023).

A oxidação de proteínas resulta na formação irreversível de grupos carbonila (aldeídos e cetonas) nos resíduos de aminoácidos, que podem ser detectados e quantificados como indicadores de danos oxidativos em proteínas, conhecido como proteínas carboniladas (PC). Espécies reativas, podem oxidar diretamente os resíduos de aminoácidos. O próprio MDA pode reagir com as proteínas e introduzir os grupos carbonila. A adição de grupos carbonilas nas proteínas pode causar perda de função, ganho de funções tóxicas e, consequentemente, marcação para degradação. A quantificação de PC é realizada por métodos como a derivatização com dinitrofenilhidrazina (DNPH), que forma hidrazonas estáveis e permite a medição espectrofotométrica (Fedorova et al., 2014).

O óxido nítrico (NO) é uma molécula sinalizadora de curta vida produzida pelo óxido nítrico sintase (NOS). O NO desempenhe funções fisiológicas importantes, como a vasodilatação. Níveis elevados de NO podem reagir com o ânion superóxido (O_2^-) formando peroxinitrito ($ONOO^-$), uma espécie altamente reativa que causa danos a lipídios, proteínas e DNA. Por exemplo, o aumento de $ONOO^-$ pode resultar em problemas nas membranas. A detecção e quantificação de NO e seus derivados, como nitritos e nitratos, são utilizadas como indicadores de estresse nitrosativo e oxidativo (Radi, 2018; Guerby et al., 2021).

2.4.3 Genotoxicidade

As análises de genotoxicidade são essenciais para avaliar os efeitos de agentes tóxicos no material genético. A combinação de diferentes métodos possibilita identificar potenciais riscos, desses agentes, para a saúde humana e ambiental. Dois métodos amplamente utilizados para essas análises são o teste de cometa e a citometria de fluxo,

que fornecem informações detalhadas, e complementares, sobre danos ao DNA e a resposta celular a diferentes tóxicos.

O teste de cometa, ou ensaio cometa alcalino, é uma técnica relativamente simples para detectar danos/quebras no DNA em células individuais. Este método envolve a suspensão das células em agarose e sua deposição em lâminas. Após a lise celular, que remove as membranas e expõe o DNA, as lâminas são submetidas à eletroforese em um tampão alcalino ou neutro. O princípio do ensaio depende da organização espacial do DNA no núcleo, ou seja, devemos considerar a ligação da molécula linear as proteínas e a formação de nucleossomos. Essa organização significa que, quando as proteínas são removidas durante a etapa de lise do ensaio, o DNA permanece em um estado superenrolado compacto. No entanto, em condições alcalinas, as quebras de fita simples e dupla relaxa esse superenrolamento, permitindo que fragmentos de DNA migrem em direção ao ânodo, formando uma estrutura semelhante a um cometa, onde a "cabeça" contém o DNA intacto e a "cauda" é composta pelos fragmentos danificados. Amostras de espermatozoides devem ter o processo de lise modificado para permitir o relaxamento do DNA (Collins et al., 2023). A coloração com um corante de DNA, como brometo de etídio, e a análise ao microscópio de fluorescência permitem quantificar a extensão do dano. O teste de cometa é amplamente utilizado devido à sua capacidade de detectar danos genéticos induzidos por uma variedade de agentes, como radiação e produtos químicos, além de sua alta sensibilidade e aplicabilidade a diferentes tipos celulares (Collins et al., 2023).

A citometria de fluxo é outra técnica empregada em análises de genotoxicidade. Através da técnica é possível a avaliação de múltiplos parâmetros celulares simultaneamente. O processo pode envolver a marcação das células com fluorocromos específicos que se ligam ao DNA ou a outras estruturas celulares de interesse. À medida

que as células marcadas passam por um feixe de laser na citômetro de fluxo, a fluorescência emitida é detectada, proporcionando informações quantitativas sobre o conteúdo de DNA e outros marcadores de interesse. A citometria de fluxo é especialmente útil para a análise de alterações no ciclo celular e outras respostas celulares ao dano genotóxico. Através da análise de subpopulações celulares, é possível identificar células em diferentes fases do ciclo celular, bem como aquelas que estão passando por apoptose ou necrose, fornecendo uma visão abrangente dos efeitos de agentes genotóxicos (Gupta et al., 2022).

2.4.4 Análises de fertilidade

Os testes de fertilidade são importantes parâmetros para avaliar o sucesso de terapias com foco em aumentar a qualidade espermática, bem como na avaliação de toxicidade reprodutiva. Trabalhando com o macho como sistema de teste, alguns índices podem nos ajudar a entender como o agente tóxico impacta a fertilidade desses animais. Os índices aqui apresentados foram apresentados por Hood e Hood, 2005.

Através do *índice de acasalamento masculino*, conseguimos avaliar, de forma geral, o sucesso de acasalamento num sistema em que o macho é testado. Pode ser influenciado por vários fatores, como comprometimento físico dos machos, intoxicação aguda, alterações na libido, desequilíbrio hormonal, entre outros. Já o *índice de fertilidade masculino* avalia a capacidade do macho de produzir espermatozoides capazes de emprenhar fêmeas. O acasalamento não implica em fecundação, sendo um parâmetro adicional ao primeiro. Aqui, fatores relacionados aos espermatozoides podem influenciar o resultado, como baixa produção e qualidade espermática, problemas na morfologia espermática, na motilidade espermática, no processo de maturação, entre outros.

Os *índices de implantação, perda pré-implantação e perda pós implantação* auxiliam no entendimento da qualidade dos espermatozoides. Ao avaliar a eficácia da implantação e o processo de perda durante o processo, considerando o macho como sistema de teste, determinamos a qualidade do espermatozoide, desde suas organelas até a integridade do seu conteúdo haploide. Além destes índices, a avaliação dos fetos também é importante na avaliação da toxicologia de compostos perante a fertilidade dos machos. Por exemplo, o peso dos fetos e a distância ânus-genital são importantes indicativos do desenvolvimento dos mesmo e, desconsiderando problemas maternos, podem estar associados a danos ao DNA espermático (Schwartz et al., 2019).

3. Referências

Abarikwu, S.O., Costa, G.M.J., de Lima, E. M.L.N., Lacerda, S.M.S.N., de França, L.R., 2021. Atrazine impairs testicular function in BalB/c mice by affecting Leydig cells. Toxicology. 15;455:152761. doi: 10.1016/j.tox.2021.152761.

Abarikwu, S.O., Ezim, O.E., Ikeji C.N., Farombi, E.O., 2023. Atrazine: cytotoxicity, oxidative stress, apoptosis, testicular effects and chemopreventive Interventions. Front Toxicol. 9;5:1246708. doi: 10.3389/ftox.2023.1246708. PMID: 37876981; PMCID: PMC10590919.

Acevedo-Rodriguez, A., Kauffman, A.S., Cherrington, B.D., Borges, C.S., Roepke, T.A., Laconi, M., 2018. Emerging insights into hypothalamic-pituitary-gonadal axis regulation and interaction with stress signalling. J Neuroendocrinol 30, 0–3. https://doi.org/10.1111/jne.12590

Allocati, N., Masulli, M., Di Ilio, C., Federici, L., 2018. Glutathione transferases: Substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. Oncogenesis 7. https://doi.org/10.1038/s41389-017-0025-3

Araújo, R.D.S., Bernardes, R.C., Martins, G.F., 2021. A mixture containing the herbicides Mesotrione and Atrazine imposes toxicological risks on workers of Partamona helleri. Sci Total Environ. doi: 10.1016/j.scitotenv.2020.142980. Epub 2020 Oct 14.

Araújo, R. dos S., Viana, T.A., Botina, L.L., Bastos, D.S.S., da Silva Alves, B.C., Machado-Neves, M., Bernardes, R.C., Martins, G.F., 2023. Investigating the effects of mesotrione/atrazine-based herbicide on honey bee foragers. Science of the Total Environment 898, 165526. https://doi.org/10.1016/j.scitotenv.2023.165526

Barchanska, H., Rusek, M., Szatkowska, A., 2011. New procedures for simultaneous determination of mesotrione and atrazine in water and soil. Comparison of the degradation processes of mesotrione and atrazine. Environmental Monitoring and Assessment, v. 184, n. 1, p. 321–334.

Bhattacharya, I., Dey, S., 2023. Emerging concepts on Leydig cell development in fetal and adult testis. Front Endocrinol (Lausanne) 13, 1–9. https://doi.org/10.3389/fendo.2022.1086276

Blackney, M.J., Cox, R., Shepherd, D., Parker, J.D., 2014. Cloning and expression analysis of Drosophila extracellular Cu Zn superoxide dismutase. Biosci Rep 34, 851–863. https://doi.org/10.1042/BSR20140133

Boopathy, R., 2017. Anaerobic degradation of atrazine. International Biodeterioration & Biodegradation, https://doi.org/10.1016/j.ibiod.2016.10.002

Breton, S., Nair, A. V., Battistone, M.A., 2019. Epithelial dynamics in the epididymis: role in the maturation, protection, and storage of spermatozoa. Andrology 7, 631–643. https://doi.org/10.1111/andr.12632

Cameron, D., 2007. Structure and function of the male reproductive system. xPharm: The Comprehensive Pharmacology. Reference 1–9. https://doi.org/10.1016/B978-008055232-3.60265-X

Castro, M.M., Gonçalves, W.G., Teixeira, S.A.M.V., Fialho, M.D.C.Q., Santos, F.C., Oliveira, J.M., Serrão, J.E., Machado-Neves, M., 2017. Ultrastructure and morphometric features of epididymal epithelium in Desmodus rotundus. Micron. Nov;102:35-43. doi: 10.1016/j.micron.2017.08.006.

Chang, J., Fang, W., Chen, L., Zhang, P., Zhang, G., Zhang, H., Liang, J., Wang, Q., Ma, W., 2022. Toxicological effects, environmental behaviors and remediation technologies of herbicide atrazine in soil and sediment: A comprehensive review. Chemosphere 307, 136006. https://doi.org/10.1016/j.chemosphere.2022.136006

Collins, A., Møller, P., Gajski, G., Vodenková, S., Abdulwahed, A., Anderson, D., Bankoglu, E.E., Bonassi, S., Boutet-Robinet, E., Brunborg, G., Chao, C., Cooke, M.S., Costa, C., Costa, S., Dhawan, A., de Lapuente, J., Bo', C. Del, Dubus, J., Dusinska, M., Duthie, S.J., Yamani, N. El, Engelward, B., Gaivão, I., Giovannelli, L., Godschalk, R., Guilherme, S., Gutzkow, K.B., Habas, K., Hernández, A., Herrero, O., Isidori, M., Jha, A.N., Knasmüller, S., Kooter, I.M., Koppen, G., Kruszewski, M., Ladeira, C., Laffon, B., Larramendy, M., Hégarat, L. Le, Lewies, A., Lewinska, A., Liwszyc, G.E., de Cerain, A.L., Manjanatha, M., Marcos, R., Milić, M., de Andrade, V.M., Moretti, M., Muruzabal, D., Novak, M., Oliveira, R., Olsen, A.K., Owiti, N., Pacheco, M., Pandey, A.K., Pfuhler, S., Pourrut, B., Reisinger, K., Rojas, E., Rundén-Pran, E., Sanz-Serrano, J., Shaposhnikov, S., Sipinen, V., Smeets, K., Stopper, H., Teixeira, J.P., Valdiglesias, V., Valverde, M., van Acker, F., van Schooten, F.J., Vasquez, M., Wentzel, J.F., Wnuk, M., Wouters, A., Žegura, B., Zikmund, T., Langie, S.A.S.,

Azqueta, A., 2023. Measuring DNA modifications with the comet assay: a compendium of protocols. Nat Protoc 18, 929–989. https://doi.org/10.1038/s41596-022-00754-y

Cordiano, R., Di Gioacchino, M., Mangifesta, R., Panzera, C., Gangemi, S., Minciullo, P.L., 2023. Malondialdehyde as a Potential Oxidative Stress Marker for Allergy-Oriented Diseases: An Update. Molecules 28, 1–22. https://doi.org/10.3390/molecules28165979

Cornwall, G.A., 2009. New insights into epididymal biology and function. Hum Reprod Update 15, 213–227. https://doi.org/10.1093/humupd/dmn055

Corradi, P.F., Corradi, R.B., Greene, L.W., 2016. Physiology of the Hypothalamic Pituitary Gonadal Axis in the Male. Urologic Clinics of North America 43, 151–162. https://doi.org/10.1016/j.ucl.2016.01.001

Cyr, D.G., Dufresne, J., Gregory, M., 2018. Cellular junctions in the epididymis, a critical parameter for understanding male reproductive toxicology. Reproductive Toxicology 81, 207–219. https://doi.org/10.1016/j.reprotox.2018.08.013

Das, S., Sakr, H., Al-Huseini, I., Jetti, R., Al-Qasmi, S., Sugavasi, R., Sirasanagandla, S.R., 2023. Atrazine Toxicity: The Possible Role of Natural Products for Effective Treatment. Plants 12. https://doi.org/10.3390/plants12122278

Demirci-Çekiç, S., Özkan, G., Avan, A.N., Uzunboy, S., Çapanoğlu, E., Apak, R., 2022. Biomarkers of Oxidative Stress and Antioxidant Defense. J Pharm Biomed Anal 209, 114477. https://doi.org/10.1016/j.jpba.2021.114477

Figueiredo, A.F.A., Hess, R.A., Batlouni, S.R., Wnuk, N.T., Tavares, A.O., Abarikwu, S.O., Costa, G.M.J., França, L.R., 2021. Insights into differentiation and function of the transition region between the seminiferous tubule and rete testis. Differentiation 120, 36–47. https://doi.org/10.1016/j.diff.2021.06.002

Fedorova, M., Bollinen,i R.C., Hoffmann, R., 2014. Protein carbonylation as a major hallmark of oxidative damage: update of analytical strategies. Mass Spectrom Rev. Mar-Apr;33(2):79-97. doi: 10.1002/mas.21381.

França, L.R., Hess, R.A., Dufour, J.M., Hofmann, M.C., and Griswold, M.D., 2016. The sertoli cell: one hundred fifty years of beauty and plasticity. Andrology 4 (2), 189–212. doi:10.1111/andr.12165

Gao, Fengyi, Li, G., Liu, C., Gao, H., Wang, H., Liu, W., Chen, M., Shang, Y., Wang, L., Shi, J., Xia, W., Jiao, J., Gao, Fei, Li, J., Chen, L., Li, W., 2018. Autophagy regulates testosterone synthesis by facilitating cholesterol uptake in Leydig cells. Journal of Cell Biology 217, 2103–2119. https://doi.org/10.1083/jcb.201710078

Gerber, J., Heinrich, J., Brehm, R., 2016. Blood–testis barrier and Sertoli cell function: lessons from SCCx43KO mice. Reproduction 151, R15–R27. DOI: 10.1530/REP-15-0366

Gianessi, L.P., 2013. The increasing importance of herbicides in worldwide crop production. Pest Manag Sci 69, 1099–1105. https://doi.org/10.1002/ps.3598

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr Rev 36, 1–150. https://doi.org/10.1210/er.2015-1010

Guerby, P., Tasta, O., Swiader, A., Pont, F., Bujold, E., Parant, O., Vayssiere, C., Salvayre, R., Negre-Salvayre, A., 2021. Role of oxidative stress in the dysfunction of the placental endothelial nitric oxide synthase in preeclampsia. Redox Biol 40. https://doi.org/10.1016/j.redox.2021.101861

Guercio, G., Saraco, N., Costanzo, M., Marino, R., Ramirez, P., Berensztein, E., Rivarola, M.A., Belgorosky, A., 2020. Estrogens in Human Male Gonadotropin Secretion

and Testicular Physiology From Infancy to Late Puberty. Front Endocrinol (Lausanne) 11, 1–14. https://doi.org/10.3389/fendo.2020.00072

Gupta, N., Mittal, A., Dadu, T., Choudhary, D., Handoo, A., 2022. Flow Cytometric DNA Ploidy Analysis in Haemato-Lymphoid Neoplasms: An Analysis of 132 Cases. Int J Hematol Oncol Stem Cell Res 16, 34–46. https://doi.org/10.18502/ijhoscr.v16i1.8440

Hailu, G., 2023. Reflections on technological progress in the agri-food industry: Past, present, and future. Canadian Journal of Agricultural Economics 71, 119–141. https://doi.org/10.1111/cjag.12325

He, H., Liu, Y., You, S., Liu, J., Xiao, H., Tu, Z., 2019. A Review on Recent Treatment Technology for Herbicide Atrazine in Contaminated Environment. Int J Environ Res Public Health 16;16(24):5129. doi: 10.3390/ijerph16245129. PMID: 31888127; PMCID: PMC6950201.

Hess, R.A., Fernandes, S.A.F., Gomes, G.R.O., Oliveira, C.A., Lazari, M.F.M., Porto, C.S., 2011. Estrogen and its receptors in efferent ductules and epididymis. J Androl 32, 600–613. https://doi.org/10.2164/jandrol.110.012872

Hood, R.D. and Hood, R.D., 2005. Developmental and reproductive toxicology: a practical approach. CRC press.

Ikeji, C.N., Adedara, I.A., Farombi, E.O., 2023. Dietary myricetin assuages atrazine-mediated hypothalamic-pituitary–testicular axis dysfunction in rats. Environmental Science and Pollution Research 30, 15655–15670. https://doi.org/10.1007/s11356-022-23033-5

James, E.R., Carrell, D.T., Aston, K.I., Jenkins, T.G., Yeste, M., Salas-Huetos, A., 2020. The Role of the Epididymis and the Contribution of Epididymosomes to

Mammalian Reproduction. Int J Mol Sci. Jul 29;21(15):5377. doi: 10.3390/ijms21155377.

Junqueira, L.C., Carneiro, J., 2013. Aparelho reprodutor Masculino. In: Junqueira, L. C.; Carneiro, J. Histologia Básica. Texto e Atlas. Rio de Janeiro: Editora Guanabara, 13 ed,p:1398-1441.

Kempinas, W.G., Klinefelter, G.R., 2014. Interpreting histopathology in the epididymis. Spermatogenesis, v. 4, n. 2, p. e979114.

Kumar, V., Upadhyay, N., Singh, S., Singh, J., Kaur, P., 2013. Thin-Layer Chromatography: Comparative Estimation of Soil's Atrazine. Current World Environment Journal 8, 469–472. https://doi.org/10.12944/cwe.8.3.17

Li, L., Lin, W., Wang, Z., Huang, R., Xia, H., Li, Z., Deng, J., Ye, T., Huang, Y., Yang, Y., 2024. Hormone Regulation in Testicular Development and Function. Int J Mol Sci 25. https://doi.org/10.3390/ijms25115805

Lin, J.B., Troyer, D., 2014. Testicular Anatomy and Physiology, Pathobiology of Human Disease: A Dynamic Encyclopedia of Disease Mechanisms. Published by Elsevier Inc. https://doi.org/10.1016/B978-0-12-386456-7.05102-9

Luaces, J.P., Toro-Urrego, N., Otero-Losada, M., Capani, F., 2023. What do we know about blood-testis barrier? current understanding of its structure and physiology. Front Cell Dev Biol. Jun 15;11:1114769. doi: 10.3389/fcell.2023.1114769.

Luccio-Camelo, D.C., Prins, G.S., 2011. Disruption of androgen receptor signaling in males by environmental chemicals. Journal of Steroid Biochemistry and Molecular Biology 127, 74–82. https://doi.org/10.1016/j.jsbmb.2011.04.004

Ma, Y., Ma, Q.W., Sun, Y., Chen, X.F., 2023. The emerging role of extracellular vesicles in the testis. Hum Reprod. Mar 1;38(3):334-351. doi: 10.1093/humrep/dead015.

Mita, P., Hinton, B.T., Dufour, J.M., 2011. The blood-testis and blood-epididymis barriers are more than just their tight junctions. Biol Reprod 84, 851–858. https://doi.org/10.1095/biolreprod.110.087452

Monageng, E., Offor, U., Takalani, N.B., Mohlala, K., Opuwari, C.S., 2023. A Review on the Impact of Oxidative Stress and Medicinal Plants on Leydig Cells. Antioxidants 12, 1–29. https://doi.org/10.3390/antiox12081559

Mruk, D.D., Cheng, C.Y., 2015. The mammalian blood-testis barrier: Its biology and regulation. Endocr Rev 36, 564–591. https://doi.org/10.1210/er.2014-1101

Nandi, A., Yan, L.J., Jana, C.K., Das, N., 2019. Role of Catalase in Oxidative Stress- And Age-Associated Degenerative Diseases. Oxid Med Cell Longev 2019. https://doi.org/10.1155/2019/9613090

Odetayo, A.F., Akhigbe, R.E., Bassey, G.E., Hamed, M.A., Olayaki, L.A., 2023. Impact of stress on male fertility: role of gonadotropin inhibitory hormone. Front Endocrinol (Lausanne) 14, 1–11. https://doi.org/10.3389/fendo.2023.1329564

Parven, A., Meftaul, I.M., Venkateswarlu, K., Megharaj, M., 2024. Herbicides in modern sustainable agriculture: environmental fate, ecological implications, and human health concerns. International Journal of Environmental Science and Technology. https://doi.org/10.1007/s13762-024-05818-y

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., Bitto, A., 2017. Oxidative Stress: Harms and Benefits for Human Health. Oxid Med Cell Longev 2017. https://doi.org/10.1155/2017/8416763

Qu, R.Y., He, B., Yang, J.F., Lin, H.Y., Yang, W.C., Wu, Q.Y., Li, Q.X., Yang, G.F., 2021. Where are the new herbicides? Pest Manag Sci 77, 2620–2625. https://doi.org/10.1002/ps.6285

Radi, R., 2018. Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. Proc Natl Acad Sci U S A 115, 5839–5848. https://doi.org/10.1073/pnas.1804932115

Robaire, B., Hinton, B.T., 2015. The Epididymis, Fourth Edi. ed, Knobil and Neill's Physiology of Reproduction: Two-Volume Set. Elsevier. https://doi.org/10.1016/B978-0-12-397175-3.00017-X

Ruthig, V.A., Lamb, D.J., 2022. Updates in Sertoli Cell-Mediated Signaling During Spermatogenesis and Advances in Restoring Sertoli Cell Function. Front Endocrinol (Lausanne) 13, 1–8. https://doi.org/10.3389/fendo.2022.897196

Schwartz, C.L., Christiansen, S., Vinggaard, A.M., Axelstad, M., Hass, U., Svingen, T., 2019. Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. Arch Toxicol 93, 253–272. https://doi.org/10.1007/s00204-018-2350-5

Setchell, B.P., Breed, W.G., 2005. Anatomy, Vasculature, and Innervation of the Male Reproductive Tract. Knobil and Neill's Physiology of Reproduction 771–825. https://doi.org/10.1016/B978-012515400-0/50022-1

Sies, H., 2017. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. Redox Biol 11, 613–619. https://doi.org/10.1016/j.redox.2016.12.035

Smith, L.B., Walker, W.H., 2014. The regulation of spermatogenesis by androgens. Semin Cell Dev Biol 30, 2–13. https://doi.org/10.1016/j.semcdb.2014.02.012

Solomon, R.D.J., Kumar, A., Satheeja Santhi, V., 2013. Atrazine biodegradation efficiency, metabolite detection, and trzD gene expression by enrichment bacterial cultures from agricultural soil. J Zhejiang Univ Sci B 14, 1162–1172. https://doi.org/10.1631/jzus.B1300001

Starrett, J.R., Moenter, S.M., 2023. Hypothalamic kisspeptin neurons as potential mediators of estradiol negative and positive feedback. Peptides (N.Y.) 163, 170963. https://doi.org/10.1016/j.peptides.2023.170963

Stradtman, S.C., Freeman, J.L., 2021. Mechanisms of neurotoxicity associated with exposure to the herbicide atrazine. Toxics 9. https://doi.org/10.3390/toxics9090207

Tudi, M., Daniel Ruan, H., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., Phung, D.T., 2021. Agriculture Development, Pesticide Application and Its Impact on the Environment. Int J Environ Res Public Health. Jan 27;18(3):1112. doi: 10.3390/ijerph18031112

Vázquez-Cuevas, F., Aguilar-Roblero, R., Arellanes-Licea, E., Macotela, Y., Vázquez-Martínez, O., Villanueva, I., & Díaz-Muñoz, M., 2017. Food Intake and Its Control by Signaling Molecules. Hormones, Brain and Behavior, 175–209. doi:10.1016/b978-0-12-803592-4.00006-7

Wang, C., Harwood, J.D., Zhang, Q., 2018. Oxidative stress and DNA damage in common carp (Cyprinus carpio) exposed to the herbicide mesotrione. Chemosphere 193, 1080–1086. https://doi.org/10.1016/j.chemosphere.2017.11.148

Wang, C., Meriggiola, C., Behre, H.M., Page, S.T., 2024. Hormonal male contraception. Andrology 1–7. https://doi.org/10.1111/andr.13699

Wang, Y., Chen, F., Ye, L., Zirkin, B., Chen, H., 2017. Steroidogenesis in leydig cells: Effects of aging and environmental factors. Reproduction 154, R111–R122. https://doi.org/10.1530/REP-17-0064

Wang, Y., Branicky, R., Noë, A., Hekimi, S., 2018. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J Cell Biol. Jun 4;217(6):1915-1928. doi: 10.1083/jcb.201708007.

Wirbisky, S.E., Freeman, J.L., 2015. Atrazine Exposure and Reproductive Dysfunction through the Hypothalamus-Pituitary-Gonadal (HPG) Axis. Toxics. Dec;3(4):414-450. doi: 10.3390/toxics3040414.

Zhao, H., Yu, C., He, C., Mei, C., Liao, A., Huang, D., 2020. The Immune Characteristics of the Epididymis and the Immune Pathway of the Epididymitis Caused by Different Pathogens. Front Immunol 11, 1–12. https://doi.org/10.3389/fimmu.2020.02115

Zhao, S., Zhu, W., Xue, S., Han, D., 2014. Testicular defense systems: Immune privilege and innate immunity. Cell Mol Immunol 11, 428–437. https://doi.org/10.1038/cmi.2014.38

Zhou, R., Wu, J., Liu, B., Jiang, Y., Chen, W., Li, J., He, Q., He, Z., 2019. The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. Cellular and Molecular Life Sciences 76, 2681–2695. https://doi.org/10.1007/s00018-019-03101-9

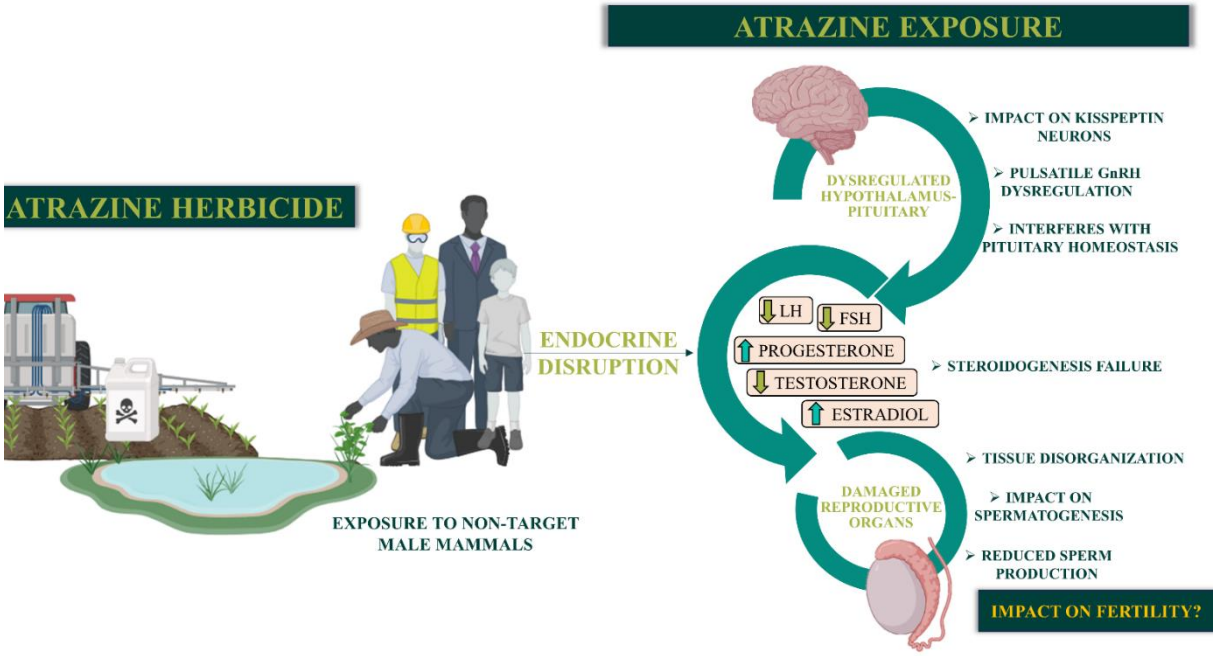
Zhou, W., De Iuliis, G.N., Dun, M.D., Nixon, B., 2018. Characteristics of the Epididymal Luminal Environment Responsible for Sperm Maturation and Storage. Front Endocrinol (Lausanne). Feb 28;9:59. doi: 10.3389/fendo.2018.00059. PMID: 29541061; PMCID: PMC5835514.

Zirkin, B.R., Papadopoulos, V., 2018. Leydig cells: Formation, function, and regulation. Biol Reprod 99, 101–111. https://doi.org/10.1093/biolre/ioy059

CAPÍTULO 2

Exploring the endocrine-disrupting potential of atrazine for male reproduction: A systematic review and meta-analysis

Graphical abstract:



Abstract

Atrazine is an herbicide widely used on plantations worldwide. Experimental studies suggest that the herbicide impairs male reproductive function in mammals. This systematic review and meta-analysis aimed to evaluate the impact of atrazine exposure on the levels of hormones from the hypothalamic-pituitary-testicular axis using murine as the animal model. After an extensive literature search, we selected 25 articles for the systematic review. Bias analysis and methodological quality assessments were examined using the SYRCLE Risk of Bias tool. Moreover, 20 out of the 25 studies were eligible for performing a meta-analysis to evaluate the intensity of atrazine damage on the levels of intratesticular testosterone and serum follicle-stimulating hormone (FSH), luteinizing

hormone (LH), testosterone, estradiol, and progesterone. The meta-analysis revealed that atrazine exposure elicited a strong negative response in serum FSH, LH, and testosterone levels, and caused an increase in serum estradiol and progesterone levels. Atrazine also exerted a negative influence on the levels of intratesticular testosterone. Exposure to atrazine in high concentrations (≥ 100 mg kg^{-1}) was the main cause of endocrine disruption, regardless of the exposure time. None of studies have tested doses with relevant importance for human health risk. Oxidative stress and inflammation are involved in atrazine toxicity, impairing the gonadotropin release by the pituitary, disturbing steroidogenesis, and affecting the male hormone regulatory system. We may conclude that hormone disturbances lead to a failure in testicular steroidogenesis, with possible implications for male reproductive function. The registration number on the Prospero platform is CRD42024495626.

Keywords: Herbicide; hypothalamic-pituitary-testicular axis; GnRH; testosterone, estradiol.

1. Introduction

Long-term pesticide exposure poses a silent global threat to human health and ecosystems. The increasing use of chemicals is indispensable due to various factors, including rising food demand, pressures to enhance agricultural productivity, and challenges related to agricultural pests and diseases (Tudi et al., 2021; Sabzevari & Hofman, 2022). Among all pesticides available for purchase, the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) has been used since the 1950s and, currently, is the second most used worldwide with an annual consumption of approximately 8,000 tons (de Albuquerque et al., 2020; Szewczyk et al., 2020; Chang et al., 2022). The advantages of using atrazine include its cost-effectiveness (De Caroli

Vizioli et al., 2023). However, the disadvantage is related to environmental pollution, with studies reporting contaminated areas in China, Brazil, Argentina, and the USA (Sun et al., 2017; Almberg et al., 2018; Dou et al., 2020; De Caroli Vizioli et al., 2023). Atrazine exhibits high mobility and persistence in soil and water, making it one of the most common chemical contaminants in drinking and surface waters (Almberg et al., 2018).

Indeed, pesticide application contaminates air, food, and water, possibly affecting target and non-target organisms (Singh et al., 2023). This triazine herbicide acts on the target plant by inhibiting photosystem II, interrupting the production of ATP and NADPH (Shimabukuro et al., 1970). In non-target individuals, the effects of atrazine have not been elucidated yet, with recent studies trying to establish potential toxic mechanisms in vertebrates (Mohammed et al., 2023; Edwards et al., 2024; Jiang et al., 2024; Zhao et al., 2024). The extensive use of atrazine raises significant concerns regarding human and animal health. Several studies have reported alterations in the liver (Jestadi et al., 2014), kidney (Abarikwu et al., 2017; Xia et al., 2018), and heart (Rajkovic et al., 2014) metabolism, in addition to impairment in the reproductive system male and female (Piazza & Urbanetz, 2019; Graceli et al., 2020; Krzastek et al., 2021).

Notwithstanding, atrazine acts as an endocrine disruptor and affects hormonal homeostasis by disrupting the production, release, or effect on target cells (Vandenberg et al., 2012; Guarnotta et al., 2022). For instance, this herbicide interacts with estrogen and androgen receptors, enzymes, and cofactors, causing receptor dysfunction and consequent hormonal signaling pathways impairment (Cooper et al., 2007). Hormonal regulation in the male reproductive system relies upon a cascade of events occurring within the hypothalamic-pituitary-gonadal (HPG) axis. The primary releasing hormone of reproduction is gonadotropin-releasing hormone (GnRH) produced by neurons in the hypothalamus. Its role is to stimulate the release of pituitary hormones, the gonadotropins

luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Senger, 2004). FSH acts on Sertoli cells from the seminiferous epithelium stimulating the synthesis of androgen binding proteins and relevant activities for supporting spermatogenesis, whereas LH promotes the testosterone synthesis by Leydig cells from the testicular interstitium (Bhattacharya et al., 2023). Testosterone is essential for the development and maintenance of male secondary sexual characteristics and spermatogenesis, being also converted into estradiol within the seminiferous epithelium. Both testosterone and estradiol exert a negative feedback effect on the hypothalamic-pituitary-testicular axis, thus regulating LH and FSH production and secretion (Li et al., 2024). This intricate hormonal regulation ensures the proper functioning of the male reproductive organs and the maintenance of fertility and sexual competence (Kaprara & Huhtaniemi, 2017; Bhattacharya et al., 2023). Alterations in any of these actors can disrupt the testicular structure and, ultimately, compromise spermatogenesis (Ndufeiya-Kumasi et al., 2022; Mgbudom-Okah et al., 2023).

Nevertheless, studies assessing the effects of atrazine on reproductive parameters in farm animals or humans are scarce. Therefore, laboratory animals are the experimental model mostly used in published studies. Wirbisky and Freeman (2015) compiled epidemiological and qualitative information regarding the effects of atrazine exposure on reproductive functions in mammalian, anuran, and fish models. Even though, no meta-analysis has focused on the potential role of this herbicide in the HPG axis. Thus, this review aimed to evaluate the magnitude of the effect of atrazine on the levels of male reproductive hormones (FSH, LH, testosterone, estradiol, and progesterone) using murine as the animal model. Also, we determined the influence of atrazine concentration, exposure period, and animal age on the intensity of its effect. To achieve our goal, we employed systematic and meta-analytical approaches to describe the key action points

and critically discuss the consequences to the organs' functionality in the HPG axis, culminating in male subfertility/infertility. A better understanding of the toxicity of chemicals such as atrazine on male hormone regulation may be a basis for a correct risk assessment (Tennekes and Sánchez-Bayo, 2013).

2. Materials and methods

This systematic review and meta-analysis were conducted following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Page et al., 2021). Details of the research protocol were registered in PROSPERO (International Prospective Register of Systematic Reviews, number CRD42024495626).

2.1 Focus question

Our research protocol was designed considering the PICOS strategy (Population, Intervention, Comparator, Outcome, and Study design). The population of interest was limited to murine exposed to the herbicide atrazine. The comparison was carried out with animals that were exposed (contaminated animals) and not exposed to the herbicide (control). The results evaluated refer to serum or intratesticular concentrations of hormones taken from experimental work. The guiding question of this work was: What is the magnitude of the effects of atrazine exposure on male reproductive hormone levels based in animal models?

2.2 Search strategy and study selection

A comprehensive and systematic literature search was conducted across electronic databases: Medline/Pubmed, Scopus, and Web of Science, up to October 27,

2023 (14:28). Two main criteria were employed in the search: (i) atrazine and (ii) male reproductive hormones. The search strategy encompassed terms such as atrazine, ATR, and triazines for atrazine, while focusing on the most important hormones for the male reproductive system: LH, FSH, testosterone, estradiol, and progesterone. The search strategy targeted titles, abstracts, and keywords. These terms (i and ii) were combined using Boolean connectors [OR] within each filter and then combined with [AND]. The detailed search strategy can be found in Supplementary Table 1.

To be included in this review, studies needed to meet eligibility criteria: (i) original articles published in peer-reviewed journals; (ii) inclusion of control groups (not exposed to atrazine) and treatment groups (exposed to atrazine); (iii) use of murine models, such as rats and mice; (iv) analysis of relevant levels of male reproductive hormones; (v) publication in English. In addition, studies were required to report the sample size, mean, and measurement of variation (SD or SEM) of the examined parameters for inclusion in the meta-analysis (Machado-Neves, 2022). Manuscripts were excluded if they: (i) were not original research articles, such as brief reports, literature reviews, comments, or book chapters; (ii) did not involve exposure to atrazine in male animals; (iii) employed other models, such as *in vitro* or *in vivo* studies, or used species other than mice and rats; (iv) did not address relevant aspects of the central question, particularly post-exposure hormonal levels; (v) involved indirect exposure to atrazine (e.g., exposure in utero). Additionally, the reference lists of included studies were manually reviewed to identify other studies eligible for review and meta-analysis.

2.3 Data extraction

Two independent researchers (LOGE and MQA) selected eligible studies by analyzing their titles and abstracts. The level of agreement between these reviewers was

assessed using the Kappa test (kappa = 0.918). After selecting studies that met our inclusion criteria, data extraction involved the recovery of publication characteristics (authors, year of publication, and country), experimental design features (animal model, age, total number of animals, number of animals per group, and number of groups), exposure aspects (formulation of atrazine, route of exposure, vehicle, concentration, and exposure time), and methods used to evaluate the hormones.

2.4 Meta-analysis

The studies eligible for inclusion in the meta-analytic study were examined to gather data on the effects of atrazine on hormone production. Response means values ($X_{control}$ and $X_{treatment}$), standard deviations ($S_{control}$ and $S_{treatment}$), and sample sizes ($N_{control}$ and $N_{treatment}$) were analyzed. When data were presented graphically, they were digitized and means and variance measurements were obtained using ImageJ® software after image calibration. Variance measures were then converted to standard deviations from the mean using the MetaWin Statistical Calculator (Rosenberg et al., 2000). A separate random-effects meta-analysis model was conducted for the six variables affected by atrazine: serum concentration of testosterone, FSH, LH, estradiol, and progesterone, besides intratesticular testosterone. These variables were established considering the outcomes extracted from the eligible studies and our focus question.

The standardized difference between the control and atrazine groups was used to interpret and summarize the effects of atrazine on hormone levels. For each study, the magnitude of the effect (d) was calculated as $d = (Xt - Xc/\mathrm{SD})*J$, where Xt is the treatment group response, Xc is the control group response, SD is the deviation-matched pattern and J is a correction term to remove bias for small sample sizes (Rosenberg et al., 2000). J reaches 1.0 in sample sizes ≥ 25.

After calculating Hedge's d for each independent comparison, the cumulative effect (d_{++}) was calculated for each variable. Then, they were analyzed using a random effects model. This model calculated the effect of atrazine regardless of differences in the experimental design. This review also used a mixed effects model to analyze methodological moderators. Relevant moderators considered for the toxic effects of atrazine included: the dose administered, duration of exposure, and age of animals. Specifically, exposure duration was categorized according to Eaton and Gilbert (2013) as follows: acute (≤ 1 day; single dose), subacute (up to 30 days), subchronic (between 31 and 89 days), and chronic exposure (≥ 90 days). Animal age followed the classification of Picut et al. (2017): peripubertal corresponding to young animals at approximately 50 days old and puberty animals older than 50 days at the exposure. Both random-effects and mixed-effects models assume that differences between studies within a class are determined by sampling error and random variation (Rosenberg et al., 2000). The upper and lower confidence intervals (CI) were calculated according to the average cumulative effect, and intervals that did not overlap with zero, with n − 1 degree of freedom (*df*), were considered significant. By convention, a d_{++} value around 0.2 indicates a weak effect, 0.5 indicates a moderate effect and 0.8 indicates a strong effect (Cohen, 1992). Positive values of d_{++} indicate a positive effect of this herbicide on each variable, while negative values indicate a negative influence of atrazine.

Finally, heterogeneity analyses (Q statistics) were employed to test whether categorical groups in mixed models were homogeneous concerning the calculated effect sizes. Total heterogeneity (Q_T) for all effects tested and heterogeneity within (Q_W) and between groups (Q_B) were calculated. The significance of these statistics was assessed according to a chi-square distribution with n – 1 *df*. Because the analyses were based only on published studies, and studies showing large, significant effects may be more likely to

be published than those with weak or no effects (Rosenthal et al., 1979), the fail-safe numbers were calculated for each tested effect. Fail-safe numbers indicate the number of nonsignificant, unpublished, or missing studies that would need to be added to the sample to change its results from significant to nonsignificant (Rosenberg et al., 2000). As a rule, results are considered robust if its value exceeds $5k + 10$, where k is the number of comparisons in the analysis. All analyses were performed in MetaWin 2.1 (Rosenberg et al., 2000) and numbers were done in Sigma Plot 10.0 software.

2.5 Risk of bias assessment

The quality of reporting of studies eligible for review was assessed using the SYRCLE (Systematic Review Center for Experimentation with Laboratory Animals) Risk of Bias tool (Hooijmans et al., 2014). This tool assesses methodological quality and possible biases in preclinical studies involving animals. Questions were designed into subtopics for selection bias analysis, performance, detection, attrition, reporting, and other sources of bias. Items were scored “yes” (low risk of bias), “no” (high risk of bias), or “unclear” (indicating that the item was not adequately reported and therefore at risk of bias). This tool helps ensure that systematic reviews and meta-analyses accurately represent available evidence from preclinical animal studies, while also considering potential biases that may affect the validity of results. The SYRCLE chart was created using the Review Manager 5.3 software system.

3. Results

3.1 Eligible studies and their characteristics

The initial search identified 933 articles through PubMed, Scopus, and Web of Science databases published between 2000 and 2024. After removing duplicates, 452

articles were screened for eligibility (Fig. 1). One article was retrieved in the reference analysis of accepted manuscripts and included in this screening after meeting the eligibility criteria. A total of 25 studies were considered eligible for analysis under systematic review (Table 1). Five out of 25 studies were removed from the meta-analysis because they missed the statistical information required for this analysis (Fig. 1).

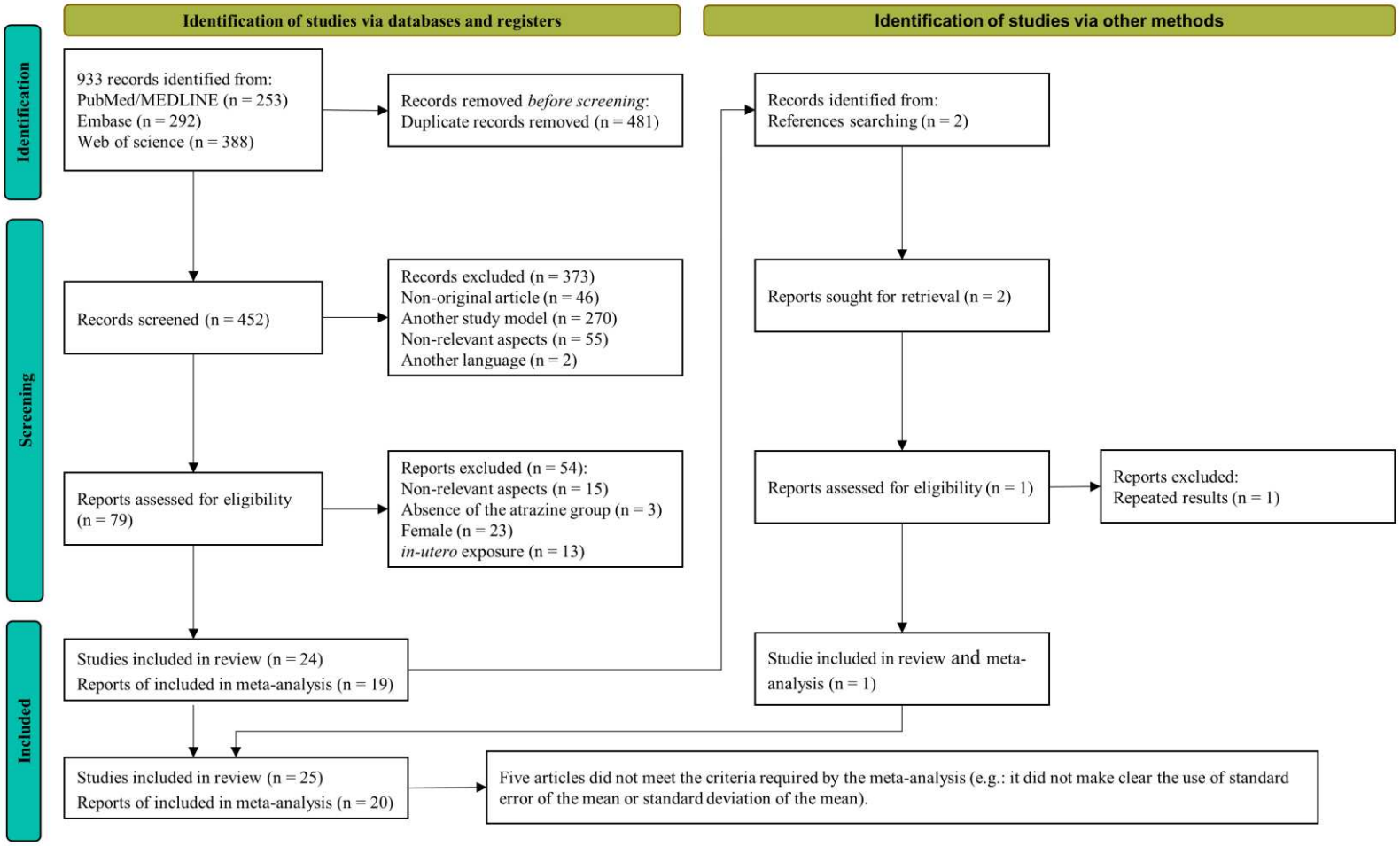


Fig. 1 Flowchart depicting the results of the systematic review and meta-analysis literature search, adhering to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement guidelines (http://www.prisma-statement.org).

Amongst the 25 studies, most of the articles were carried out in Nigeria, the United States, China, and Iran. Brazil, Egypt, France, Japan, and Serbia conducted one study each (Fig. 2A). Rat was the animal model commonly used, followed by mice. The strains mostly used were Wistar and Sprague-Dawley rats (Fig. 2B). The animals were mostly adults, with few studies performing the analysis in peripubertal animals and one not reporting the animal age (Fig. 2E; Supplementary Table 2). Authors sought to evaluate the effect of exposure to atrazine only (n = 19), whereas others tested the herbicide and therapeutic substances (n = 6; Table 1). Twenty-two articles used atrazine as the herbicide, followed by three studies that tested a commercial formulation. Corn oil was the vehicle often used to dilute the herbicide (n = 10), followed by methylcellulose (n = 6), olive oil (n = 2), saline, water, mineral oil, dimethylsulfoxide, and diet (n = 1 study each). One article did not report the vehicle (Table 1). The atrazine exposure routes included gavage, drinking water, diet, and intraperitoneal injection (Fig. 2C; Table 1). Twelve articles tested only one concentration of atrazine, whereas 13 studies evaluated two or more concentrations (Table 1). These concentrations ranged from 0.5 to 1,000 mg kg^{-1}, predominating 50 and 200 mg kg^{-1} (n = 11/each), followed by 100 mg kg^{-1} (n = 8; Fig. 2D). Seven studies have tested atrazine concentrations < 10 mg kg^{-1}, with two articles evaluating 0.5 and 1.0 mg kg^{-1} (Table 1). One study performed an acute exposure to evaluate the atrazine effect, whereas 18 evaluated subacute and seven subchronic exposures. Only one study exposed animals to a chronic period (Table 1). One study euthanized the animals two weeks after the removal of atrazine (Gely-Pernot et al., 2015), while other studies euthanized the animals within 24 h after the end of the exposure period to the herbicide. The exposure frequency mostly tested was daily (n = 20), three times a week (n = 1), and once a week (n = 1). One study administered four doses during the experimental period and another article did not report this information (Table 1). The

methods used to determine the hormone levels were the enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA; Table 1). Supplementary Table 2 summarizes the total number of animals, the number of animals per group, and the number of groups used in each article.

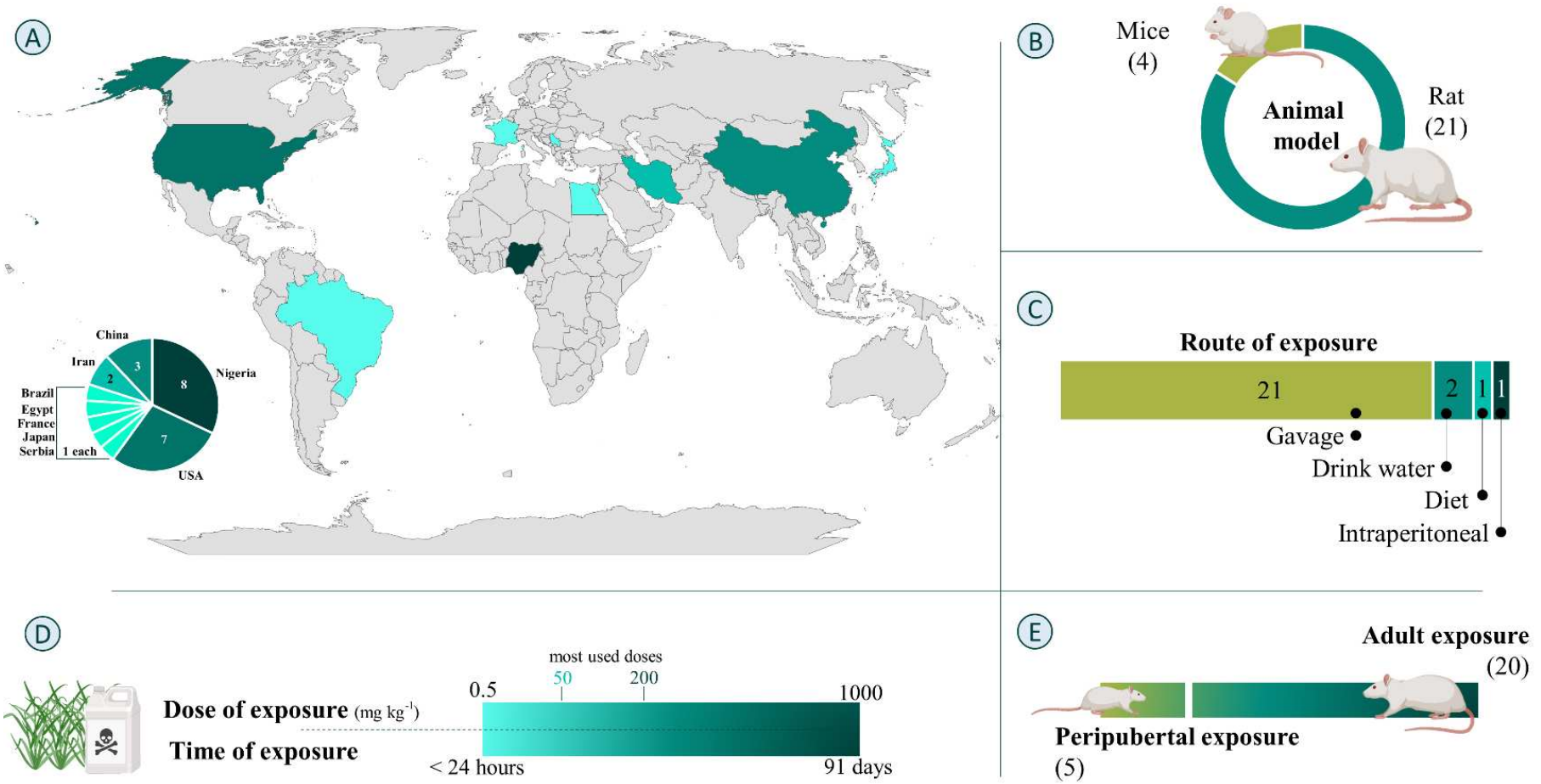


Fig. 2 Characteristics of the articles included in the systematic review. (A) Country. (B) Animal model. (C) Exposure route. (D) Dose and time of exposure. (E) Animal age.

Table 1. Characteristics of atrazine exposure from studies that evaluated its effect on the levels of different hormones.

	Article	Atrazine formulation	Exposure route	Vehicle	Dose (mg kg^{-1})	Duration of exposure (days)	Frequency of exposure	Outcomes	Hormone assays
1	Abarikwu et al., 2015	Commercial formulation	Oral (gavage)	Corn oil	12.5	52	3x week	Serum T, LH, and FSH	ELISA
2	Abarikwu et al., 2020#	Atrazine	Oral (gavage)	Corn oil	50	60	1x week	Serum and intratesticular T	ELISA
3	Abarikwu et al., 2021	Atrazine	Oral (gavage)	Saline	0.5, 25, and 50	3	Daily	Serum and intratesticular T	ELISA
4	Adedara et al., 2021	Atrazine	Oral	-	40	42	Daily	LH, FSH, serum and intratesticular T	ELISA
5	Agdam et al., 2017	Atrazine	Oral (gavage)	Corn oil	200	48	Daily	Serum T and LH	RIA
6	Aziz et al., 2018	Atrazine	Oral (gavage)	Corn oil	120	21	Daily	Serum T	RIA
7	Feyzi-Dehkhargani et al., 2012	Atrazine	Oral (gavage)	Corn oil	100, 200, and 300	12, 24, and 48	Daily	Serum T, LH, and FSH	RIA
8	Foradori et al., 2017	Atrazine	Oral (gavage)	Methylcellulose	6.5, 25, and 100	1, 7, 14, and 28	Daily	Serum T	RIA
9	Friedmann et al., 2002*	Atrazine	Oral (gavage)	Mineral oil	50	26	Daily	Serum and intratesticular T	RIA
10	Gely-Pernot et al., 2015#	Atrazine	Oral (drinking water)	Water	100	21 + 14 recovery	Daily	Serum T and FSH	ELISA and RIA
11	Ikeji et al., 2023	Atrazine	Oral (gavage)	Corn oil	50	30	Daily	Serum and intratesticular T, LH, and FSH	ELISA

12	Jin et al., 2013*	Atrazine	Oral (drinking water)	-	50, 100, and 200	21	Daily	Serum T and E_2	ELISA
13	Jin et al., 2014#	Atrazine	Intraperitoneal	Corn oil	100 and 200	8	4 doses	Serum and intratesticular T	ELISA
14	Kale et al., 2018	Atrazine	Oral (gavage)	Corn oil	300	7	Daily	Serum T, LH, and FSH	ELISA
15	Kandori et al., 2005	Atrazine	Oral (diet)	Diet	500 and 1000	91	Daily	Serum T	RIA
16	Laws et al., 2009#	Atrazine	Oral (gavage)	Methylcellulose	5, 50, 100, and 200	0	-	P_4	RIA
17	Ndufeiya-Kumasi et al., 2022	Atrazine	Oral (gavage)	Dimethylsulfoxide	50	60	Every other day	Serum T, FSH, and LH	ELISA
18	Pogrmic et al., 2009*	Atrazine	Oral (gavage)	Olive oil	50 and 200	27	Daily	Serum T and LH	RIA
19	Riffle et al., 2013	Atrazine	Oral (gavage)	Methylcellulose	200	5	Daily	Serum T, E_2, and P_4	RIA
20	Riffle et al., 2014	Atrazine	Oral (gavage)	Methylcellulose	5, 25, 75, and 200	3	Daily	Serum T, LH, and P_4	RIA
21	Rotimi et al., 2023	Commercial formulation	Oral (gavage)	Olive oil	120	7	Daily	Serum T, FSH, and LH	ELISA
22	Stoker et al., 2000*	Atrazine	Oral (gavage)	Methylcellulose	12.5, 25, 50, 100, 150, and 200	30	Daily	Serum and intratesticular T, LH, and E_2	RIA
23	Trentacoste et al., 2001*	Atrazine	Oral (gavage)	Carboxymethylcellulose	1, 2,5, 5, 10, 25, 50, 100, and 200	26	Daily	E_2 and LH	RIA
24	Victor-Costa et al., 2010	Commercial formulation	Oral (gavage)	Corn oil	50, 200, and 300	7, 15, and 40	Daily	Serum and intratesticular T and E_2	ELISA and RIA

25	Yang et al., 2014	Atrazine	Oral (gavage)	Corn oil	38.5, 77, and 154	30	Daily	Serum T, LH, and FSH	ELISA

ELISA: Enzyme-Linked Immunosorbent Assay; RIA: Radioimmunoassays; T: testosterone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; P_4: progesterone; E_2: estradiol. #Studies not included in the meta-analysis. *Studies performed in peripubertal animals.

3.2 Meta-analysis

Twenty studies eligible for the meta-analysis generated 142 independent comparisons. The random-effects model showed that atrazine exerted a strong and negative effect on serum FSH, LH, and testosterone (Fig. 3). Rosenthal's fail-safe numbers were fairly high for these variables (Supplementary Table 3). Also, atrazine elicited a strong and negative effect on intratesticular testosterone levels and a strong and positive effect on serum estradiol and progesterone levels (Fig. 3). Rosenthal's fail-safe numbers for these effects were described in Supplementary Table 3.

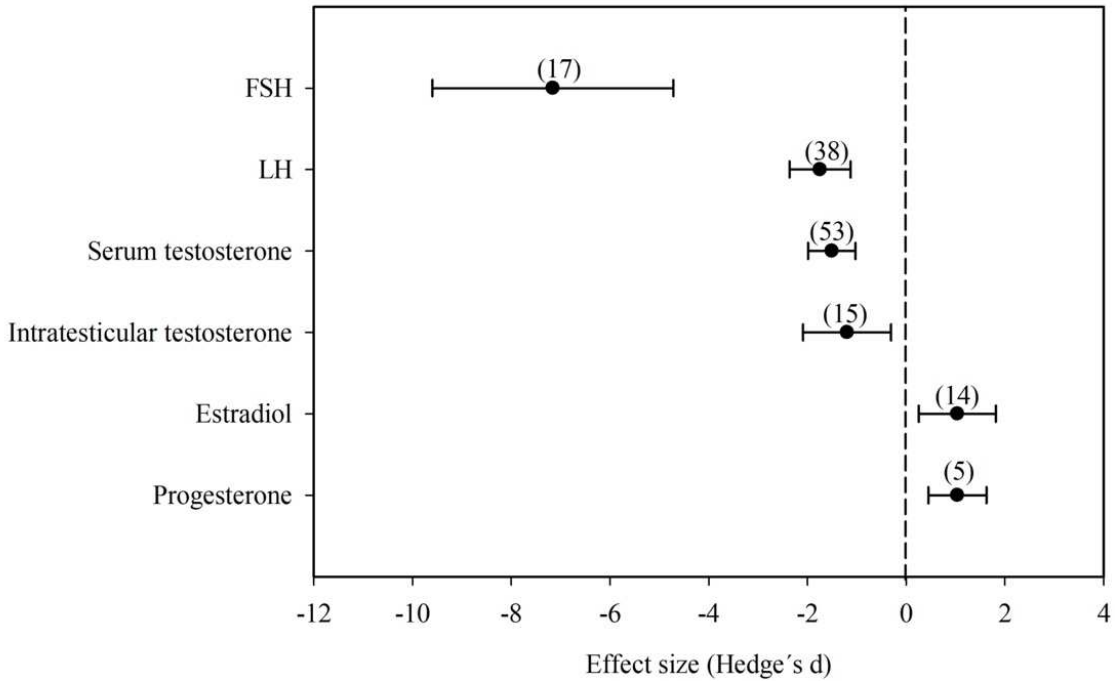


Fig. 3 Effects of atrazine on hormone variables according to a random-effect model of meta-analysis. The cumulative effect size is reported with its 95% confidence interval. The effect is significant if confidence intervals do not overlap with zero. (n) = number of independent comparisons.

The mixed-effects model of meta-analysis revealed that atrazine influenced serum testosterone and LH levels at concentrations < and ≥ 100 mg kg^{-1} (Fig. 4A). This herbicide altered FSH and estradiol levels at concentrations ≥ 100 mg kg^{-1}. Conversely, atrazine concentration did not exert any influence on intratesticular testosterone levels

(Fig. 4A) and progesterone levels (< 100 mg kg^{-1}: d_{++} = 0.95; *df* = 2; CI = -0.23 to 2.12; ≥ 100 mg kg^{-1}: d_{++} = 1.17; *df* = 1; CI = -3.18 to 5.52). Moreover, subchronic and subacute exposure exhibited strong and negative effects on serum testosterone, FHS, and LH levels (Fig. 4B). Data of intratesticular testosterone, estradiol, and progesterone were not evaluated because of the low number of independent comparisons. Ultimately, the results showed that intratesticular testosterone and serum LH and testosterone levels measured in adults were negatively influenced by atrazine rather than in young animals. In contrast, estradiol concentration increased in young animals after atrazine exposure than in adult animals (Fig. 5). FSH and progesterone were not evaluated because of the low number of independent comparisons.

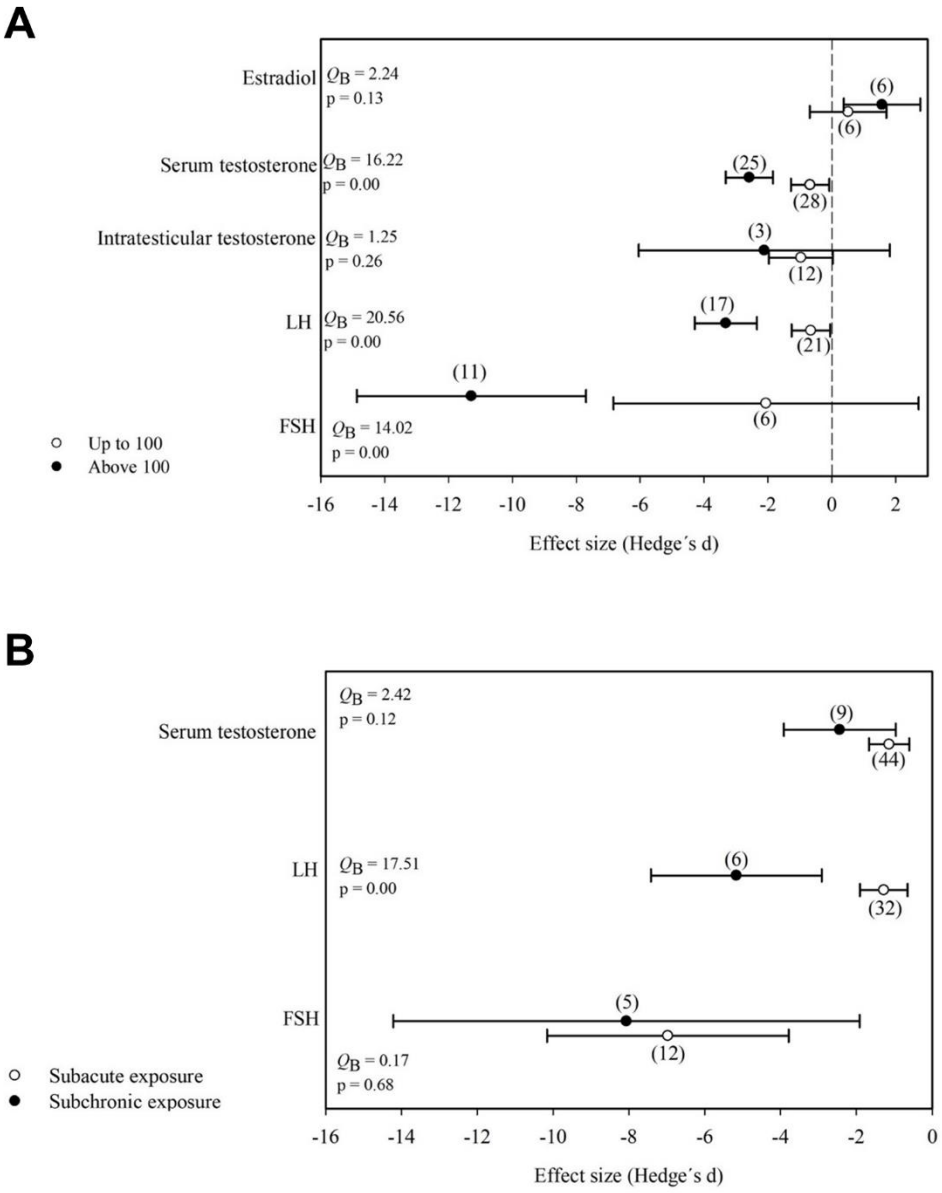


Fig. 4 Effects of atrazine concentration (A; < and ≥ 100 mg Kg^{-1}) and exposure period (B; subacute and subchronic exposure) on hormone variables. The cumulative effect size is presented for each measured effect alongside its 95% confidence intervals. Effects are considered significant if the confidence intervals do not overlap zero. "(n)" denotes the number of independent comparisons for each effect. Q_B indicates heterogeneity between variables.

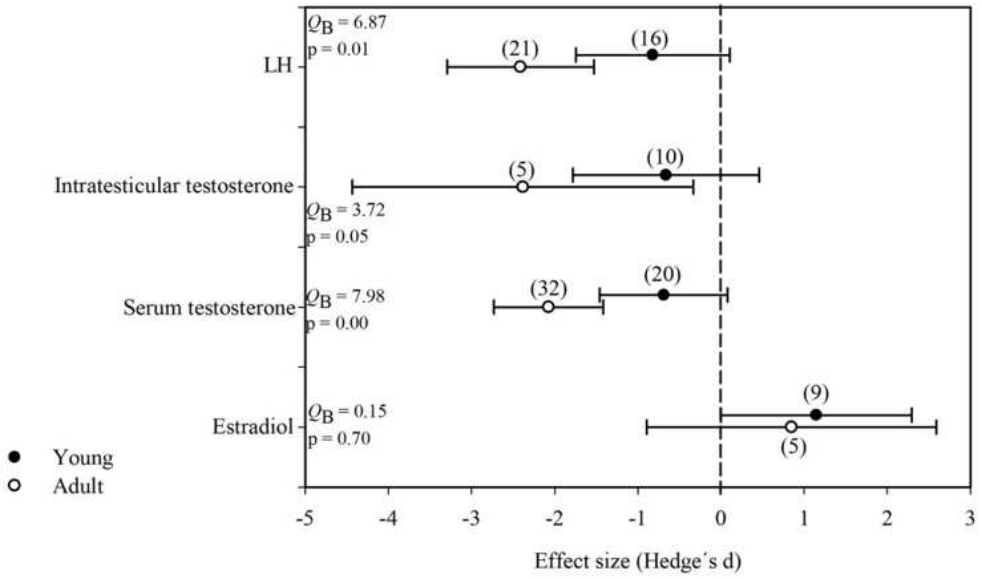


Fig. 5 Effects of the animal age (young or adult) on hormone variables after exposure to atrazine. The cumulative effect size is presented for each measured effect alongside its 95% confidence intervals. Effects are considered significant if the confidence intervals do not overlap zero. "(n)" denotes the number of independent comparisons for each effect. Q_B indicates heterogeneity between variables.

3.3 Data reporting and bias analysis

The methods applied to set up random sequence generation, allocation concealment, random housing, and random outcome evaluation were not mentioned clearly in any of the articles and were often defined as unclear. Baseline similarities were reported in some of the studies and all reports were free of selective reporting of results and free of other issues that could result in a high risk of bias (Fig. 6).

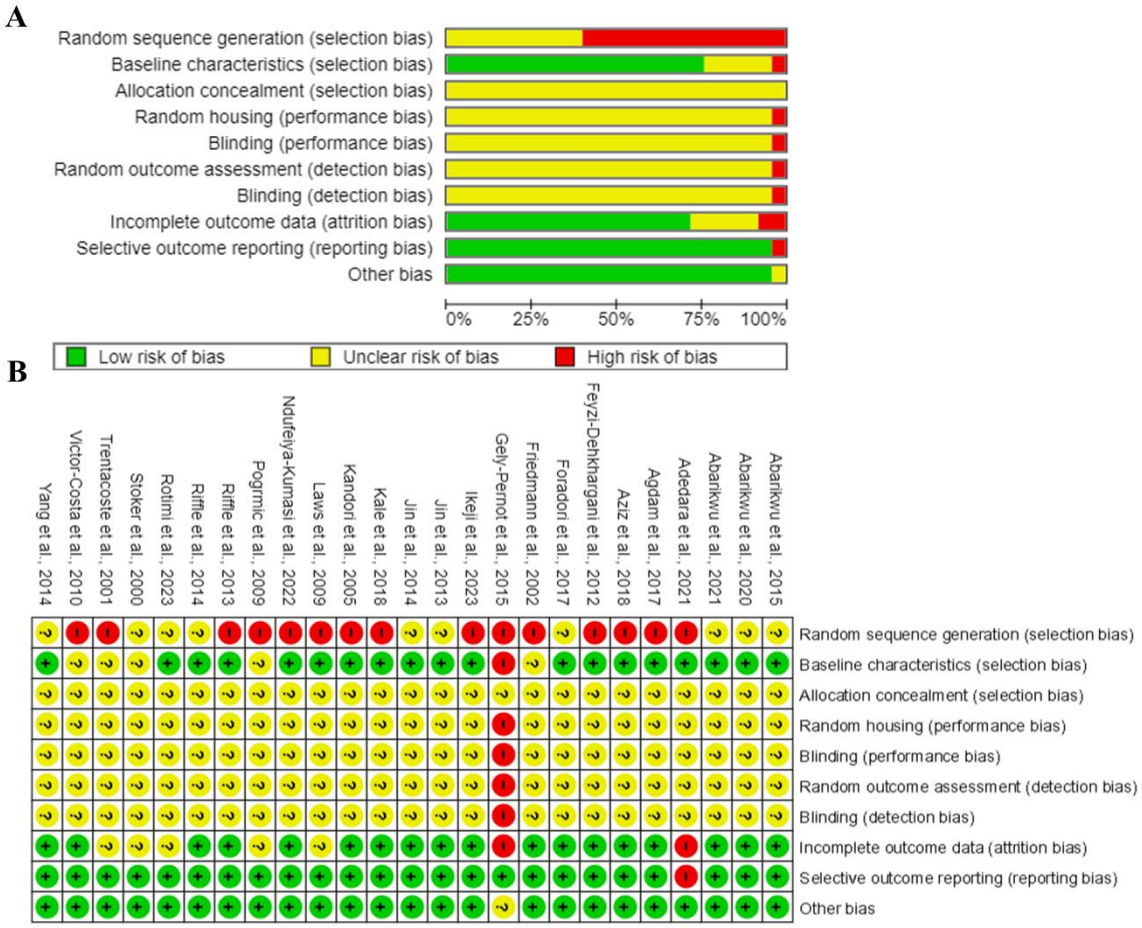


Fig. 6 Risk of bias assessment and methodological quality indicators for all included studies evaluating the effects of atrazine on serum levels of sex hormones and intratesticular testosterone in murine models. The SYRCLE RoB tool items were categorized as "yes" (indicating low risk of bias), "no" (indicating high risk of bias), or "unclear" (indicating insufficient reporting, thus the risk of bias remained unknown).

4. Discussion

The current review evidenced the harmful effect of atrazine on the levels of crucial hormones for male reproduction, and the potential influence of exposure time, concentration, and age of animals on the alterations. Our results revealed a significant reduction in serum levels of testosterone, FSH, LH, and intratesticular testosterone levels in mice after subacute and subchronic exposure to atrazine. The ingestion of concentrations > 100 mg kg^{-1} was more harmful to gonadotrophic and steroid hormones than concentrations < 100 mg kg^{-1}. The herbicide caused an increase in serum progesterone and estradiol levels, especially the estradiol in young males. Collectively,

these findings suggest hormonal imbalance indicating a testicular steroidogenesis failure. Human reproduction, including the expression of secondary sexual characteristics, depends on an intact HPG axis (Maggi et al., 2016). Hence, any disturbance in the production and action of androgens can have serious consequences for male reproductive function. A better understanding of the toxicity of chemicals such as atrazine to organisms is also the basis for a correct risk assessment (Tennekes and Sánchez-Bayo, 2013).

Most of the studies included in the systematic review were carried out by authors from Nigeria and the USA, which are among the main users of atrazine in the world (de Albuquerque et al., 2020). The eligible studies often used rats to evaluate atrazine effects. Rats exhibit nice behavior and big size, providing sufficient organ mass to be shared between laboratory analyses. Despite rats and mice metabolizing atrazine into desethylatrazine and deisopropylatrazine through distinct mechanisms, this fact did not represent a relevant difference between them. There is a greater tendency for atrazine to conjugate with glutathione in rats, whereas mice exhibit a greater propensity for hydroxylation reactions, generating different secondary metabolites and activating detoxification pathways (Timchalk et al., 1990). Moreover, atrazine was offered to animals through oral and gavage routes to mimic environmental situations by which humans and animals may pass through involuntary atrazine contamination (ATSDR, 2003).

The findings from the meta-analysis evidenced the impact of atrazine on the pituitary gland's role in stimulating testis functions. As described before, the HPG axis involves the hypothalamus, pituitary, and testis (Fig. 7) and orchestrates the hormonal regulation of male reproduction. The studies included in this review have not assessed hypothalamic parameters after atrazine exposure. Undoubtedly, GnRH is the key regulator of reproductive function (Senger, 2004). The absence of GnRH analysis may be

related to its biochemical properties. GnRH is highly diluted and rapidly degraded in the bloodstream, making it impossible to measure it accurately after it leaves the pituitary-portal circulation (Maggi et al., 2016). Nonetheless, previous studies suggested that atrazine may indirectly affect GnRH function without altering its gene expression in the hypothalamus (Foradori et al., 2013, 2009). Precisely, atrazine disturbed the expression and production of kisspeptin 1 (Kimura et al., 2019) and the activity of excitatory (e.g., glutamate) and inhibitory neurotransmitters, including gamma-aminobutyric acid, dopamine, and norepinephrine (Lin et al., 2013; Chávez-Pichardo et al., 2020; Stradtman and Freeman, 2021), besides inhibited the activity of GnRH neurons affecting the secretion of GnRH and gonadotropins (Fig. 7A; Lin et al., 2013; Chávez-Pichardo et al., 2020; Stradtman and Freeman, 2021). The effects of the herbicide on KNDy neurons, important regulators of GnRH neurons, are unknown (Fig. 7A). The neurochemical/hormonal disorders identified so far may be strongly related to the oxidative stress generated by atrazine in the brain. The herbicide impairs the function of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GST), generating reactive oxygen species (ROS) (Genovese et al., 2021; Rotimi et al., 2023).

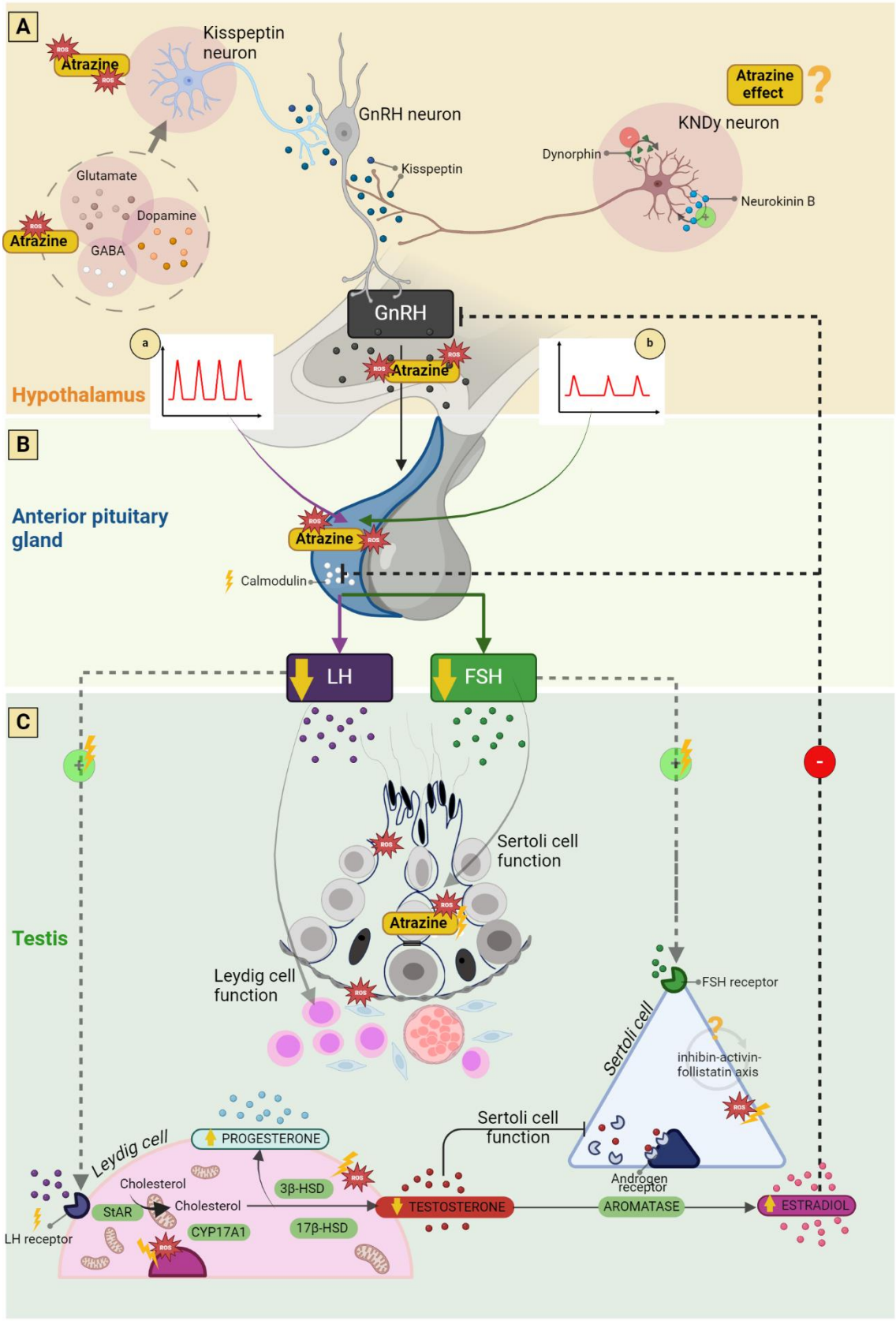


Fig. 7 A schematic representation of the functioning of the hypothalamic-pituitary-gonadal (HPG) axis and potential mechanism of atrazine toxicity. **A:** In the hypothalamus, atrazine interferes with the pulsatile secretion of Gonadotropin hormone-releasing (GnRH) neurons, essential for releasing luteinizing hormone (LH) and follicle-

stimulating hormone (FSH) by the pituitary gland. Despite low testosterone levels, elevated progesterone and estradiol can cause negative feedback on GnRH neurons. Furthermore, atrazine suppresses the production of kisspeptin, directly affecting the secretion of GnRH without altering the expression of the GnRH gene. Moreover, the herbicide deregulates the complex system of excitatory (e.g., glutamate) and inhibitory (e.g., GABA and dopamine) neurotransmitters, affecting kisspeptin neurons and indirectly influencing the production of gonadotropins. These effects are linked to the oxidative stress caused in the hypothalamic parenchyma by atrazine, damaging cellular components and compromising the hypothalamic-pituitary axis. The effects of atrazine on Kisspeptin, neurokinin B, and dynorphin (KNDy) neurons, which produce important molecules regulating kisspeptin release, such as dynorphin and neurokinin B, are still unknown. **B:** Atrazine's action on the hypothalamus interferes with pituitary homeostasis, affecting the release of gonadotropins. (*a*) High frequency and amplitude of GnRH release result in LH release, (*b*) while low frequency and amplitude favor the release of FSH. By compromising the hypothalamus, atrazine alters the dynamics of pulsatile GnRH release, affecting the adequate release of gonadotropins. Additionally, the herbicide can influence calcium/calmodulin-dependent pathways necessary for the release of gonadotropins. Atrazine also affects the inhibin-activin-follistatin axis, reducing the availability of inhibin, which could increase the production of FSH. However, the damage caused to the hypothalamus-pituitary gland appears to negate the action of inhibin. High estradiol levels cause negative feedback on the pituitary gland, reducing the secretion of LH and FSH. **C:** Insufficient secretion of LH and FSH impacts testosterone production. In addition to low LH levels, atrazine also decreases the expression of the LH receptor in the testes, compromising the signaling cascade for testosterone production. Despite the increase in steroidogenic acute regulatory protein (StAR) expression, atrazine generally impairs the expression of key enzymes involved in steroidogenesis, such as Cytochrome P45017A1 (CYP17A1) and 3β-Hydroxysteroid dehydrogenase (3β-HSD). This results in increased progesterone production and decreased testosterone production, affecting serum and intratesticular levels of the hormone. Low serum concentrations of LH and FSH appear to not provide positive physiological feedback for the function of Leydig and Sertoli. Atrazine also increases the expression of aromatase, contributing to the conversion of testosterone into estradiol, thereby increasing serum levels of the latter. Furthermore, it can reduce the number of Leydig cells due to inflammatory processes and oxidative stress, further compromising testosterone production. Yellow arrows indicate the effect of atrazine based on meta-analysis. Lightning bolts, ROS, and question marks indicate the action of atrazine based on the literature.

4.1 Effect of atrazine on gonadotropin hormones

The results confirmed the negative impact of atrazine on serum LH and FSH levels. Gonadotropic cells from the pituitary gland produce these glycoprotein polypeptide hormones (Fig. 7B), differing in their biochemical structure according to their protein subunits. LH and FSH present identical α-subunit and distinct β-subunit. The length of the β-subunit varies between FSH (111 amino acids) and LH (121 amino acids)

and confers the specific biological action (Cahoreau et al., 2015). Hence, LH and FSH act respectively on Leydig and Sertoli cells (Fig. 7C) by binding to membrane receptors and supporting steroidogenesis and spermatogenesis (Griswold, 2018; Mattos et al., 2022). The low levels of FSH and LH induced by atrazine may follow two ways. First, atrazine can interrupt the excitatory GnRH signal (Fig. 7A) with consequences to the gonadotropin release. It is known that, under health conditions, LH release is stimulated at high GnRH pulse frequencies, whereas FSH release depends on its low frequencies (Tsutsumi and Webster, 2009). The inhibin-activin-follistatin axis is another regulatory pathway of FSH secretion and paracrine regulation of testis function susceptible to atrazine action. FSH stimulates the expression and production of inhibin by Sertoli cells which, in turn, provides negative feedback for pituitary FSH production (Namwanje and Brown, 2016; Griswold, 2018). Activin can stimulate FSH release, whereas follistatin suppresses it. Studies have shown that atrazine decreases the availability and concentration of inhibin (Feyzi-Dehkhargani et al., 2012; Yang et al., 2014; Agdam et al., 2017), with no information regarding the expression and action of testicular activin and follistatin. Also, the secretion of gonadotropins relies on calcium and calmodulin pathways, which can be disturbed by atrazine (Gao et al., 2016; Li and Bi, 2023). An *in vitro* study using murine LβT2 cells revealed a negative effect of atrazine on proteins related to the calcium handling within the endoplasmic reticulum, causing a loss of the intracellular calcium content and impairment of LH secretion (Dooley et al., 2013).

4.2 Effect of atrazine on sexual hormone levels

According to our meta-analysis, atrazine reduced testosterone levels and increased serum estradiol and progesterone levels. The negative effect of atrazine on LH levels directly impacts the testosterone synthesis by Leydig cells because of dysregulation

of LH pulses and deficiency of LH binding in the choriogonadotropin receptor (LHR; Lawrence et al., 2022). The harmful effect of atrazine on LHR is still controversial (Pogrmic et al., 2009; Stoker et al., 2000). A decrease in the number and activity of LHR inhibits the signaling cascade that transports cholesterol to mitochondria, compromising the steroidogenic process (Wang et al., 2017). The expression of the scavenger receptor class B type 1 (SR-B1) decreased in animals receiving intraperitoneal injections of atrazine (Jin et al., 2014), without changing when the herbicide was provided orally (Jin et al., 2013). SR-B1 expression is dramatically altered in steroidogenic tissues after depletion of cholesterol reservoirs (Shen et al., 2018). Moreover, the expression of the StAR protein (Fig. 7C) increased after atrazine exposure in cell culture (Pogrmic et al., 2009; Pogrmic-Majkic et al., 2016; Abarikwu et al., 2012), with no change after exposure in prepubertal animals (Jin et al., 2013).

Indeed, steroid hormone biosynthesis is another target for atrazine. A cascade of suppression begins in the gene expression of enzymes important for steroidogenesis (Fig. 7C). For instance, this herbicide impairs the initial steps in converting cholesterol to testosterone by downregulating the expression of P450scc (Jin et al., 2013, 2014). The expression of the CYP17A1 enzyme is also affected by atrazine in *in vitro* (Pogrmic et al., 2009) and *in vivo* studies (Aziz et al., 2018). The main consequence is the increase in progesterone levels, which was pointed out by our findings. The expression of 3β-HSD type 2, which converts androstenedione to androstenediol and subsequently to testosterone, decreased in adult animals after atrazine poisoning (Victor-Costa et al., 2010; Martins-Santos et al., 2017; Abarikwu et al., 2021) but not in atrazine-exposed prepubertal rats and peripubertal mice (Pogrmic et al., 2009; Jin et al., 2013). The expression of the 17β-HSD enzyme, involved in the conversion of androstenedione into testosterone, was also downregulated in the testis of prepubertal animals exposed to

atrazine (Pogrmic et al., 2009; Jin et al., 2013). Notwithstanding, studies reported a low number of Leydig cells mediated by atrazine because of inflammation and oxidative stress processes, culminating in apoptosis (Feyzi-Dehkhargani et al., 2012; Agdam et al., 2017; Krzastek et al., 2021; Abarikwu et al., 2021; Mgbudom-Okah et al., 2023). All these biochemical and cellular changes culminate in low testosterone production, impacting its serum and intratesticular levels.

This current review evidenced an increase of serum estradiol in atrazine-exposed animals (Fig. 7C). Previous studies documented upregulation of aromatase (CYP19A1) in the testis (Jin et al., 2013; Martins-Santos et al., 2017), efferent ducts (Martins-Santos et al., 2018), and other body tissues (Holloway et al., 2008; Jin et al., 2013; Martins-Santos et al., 2018) from atrazine-exposed animals. This fact may contribute to the depletion of intratesticular and serum testosterone and the elevation of serum estradiol levels. Serum estradiol produces negative feedback in the hypothalamus, altering the pulse frequency and in the pituitary gland (Rochira et al., 2006), decreasing GnRH receptors and reducing the release/production of gonadotropins (Pitteloud et al., 2008). However, the effects of atrazine on estradiol receptors in the hypothalamus and pituitary gland have not yet been elucidated.

4.3 The influence of exposure duration, concentration, and age of animals on atrazine effects

The selection of exposure duration, frequency, and concentration constitutes fundamental aspects of toxicological investigations (Eaton and Gilbert, 2013). Herein, the results showed that both short- and long-term exposure contributed to the negative effect of atrazine on serum LH, FSH, and testosterone levels. Determining the duration of exposure depends on the main objectives of the research. For example, short-term exposures can reveal the initial effects of the contaminant on the organs' functionality,

while prolonged exposures can show more pronounced tissue damage (Eaton and Gilbert, 2013). In the eligible studies, the exposure mainly occurred with daily doses administered at subacute and subchronic periods. These periods allowed a rapid interaction of atrazine with neurons upstream of GnRH, which interfered with its stimuli and impaired its pulsatile release (Tsutsumi and Webster, 2009). As a result, the release of both LH and FSH is impaired, interfering with testosterone production.

It is worth noting that all studies tested concentrations of atrazine much higher than the environmental concentration, or above the limit of detection of atrazine in drinking water determined by the World Health Organization (0.1 mg L^{-1}; WHO, 2022). Our findings revealed a strong reduction of serum testosterone, estradiol, FSH, and LH levels after atrazine poisoning at concentrations ≥ 100 mg kg^{-1}, causing consequences for testis and epididymis morphology and function. The diversity of experimental designs hampers the best comprehension of atrazine toxicity. For instance, the highest dose of 1,000 mg kg^{-1} was tested in rats for 91 days (Kandori et al., 2005), while the lowest dose of 0.5 mg kg^{-1} was offered to BALB/c mice for 3 days (Abarikwu et al., 2021). Therefore, we may suggest that future experimental studies should seek an environmentally relevant assessment in order to understand the true impact of relevant and low doses of atrazine on male reproduction.

According to the meta-analysis, the hormones testosterone and LH were disturbed by atrazine in adult animals rather than young individuals. The concentration of these hormones is regularly lower in juvenile animals than in adults because they have not reached puberty yet. Adults, in turn, present a sexually mature status with an HPG axis fully established (Picut et al., 2017). Nevertheless, the hormonal machinery of young individuals is more susceptible to the toxic mechanisms of atrazine, causing damage in the development of the HPG axis (Stoker et al., 2000; Trentacoste et al., 2001: Friedmann

et al., 2002; Pogrmic et al., 2009; Jin et al., 2013) and disturbing the age of puberty onset and the male reproductive lifetime. For that reason, investigating the action of atrazine during the peripubertal development window is relevant and should be better explored in future studies.

4.4 General mechanism of atrazine toxicology

The adverse effects of atrazine on the hormonal variables evaluated here were probably driven by molecular mechanisms involving redox metabolism and inflammation in the HPG axis. The atrazine absorbed in the intestine reaches the liver through circulatory system and disturb the activity of cytochrome CYP450 enzymes (e.g., CYP1A1, CYP1A2, and CYP2E1), responsible for successive N-dealkylation of atrazine to desethylatrazine and deisopropylatrazine (Mohammed et al., 2023), associated with the detoxification process. Hence, the remaining atrazine and its metabolites enter the cells and elicit a mitochondrial dysfunction by decreasing mitochondrial membrane potential and causing cristae fragmentation (Li et al., 2024). Investigations in brain, liver, and testis tissues showed the potential of atrazine to trigger oxidative stress by inactivating antioxidant enzymes and producing excessive ROS (Ikeji et al., 2023; Mohammed et al., 2023; Rotimi et al., 2023; Edwards et al., 2024). ROS may interfere with the nuclear factor erythroid-2-related factor 2 (Nrf2) pathway, damaging antioxidant and detoxification systems, and activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Edwards et al., 2024). Likewise, ROS signals activate the transcription of genes involved in inflammatory processes, with an increase in interleukin -1β, -6, and tumor necrosis factor-α after exposure to high concentrations of atrazine (Mohamed et al., 2023; Edwards et al., 2024; Jiang et al., 2024; Zhao et al., 2024). Finally, mitochondrial dysfunction induced by atrazine results in apoptosis by activation

the mitogen-activated protein kinase (MAPK) pathway. The cascade of events includes upregulation of MAPK14, Bcl-2-associated X protein, and caspase-3 along with downregulation of B-cell lymphoma 2 (Mohammed et al., 2023; Edwards et al., 2024; Zhao et al., 2024). Collectively, the initial process of oxidative stress and subsequent inflammation destabilize the antioxidant defenses of the HPG axis, affecting the hormone synthesis, secretion, and release.

Beyond the hormonal alterations in the HPG axis described here, changes in the testis morphology and function also occurred after exposure to atrazine. Several studies have documented low testicular weight, high sensitivity of AR-expressing cells, alteration in seminiferous tubule morphometry, meiosis acceleration, germ cell population declination, spermiation retardation, and accentuated autophagy by Sertoli cells (Victor-Costa et al., 2010; Jin et al., 2013; Yang et al., 2014; Gely-Pernot et al., 2015; Agdam et al., 2017; Kale et al., 2018; Abarikwu et al., 2021; Ndufeiya-Kumasi et al., 2022; Mgbudom-Okah et al., 2023; Rotimi et al., 2023). The reduction in daily sperm production in the testis culminated in low number of epididymal sperm (Yang et al., 2014; Abarikwu et al., 2015; Gely-Pernot et al., 2015; Kale et al., 2018; Rotimi et al., 2023). These gametes, in turn, exhibited low viability and motility when harvested from the cauda epididymis (Krzastek et al., 2021; Zhu et al., 2021; Rotimi et al., 2023). Alterations in the epididymal duct morphometry and epithelial integrity were also described in atrazine-exposed animals (Aziz et al., 2018; Rotimi et al., 2023). Altogether, these factors end up generating tissue damage in androgen-dependent organs (Kale et al., 2018; Ikeji et al., 2023; Rotimi et al., 2023).

5. Review limitations and recommendations

The bias analysis revealed that essential methodological characteristics, such as the blinding of participants (caregivers and outcome assessors), were frequently not reported across several studies. Additionally, heterogeneity in factors like the frequency of atrazine administration and exposure time necessitated the exclusion of some articles from the statistical analysis. To mitigate this heterogeneity, studies were grouped into more homogeneous subgroups based on atrazine dose classifications (e.g., doses lower than 100 mg kg^{-1} and doses higher than 100 mg kg^{-1}). Future research should focus on exposing peripubertal animals to atrazine and monitoring them into adulthood to determine if the effects observed during the peripubertal development phase persist. This will enhance the understanding of atrazine's role as an endocrine disruptor and its long-term impacts on male reproductive health.

6. Conclusion

This review evidenced the strong negative effect of atrazine in the HPG axis, affecting serum levels of FSH, LH, and testosterone due to a failure in their synthesis and secretion by the pituitary and testis of adult rats. The impairment of the HPG axis may involve oxidative stress and inflammation driven by atrazine toxicity, altering the hormone regulation control in males. Disruption of GnRH pulsative release reduces gonadotropin secretion and deregulate the steroidogenesis. This cascade of events compromises the testis functionality, causing irreversible damage to spermatozoa. Nevertheless, there is still no data in the literature that reveals the impairment of the fertility of atrazine-exposed animals. Moreover, atrazine exposure at high concentrations were the main cause of the endocrine disruption observed here, regardless of the exposure time. Unfortunately, the effect of environmental concentrations or relevant doses for

human health risk remains uncertain. The next step in understanding the effects of atrazine on the male reproductive system is the evaluation of parameters related to male fertility, data that is still missing in the literature.

7. References

Abarikwu, S.O., Akiri, O.F., Durojaiye, M.A., Adenike, A., 2015. Combined effects of repeated administration of Bretmont Wipeout (glyphosate) and Ultrazin (atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats. Toxicol Mech Methods 25, 70–80. https://doi.org/10.3109/15376516.2014.989349

Abarikwu, S.O., Costa, G.M.J., de Lima e Martins Lara, N., Lacerda, S.M.S.N., de França, L.R., 2021. Atrazine impairs testicular function in BalB/c mice by affecting Leydig cells. Toxicology 455, 152761. https://doi.org/10.1016/j.tox.2021.152761

Abarikwu, S.O., Duru, Q.C., C, R., A, N.B., Amadi, Tamunoibuomie, A., Keboh, E., 2017. Effects of co-exposure to atrazine and ethanol on the oxidative damage of kidney and liver in Wistar rats. Renal Failure, 39:1, 588-596.

Abarikwu, S.O., Pant, A.B., Farombi, E.O., 2012. The protective effects of quercetin on the cytotoxicity of atrazine on rat Sertoli-germ cell co-culture. Int J Androl 35, 590–600. https://doi.org/10.1111/j.1365-2605.2011.01239.x

Agdam, H.R., Razi, M., Amniattalab, A., Malekinejad, H., Molavi, M., 2017. Co-administration of Vitamin E and testosterone attenuates the atrazine-induced toxic effects on sperm quality and testes in rats. Cell J 19, 292–305. https://doi.org/10.22074/cellj.2016.490

Agency for Toxic Substances and Disease Registry (US), 2003. Toxicological Profile for Atrazine. Atlanta (GA) Sep. 3, HEALTH EFFECTS.

Almberg, K.S., Turyk, M.E., Jones, R.M., Rankin, K., Freels, S., Stayner, L.T., 2018. Atrazine contamination of drinking water and adverse birth outcomes in community water systems with elevated atrazine in Ohio, 2006–2008. Int J Environ Res Public Health 15, 12–15. https://doi.org/10.3390/ijerph15091889

Aziz, A.R.L., Abdel-Wahab, A., Abo El-Ela, F.I., Hassan, N.E.H.Y., El-Nahass, E.S., Ibrahim, M.A., Khalil, A.T.A.Y., 2018. Dose- dependent ameliorative effects of quercetin and L-Carnitine against atrazine- induced reproductive toxicity in adult male Albino rats. Biomedicine and Pharmacotherapy 102, 855–864. https://doi.org/10.1016/j.biopha.2018.03.136

Bhattacharya, I., Dey, S., Banerjee, A., 2023. Revisiting the gonadotropic regulation of mammalian spermatogenesis: evolving lessons during the past decade. Front. Endocrinol Apr 14;14:1110572. doi: 10.3389/fendo.2023.1110572.

Cahoreau, C., Klett, D., Combarnous, Y., 2015. Structure-function relationships of glycoprotein hormones and their subunits' ancestors. Front Endocrinol (Lausanne) 6, 1–14. https://doi.org/10.3389/fendo.2015.00026

Chang, J., Fang, W., Chen, L., Zhang, P., Zhang, G., Zhang, H., Liang, J., Wang, Q., Ma, W., 2022. Toxicological effects, environmental behaviors and remediation technologies of herbicide atrazine in soil and sediment: A comprehensive review. Chemosphere 307, 136006. https://doi.org/10.1016/j.chemosphere.2022.136006

Chávez-Pichardo, M.E., Reyes-Bravo, D.Y., Mendoza-Trejo, M.S., Marín-López, A.G., Giordano, M., Hernández-Chan, N., Domínguez-Marchan, K., Ortega-Rosales, L.C., Rodríguez, V.M., 2020. Brain alterations in GABA, glutamate and

glutamine markers after chronic atrazine exposure in the male albino rat. Arch Toxicol 94, 3217–3230. https://doi.org/10.1007/s00204-020-02806-2

Cohen, J., 1992. A power primer. Psychol. Bull., 112, pp. 155-159.

Cooper, R.L., Laws, S.C., Das, P.C., Narotsky, M.G., Goldman, J.M., Tyrey, E.L., Stoker, T.E., 2007. Atrazine and reproductive function: Mode and mechanism of action studies. Birth Defects Res B Dev Reprod Toxicol 80, 98–112. https://doi.org/10.1002/bdrb.20110

de Albuquerque, F.P., de Oliveira, J.L., Moschini-Carlos, V., Fraceto, L.F., 2020. An overview of the potential impacts of atrazine in aquatic environments: Perspectives for tailored solutions based on nanotechnology. Science of the Total Environment 700. https://doi.org/10.1016/j.scitotenv.2019.134868

De Caroli Vizioli, B., Silva da Silva, G., Ferreira de Medeiros, J., Montagner, C.C., 2023. Atrazine and its degradation products in drinking water source and supply: Risk assessment for environmental and human health in Campinas, Brazil. Chemosphere 336. https://doi.org/10.1016/j.chemosphere.2023.139289

Dooley, G.P., Tjalkens, R.B., Hanneman, W.H., 2013. The atrazine metabolite diaminochlorotriazine suppresses LH release from murine LβT2 cells by suppressing GnRH-induced intracellular calcium transients. Toxicol Res (Camb) 2, 180–186. https://doi.org/10.1039/c3tx20088d

Dou, R., Sun, J., Deng, F., Wang, P., Zhou, H., Wei, Z., Chen, M., He, Z., Lai, M., Ye, T., Zhu, L., 2020. Contamination of pyrethroids and atrazine in greenhouse and open-field agricultural soils in China. Science of the Total Environment 701, 134916. https://doi.org/10.1016/j.scitotenv.2019.134916

Eaton, D.L. and Gilbert, S.G., 2013. Principles of toxicology. C.D. Klaassen (Ed.), Casarett & Doull's Toxicology the Basic Science of Poisons (eighth ed.), McGraw Hill, New York, pp. 16-48

Edwards, H., Javed, K., Yadev, K., Ara, C., Omer, A.M., 2024. Therapeutic potential of salvigenin to combat atrazine induced liver toxicity in rats via regulating Nrf-2/Keap-1 and NF-κB pathway. Pestic Biochem Physiol. Jun;202:105966.

Feyzi-Dehkhargani, S., Shahrooz, R., Malekinejad, H., Sadrkhanloo, R.-A., 2012. Atrazine in sub-acute exposure results in sperm DNA disintegrity and nuclear immaturity in rats. Vet Res Forum 3, 19–26.

Foradori, C.D., Hinds, L.R., Hanneman, W.H., Legare, M.E., Clay, C.M., Handa, R.J., 2009. Atrazine inhibits pulsatile luteinizing hormone release without altering pituitary sensitivity to a gonadotropin-releasing hormone receptor agonist in female wistar rats. Biol Reprod 81, 40–45. https://doi.org/10.1095/biolreprod.108.075713

Foradori, C.D., Zimmerman, A.D., Hinds, L.R., Zuloaga, K.L., Breckenridge, C.B., Handa, R.J., 2013. Atrazine inhibits pulsatile gonadotropin-releasing hormone (GnRH) release without altering GnRH messenger RNA or protein levels in the female rat. Biol Reprod 88, 1–7. https://doi.org/10.1095/biolreprod.112.102277

Friedmann, A.S., 2002. Atrazine inhibition of testosterone production in rat males following peripubertal exposure. Reproductive Toxicology 16, 275–279. https://doi.org/10.1016/S0890-6238(02)00019-9

Gao, S., Wang, Z., Zhang, C., Jia, L., Zhang, Y., 2016. Oral exposure to atrazine induces oxidative stress and calcium homeostasis disruption in spleen of mice. Oxid Med Cell Longev 2016. https://doi.org/10.1155/2016/7978219

Gely-Pernot, A., Hao, C., Becker, E., Stuparevic, I., Kervarrec, C., Chalmel, F., Primig, M., Jégou, B., Smagulova, F., 2015. The epigenetic processes of meiosis in male mice are broadly affected by the widely used herbicide atrazine. BMC Genomics 16, 1–22. https://doi.org/10.1186/s12864-015-2095-y

Genovese, T., Siracusa, R., Fusco, R., D'amico, R., Impellizzeri, D., Peritore, A.F., Crupi, R., Gugliandolo, E., Morabito, R., Cuzzocrea, S., Salinaro, A.T., Cordaro, M., Di Paola, R., 2021. Atrazine inhalation causes neuroinflammation, apoptosis and accelerating brain aging. Int J Mol Sci 22. https://doi.org/10.3390/ijms22157938

Graceli, J.B., Dettogni, R.S., Merlo, E., Niño, O., da Costa, C.S., Zanol, J.F., Ríos Morris, E.A., Miranda-Alves, L., Denicol, A.C., 2020. The impact of endocrine-disrupting chemical exposure in the mammalian hypothalamic-pituitary axis. Mol Cell Endocrinol 518. https://doi.org/10.1016/j.mce.2020.110997

Griswold, M.D., 2018. 50 years of spermatogenesis: Sertoli cells and their interactions with germ cells. Biol Reprod 99, 87–100. https://doi.org/10.1093/biolre/ioy027

Guarnotta, V., Amodei, R., Frasca, F., Aversa, A., Giordano, C., 2022. impact of chemical endocrine disruptors and hormone modulators on the endocrine system. Int J Mol Sci 23. https://doi.org/10.3390/ijms23105710

Guidelines for drinking-water quality: fourth edition incorporating the first and second addenda. Geneva: World Health Organization; 2022

Holloway, A.C., Anger, D.A., Crankshaw, D.J., Wu, M., Foster, W.G., 2008. Atrazine-induced changes in aromatase activity in estrogen sensitive target tissues. Journal of Applied Toxicology 28, 260–270. https://doi.org/10.1002/jat.1275

Hooijmans, C.R., Rovers, M.M., de Vries, R.B., Leenaars, M., Ritskes-Hoitinga, M., Langendam, M.W., 2014. SYRCLE's risk of bias tool for animal studies. BMC Med Res Methodol 14, 43. https://doi.org/10.1186/1471-2288-14-43

Ikeji, C.N., Adedara, I.A., Farombi, E.O., 2023. Dietary myricetin assuages atrazine-mediated hypothalamic-pituitary–testicular axis dysfunction in rats. Environmental Science and Pollution Research 30, 15655–15670. https://doi.org/10.1007/s11356-022-23033-5

Jestadi, D.B., Phaniendra, A., Babji, U., Srinu, T., Shanmuganathan, B., Periyasamy, L., 2014. Effects of short-term exposure of atrazine on the liver and kidney of normal and diabetic rats. J Toxicol. 2014;2014:536759.

Jiang, F.W., Guo, J.Y., Lin, J., Zhu, S.Y., Dai, X.Y., Saleem, M.A.U., Zhao, Y., Li, J.L., 2024. MAPK/NF-κB signaling mediates atrazine-induced cardiorenal syndrome and antagonism of lycopene. Sci Total Environ. Apr 20;922:171015.

Jin, Y., Wang, L., Chen, G., Lin, X., Miao, W., Fu, Z., 2014. Exposure of mice to atrazine and its metabolite diaminochlorotriazine elicits oxidative stress and endocrine disruption. Environ Toxicol Pharmacol 37, 782–790. https://doi.org/10.1016/j.etap.2014.02.014

Jin, Y., Wang, L., Fu, Z., 2013. Oral exposure to atrazine modulates hormone synthesis and the transcription of steroidogenic genes in male peripubertal mice. Gen Comp Endocrinol 184, 120–127. https://doi.org/10.1016/j.ygcen.2013.01.010

Kale, O.E., Oyesola, T.O., Raji, F.S., 2018. Celecoxib, a cyclooxygenase-2 inhibitor, offers chemoprevention against reproductive and neurobehavioural abnormalities induced by atrazine in male Wistar rats. Environ Toxicol Pharmacol 58, 84–97. https://doi.org/10.1016/j.etap.2017.12.026

Kandori, H., Suzuki, S., Asamoto, M., Murasaki, T., Mingxi, T., Ogawa, K., Shirai, T., 2005. Influence of atrazine administration and reduction of calorie intake on prostate carcinogenesis in probasin/SV40 T antigen transgenic rats. Cancer Sci 96, 221–226. https://doi.org/10.1111/j.1349-7006.2005.00041.x

Kaprara, A., Huhtaniemi, I.T., 2017. The hypothalamus-pituitary-gonad axis: tales of mice and men. Metabolism Sep;86:3-17. doi: 10.1016/j.metabol.2017.11.018

Kimura, M., Ishii, M.N., Seki, N., Sakai, Y., Yamashita, T., Awatsuji, H., Kanda, K., Matsumoto, K., Matsui, H., 2019. Reduction of Kiss1 expression in the anteroventral periventricular nucleus is associated with atrazine-induced attenuation of the luteinizing hormone surge in female rats. Biol Reprod 100, 41–48. https://doi.org/10.1093/biolre/ioy159

Krzastek, S.C., Farhi, J., Gray, M., Smith, R.P., 2021. Impact of environmental toxin exposure on male fertility potential. Transl Androl Urol 9, 2797–2813. https://doi.org/10.21037/tau-20-685

Lawrence, B.M., O'Donnell, L., Smith, L.B., Rebourcet, D., 2022. New Insights into Testosterone Biosynthesis: Novel Observations from HSD17B3 Deficient Mice. Int J Mol Sci 23. https://doi.org/10.3390/ijms232415555

Li, J., Bi, H., 2023. Molecular mechanisms of atrazine toxicity on H19-7 hippocampal neurons revealed by integrated miRNA and mRNA "omics." Ecotoxicol Environ Saf 253, 114681. https://doi.org/10.1016/j.ecoenv.2023.114681

Li, L., Lin, W., Wang, Z., Huang, R., Xia, H., Li, Z., Deng, J., Ye, T., Huang, Y., Yang, Y., 2024. Hormone Regulation in Testicular Development and Function. International Journal of Molecular Sciences. 25(11):5805. https://doi.org/10.3390/ijms25115805

Lin, Z., Dodd, C.A., Filipov, N.M., 2013. Short-term atrazine exposure causes behavioral deficits and disrupts monoaminergic systems in male C57BL/6 mice. Neurotoxicol Teratol 39, 26–35. https://doi.org/10.1016/j.ntt.2013.06.002

Machado-Neves, M., 2022. Effect of heavy metals on epididymal morphology and function: An integrative review. Chemosphere 291. https://doi.org/10.1016/j.chemosphere.2021.133020

Maggi, R., Cariboni, A.M., Marelli, M.M., Moretti, R.M., Andrè, V., Marzagalli, M., Limonta, P., 2016. GnRH and GnRH receptors in the pathophysiology of the human female reproductive system. Hum Reprod Update 22, 358–381. https://doi.org/10.1093/humupd/dmv059

Martins-Santos, E., Pimenta, C.G., Campos, P.R.N., Franco, M.B., Gomes, D.A., Mahecha, G.A.B., Oliveira, C.A., 2017. Persistent testicular structural and functional alterations after exposure of adult rats to atrazine. Reproductive Toxicology 73, 201–213. https://doi.org/10.1016/j.reprotox.2017.08.010

Martins-Santos, E., Pimenta, C.G., Campos, P.R.N., Oliveira, A.G., Mahecha, G.A.B., Oliveira, C.A., 2018. Atrazine affects the morphophysiology, tissue homeostasis and aromatase expression in the efferent ductules of adult rats with mild alterations in the ventral prostate. Chemosphere 193, 958–967. https://doi.org/10.1016/j.chemosphere.2017.11.124

Mattos, K., Viger, R.S., Tremblay, J.J., 2022. Transcription Factors in the Regulation of Leydig Cell Gene Expression and Function. Front. Endocrinol. https://doi.org/10.3389/fendo.2022.881309

Mgbudom-Okah, C.J., Abarikwu, S.O., Wegwu, M.O., Henkel, R., 2023. Testosterone restores TM3 and TM4 cell viability, reduces reactive oxygen species generation, and protects against atrazine-induced stereological changes in rat testes. J Biochem Mol Toxicol. https://doi.org/10.1002/jbt.23562

Mohammed, E.T., Safwat, G.M., Bahnasawy, E.A., Abdel-Razik, A.H., Mohamed, D.S., 2023. Zinc Oxide Nanoparticles and Vitamin C Ameliorate Atrazine-Induced

Hepatic Apoptosis in Rat via CYP450s/ROS Pathway and Immunomodulation. Biol Trace Elem Res. Nov;201(11):5257-5271.

Namwanje, M., Brown, C.W., 2016. Activins and inhibins: Roles in development, physiology, and disease. Cold Spring Harb Perspect Biol 8, 1–55. https://doi.org/10.1101/cshperspect.a021881

Ndufeiya-Kumasi, L.C., Abarikwu, S.O., Ohanador, R., Omoregie, E.S., 2022. Curcumin improves the protective effects of quercetin against atrazine-induced testicular injury in adult Wistar rats. Andrologia 54, 1–12. https://doi.org/10.1111/and.14445

Page, M.J., McKenzie, J.E., Bossuyt, P.M., Boutron, I., Hoffmann, T.C., Mulrow, C.D., Shamseer, L., Tetzlaff, J.M., Akl, E.A., Brennan, S.E., Chou, R., Glanville, J., Grimshaw, J.M., Hróbjartsson, A., Lalu, M.M., Li, T., Loder, E.W., Mayo-Wilson, E., McDonald, S., McGuinness, L.A., Stewart, L.A., Thomas, J., Tricco, A.C., Welch, V.A., Whiting, P., Moher, D., 2021. The PRISMA 2020 Statement: An Updated Guideline for Reporting Systematic Reviews. BMJ, 89, 11.

Piazza, M.J., Urbanetz, A.A., 2019. Environmental toxins and the impact of other endocrine disrupting chemicals in women's reproductive health. J Bras Reprod Assist 23, 154–164. https://doi.org/10.5935/1518-0557.20190016

Picut, C.A., Ziejewski, M.K., Stanislaus, D., 2017. Comparative Aspects of Pre- and Postnatal Development of the Male Reproductive System. Birth Defects Res 15;110(3):190-227. doi: 10.1002/bdr2.1133

Pitteloud, N., Dwyer, A.A., DeCruz, S., Lee, H., Boepple, P.A., Crowley, W.F., Hayes, F.J., 2008. Inhibition of luteinizing hormone secretion by testosterone in men requires aromatization for its pituitary but not its hypothalamic effects: Evidence from the tandem study of normal and gonadotropin-releasing hormone-deficient

men. Journal of Clinical Endocrinology and Metabolism 93, 784–791. https://doi.org/10.1210/jc.2007-2156

Pogrmic, K., Fa, S., Dakic, V., Kaisarevic, S., Kovacevic, R., 2009. Atrazine oral exposure of peripubertal male rats downregulates steroidogenesis gene expression in Leydig cells. Toxicological Sciences 111, 189–197. https://doi.org/10.1093/toxsci/kfp135

Pogrmic-Majkic, K., Fa, S., Samardzija, D., Hrubik, J., Kaisarevic, S., Andric, N., 2016. Atrazine activates multiple signaling pathways enhancing the rapid hCG-induced androgenesis in rat Leydig cells. Toxicology 368–369, 37–45. https://doi.org/10.1016/j.tox.2016.08.016

Rajkovic, V., Kovac, R., Koledin, I., Matavulj, M., 2014. Atrazine-induced changes in the myocardial structure of peripubertal rats. Toxicol Ind Health. Apr;30(3):250-8.

Rochira, V., Zirilli, L., Genazzani, A.D., Balestrieri, A., Aranda, C., Fabre, B., Antunez, P., Diazzi, C., Carani, C., Maffei, L., 2006. Hypothalamic-pituitary-gonadal axis in two men with aromatase deficiency: Evidence that circulating estrogens are required at the hypothalamic level for the integrity of gonadotropin negative feedback. Eur J Endocrinol 155, 513–522. https://doi.org/10.1530/eje.1.02254

Rosenberg, M.S., Adams, D.C., Gurevitch, J., 2000. MetaWin: Statistical Software for Meta-Analysis Version 2.0Sinauer Associates, Sunderland.

Rosenthal, R., Kleid, J.J., Cohen, M. V., 1979. Abnormal mitral valve motion associated with ventricular septal defect following acute myocardial infarction. Am Heart J 98, 638–641. https://doi.org/10.1016/0002-8703(79)90291-6

Rotimi, D.E., Ojo, O.A., Adeyemi, O.S., 2023. Atrazine exposure caused oxidative stress in male rats and inhibited brain-pituitary-testicular functions. J Biochem Mol Toxicol 1–11. https://doi.org/10.1002/jbt.23579

Sabzevari, S., Hofman, J., 2022. A worldwide review of currently used pesticides' monitoring in agricultural soils. Science of the Total Environment 812, 152344. https://doi.org/10.1016/j.scitotenv.2021.152344

Senger, P.L., 2012. Pathways to pregnancy & parturition. 3rd edition. Redmond, OR: Current Conceptions.

Shen, W.-J., Azhar, S., Kraemer, F.B., 2018. SR-B1: A Unique Multifunctional Receptor for Cholesterol Influx and Efflux. Annu Rev Physiol 80, 95–116. https://doi.org/10.1146/annurev-physiol-021317-121550

Shimabukuro, R.H., Swanson, H.R., Walsch, W.C., 1970. Glutathione conjugation: Atrazine detoxication mechanism in corn Plant Physiol., 46, pp. 103-107

Singh, S., Rawat, M., Malyan, S.K., Singh, R., Tyagi, V.K., Singh, K., Kashyap, S., Kumar, S., Sharma, M., Panday, B.K., Pandey, R.P., 2023. Global distribution of pesticides in freshwater resources and their remediation approaches. Environ Res 225, 115605. https://doi.org/10.1016/j.envres.2023.115605

Stoker, T.E., Laws, S.C., Guidici, D.L., Cooper, R.L., 2000. The effect of atrazine on puberty in male Wistar rats: An evaluation in the protocol for the assessment of pubertal development and thyroid function. Toxicological Sciences 58, 50–59. https://doi.org/10.1093/toxsci/58.1.50

Stradtman, S.C., Freeman, J.L., 2021. Mechanisms of neurotoxicity associated with exposure to the herbicide atrazine. Toxics 9. https://doi.org/10.3390/toxics9090207

Sun, J.T., Pan, L.L., Zhan, Y., Tsang, D.C.W., Zhu, L.Z., Li, X.D., 2017. Atrazine contamination in agricultural soils from the Yangtze River Delta of China and associated health risks. Environ Geochem Health 39, 369–378. https://doi.org/10.1007/s10653-016-9853-x

Szewczyk, R., Różalska, S., Mironenka, J., Bernat, P., 2020. Atrazine biodegradation by mycoinsecticide Metarhizium robertsii: Insights into its amino acids and lipids profile. J Environ Manage 262. https://doi.org/10.1016/j.jenvman.2020.110304

Tennekes, H.A., Sánchez-Bayo, F., 2013. The molecular basis of simple relationships between exposure concentration and toxic effects with time. Toxicology 309, 39–51. https://doi.org/10.1016/j.tox.2013.04.007

Timchalk, C., Dryzga, M.D., Langvardt, P.W., Kastl, P.E., Osborne, D.W., 1990. Determination of the effect of tridiphane on the pharmacokinetics of [14C]-atrazine following oral administration to male Fischer 344 rats. Toxicology. Mar 30;61(1):27-40. doi: 10.1016/0300-483x(90)90004-z.

Trentacoste, S. V., Friedmann, A.S., Youker, R.T., Breckenridge, C.B., Zirkin, B.R., 2001. Atrazine effects on testosterone levels and androgen-dependent reproductive organs in peripubertal male rats. J Androl 22, 142–148. https://doi.org/10.1002/j.1939-4640.2001.tb02164.x

Tsutsumi, R., Webster, N.J.G., 2009. GnRH pulsatility, the pituitary response and reproductive dysfunction. Endocr J 56, 729–737. https://doi.org/10.1507/endocrj.K09E-185

Tudi, M., Ruan, H.D., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., 2021. Agriculture Development, Pesticide Application and Its Impact on the Environment. Environmental Research and public health 18, 1–23.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W. V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev 33, 378–455. https://doi.org/10.1210/er.2011-1050

Victor-Costa, A.B., Bandeira, S.M.C., Oliveira, A.G., Mahecha, G.A.B., Oliveira, C.A., 2010. Changes in testicular morphology and steroidogenesis in adult rats exposed to Atrazine. Reproductive Toxicology 29, 323–331. https://doi.org/10.1016/j.reprotox.2009.12.006

Wang, Y., Chen, F., Ye, L., Zirkin, B., Chen, H., 2017. Steroidogenesis in leydig cells: Effects of aging and environmental factors. Reproduction 154, R111–R122. https://doi.org/10.1530/REP-17-0064

Wirbisky, S.E., Freeman, J.L., 2015. Atrazine exposure and reproductive dysfunction through the Hypothalamus-Pituitary-Gonadal (HPG) axis. Toxics 3, 414–450. https://doi.org/10.3390/toxics3040414

Xia, J., Lin, J., Li, X.N., Zhang, C., Li, N., Du, Z.H., Li, Y.H., Li, J.L., 2018. Atrazine-induced environmental nephrosis was mitigated by lycopene via modulating nuclear xenobiotic receptors-mediated response. J Nutr Biochem. Jan;51:80-90.

Yang, S., Jia, Z.C., Chen, J.Y., Hu, J.X., Zhang, L.S., 2014. Toxic effects of atrazine on reproductive system of male rats. Biomedical and Environmental Sciences 27, 281–288. https://doi.org/10.3967/bes2014.050

Zhao, X., Luo, H., Yao, S., Yang, T., Fu, F., Yue, M., Ruan, H., 2024. Atrazine exposure promotes cardiomyocyte pyroptosis to exacerbate cardiotoxicity by activating NF-κB pathway. Sci Total Environ. Mar 10;915:170028.

Zhu, S., Zhang, T., Wang, Y., Zhou, X., Wang, S., Wang, Z., 2021. Meta-analysis and experimental validation identified atrazine as a toxicant in the male reproductive system. Environmental Science and Pollution Research 28, 37482–37497. https://doi.org/10.1007/s11356-021-13396-6

8. Appendices

Supplementary Table 1. Search strategy for database search of the systematic and meta-analysis review.

Data base	Descriptors/Filters	Items found	Time	Date
PUBMED	#1. ATRAZINE (atrazine[Title/Abstract] OR ATR[Title/Abstract] OR triazines[Title/Abstract])	22.533	13h52	10/27/2023
	#2. HORMONE (testosterone[Title/Abstract] OR estradiol[Title/Abstract] OR progesterone[Title/Abstract] OR "hormone luteinizing"[Title/Abstract] OR "follicle-stimulating hormone"[Title/Abstract])	242.643	13h54	10/27/2023
	#1 AND #2	253	13h59	10/27/2023
Embase	#1. ATRAZINE atrazine:ab,ti OR atr:ab,ti OR triazines:ab,ti	26.889	14h30	10/27/2023
	#2. HORMONE testosterone:ab,ti OR estradiol:ab,ti OR progesterone:ab,ti OR 'hormone luteinizing':ab,ti OR 'follicle-stimulating hormone':ab,ti	297.915	14h31	10/27/2023
	#1 AND #2	292	14h30	10/27/2023
Web of Science	#1. ATRAZINE TS=(atrazine OR ATR OR triazines)	70.428	14h25	10/27/2023
	#2. HORMONE TS=(testosterone OR estradiol OR progesterone OR "hormone luteinizing" OR "follicle-stimulating hormone")	278.421	14h26	10/27/2023
	#1 AND #2	388	14h28	10/27/2023

Supplementary Table 2. Characteristics of experimental studies evaluating the effect of atrazine on hormonal parameters in male animals.

	Article	Country	Experimental design	Animal model	Age (days)	N of animals	Animals per group	Control + treated groups (n of Atrazine group)
1	Abarikwu et al., 2015	Nigeria	TOX	Wistar rats	21-28	20	5	1 + 3 (1)
2	Abarikwu et al., 2020#	Nigeria	THERAPY	Wistar rats	Adult	30	5	1 + 3 (1)
3	Abarikwu et al., 2021	Nigeria	TOX	BALB/c mice	42	40	10	1 + 3 (3)
4	Adedara et al., 2021#	Nigeria	TOX	Wistar rats	70	60	10	1 + 1 (5)
5	Agdam et al., 2017	Iran	THERAPY	Wistar rats	56	30	6	1 + 4 (1)
6	Aziz et al., 2018	Egypt	THERAPY	Wistar rats	90-120	36	6	1 + 5 (1)
7	Feyzi-Dehkhargani et al., 2012	Iran	TOX	Wistar rats	56	72	6	1 + 3 (3)
8	Foradori et al., 2017	USA	TOX	Sprague-Dawley rats	70	40	10	1 + 3 (3)
9	Friedmann et al., 2002*	USA	TOX	Sprague-Dawley rats	22	11	5-6	1 + 1 (1)
10	Gely-Pernot et al., 2015#	France	TOX	C57BL/6 mice	35	-	-	1 + 1 (1)
11	Ikeji et al., 2023	Nigeria	THERAPY	Wistar rats	84	48	8	1 + 5 (1)
12	Jin et al., 2013*	China	TOX	ICR mice	21	28	7	1 + 3 (1)
13	Jin et al., 2014#	China	TOX	ICR mice	42	30	6	1 + 4 (2)
14	Kale et al., 2018	Nigeria	THERAPY	Wistar rats	-	30	6	1 + 4 (1)
15	Kandori et al., 2005	Japan	TOX	Sprague-Dawley rats	42	40	10	1 + 3 (2)
16	Laws et al., 2009#	USA	TOX	Wistar rats	50-60	-	-	1 + 4 (4)
17	Ndufeiya-Kumasi et al., 2022	Nigeria	THERAPY	Wistar rats	Adult	35	5	1 + 6 (1)
18	Pogrmic et al., 2009*	Serbia	TOX	Wistar rats	22	-	-	1 + 2 (2)
19	Riffle et al., 2013	USA	TOX	Wistar rats	73	20	10	1 + 1 (1)
20	Riffle et al., 2014	USA	TOX	Wistar rats	71	50	10	1 + 4 (4)

21	Rotimi et al., 2023	Nigeria	TOX	Wistar rats	-	12	6	1 + 1 (1)
22	Stoker et al., 2000*	USA	TOX	Wistar rats	22	-	-	1 + 8 (8)
23	Trentacoste et al., 2001*	USA	TOX	Sprague-Dawley rats	21	90-100	9-12	1 + 8 (8)
24	Victor-Costa et al., 2010	Brazil	TOX	Wistar rats	120	-	-	1 + 3 (3)
25	Yang et al., 2014	China	TOX	Sprague-Dawley rats	28	40	10	1 + 3 (3)

[#]Studies not included in the meta-analysis. [*]Studies performed in peripubertal animals. TOX: Toxicological effects of atrazine; THERAPY: Therapeutic substances tested against atrazine effects.

Supplementary Table 3. Fail-safe numbers of atrazine meta-analyses.

Variable	d_{++}	Heterogeneity	df	95% CI	Rosenthal´s Method
FSH	-7.1571	0.000	16	-9.6061 to -4.7082	4,189
LH	-1.7460	0.0000	37	-2.3633 to -1.1287	1,6751
Serum testosterone	-1.5068	0.0000	52	-1.9889 to -1.0246	1,121
Intratesticular testosterone	-1.1972	0.0969	14	-2.0901 to -0.3042	36.9
Estradiol	1.0356	0.2774	13	0.2535 to 1.8176	32.3
Progesterone	1.0363	0.8577	4	0.4430 to 1.6297	38.9

FSH: follicle stimulating hormone; LH: luteinizing hormone; d_{++}: cumulative effect; df: n − 1 degree of freedom, CI: confidence interval.

CAPÍTULO 3

Atrazine/mesotrione-based herbicide subchronic testicular toxicity: oxidative/nitrosative stress, DNA damage, and fertility decline in male Wistar rats

Abstract

The Calaris® herbicide, a combination of atrazine and mesotrione, holds promise for large-scale production, considering cost/benefit. Data regarding the toxicology involved in mixing the herbicides atrazine and mesotrione are lacking in mammals. Thus, we evaluated subchronic exposure, for 80 days, to two concentrations of the herbicide (2 and 20 mg Kg^{-1}) in the hypothalamic-testicular axis of male Wistar rats, considering the redox balance and fertility of these animals after exposure to the agrochemical. We highlight the toxic impact of Calaris® on testicular and epididymal parameters, including oxidative and nitrosative stress, mineral imbalance, and DNA damage in testicular cells. These changes result in decreased levels of follicle-stimulating hormone, remodeling of the testicular parenchyma, sperm mitochondrial dysfunction, and a general reduction in fertility rates. Despite maintaining normal testosterone levels, exposure to 20 mg/Kg^{-1} of Calaris® for 80 days increased preimplantation losses and reduced anogenital distance in fetuses, indicating compromised reproductive efficiency and possible epigenetic problems. We emphasize the need for additional toxicological evaluations of herbicide mixtures such as Calaris®, given their increasing agricultural use and potential health risks.

Keywords: Calaris, testis, epididimys, sperm quality, endocrine disruption.

1. Introduction

Animals and humans are frequently exposed to environmental contaminants from natural and anthropogenic sources (Năstăsescu et al., 2020; Smith et al., 2021; Giglio and Vommaro, 2022). Pollutants pose a significant threat to human health because they may develop several diseases and cancers, exacerbate pre-existing conditions, and reduce male and female fertility (Akinola et al., 2016; Zhang et al., 2019; Guvvala et al., 2020; Costas-Ferreira et al., 2022; Mendaš et al., 2023). Exposure to these agents can occur through contact with air, soil, and water, besides consuming animal and plant-derived products (Mostafalou and Abdollahi, 2017; Năstăsescu et al., 2020). Agricultural pesticides stand out among toxic agents as they are directly related to agriculture and large-scale food production. For instance, herbicides prevent crop losses due to weeds (Gianessi, 2013). The application of herbicides is a simple and effective technology to control crop losses, reduce production costs, and increase food yields. However, the effects of these substances are not limited to target organisms alone (Mostafalou and Abdollahi, 2017; Van Bruggen et al., 2018). The imprudent use of herbicides may affect animal and human health due to the compounds' characteristics, such as chemical instability, incorporation ability into the cellular environment, and endocrine disruption activity (Gore et al., 2015; Ikeji et al., 2023).

The development of new formulations and the updating of commercial products have been the focus currently (Qu et al., 2021). Among the combinations recently launched in the market, Calaris® herbicide stands out, being a mixture of atrazine and mesotrione in a ratio of 10:1. Specifically, this new product comprises a mix of the active ingredient's atrazine (500 g L^{-1}), mesotrione (50 g L^{-1}), and formulation adjuvants (597 g L^{-1}), formulated as a concentrated suspension (Syngenta, São Paulo, SP, Brazil). This combination presents an attractive commercial potential for being more effective in

combating weeds and grasses than the separate use of these two base compounds. Atrazine (6-chloro-N_2-ethyl-N_4-isopropyl-1,3,5-triazine-2,4-diamine) is a broad-spectrum herbicide belonging to the triazine family and is the second most used in Brazil in crops such as corn, sorghum, sugarcane, cotton, and pineapple (Kumar et al., 2013; Solomon et al., 2013; Ikeji et al., 2023). Mesotrione [2-(4-mesyl-2-nitrobenzoyl) cyclohexane-1,3-dione], in turn, is a selective herbicide commonly used to control broadleaf weeds in crops such as corn, sorghum, cotton, and potatoes.

In the context of animal reproduction, the published studies have highlighted the role of atrazine as an endocrine disruptor capable of affecting the hypothalamic-pituitary-gonadal axis (Victor-Costa et al., 2010; Gely-Pernot et al., 2015; Stradtman and Freeman, 2021; Rotimi et al., 2023). Such effect directly disrupts the functionality of male reproductive organs, including the testis and epididymis, negatively impacting sperm parameters (Cai et al., 2017). Nonetheless, few studies have assessed the toxic effect of mesotrione in fauna. To date, studies have shown damage caused by mesotrione on oxidative and cellular parameters in nematodes (Zhang et al., 2019; Moin et al., 2023). Changes in biochemical and oxidative parameters with consequent DNA damage were also observed in *in vitro* studies using hepatic cell lines and *in vivo* studies analyzing the liver, gills, and muscles of fishes (Piancini et al., 2015; Mesnage et al., 2018; Wang et al., 2018).

Given the challenge of assessing the risks posed by toxic combinations of herbicide mixtures, experimental studies become crucial in understanding the processes by which these substances can cause endocrine and reproductive disturbances, as well as their consequences for male fertility. Therefore, we hypothesized that Calaris® affects the fertile potential of males. Here, we aimed to evaluate the effects of exposure to Calaris®, at two concentrations, on relevant parameters for male reproduction. To this end, we

evaluated the fertile potential of adult Wistar rats and the consequences to the litter. Histological, hormonal, oxidative, functional, and reproductive parameters were evaluated in the hypothalamus, testis, epididymis, and sperm of these animals.

2. Materials and methods

2.1 Animals and ethics statement

Thirty male Wistar rats (70 days old; 200 ± 50g) and 24 female Wistar rats (70 days old; 150 ± 50g) were provided by the Central Animal Facility of the Center of Biological and Health Sciences of the Universidade Federal de Viçosa (UFV). The animals were housed in individual polypropylene cages and maintained under controlled conditions of temperature (21 °C) and photoperiod (12 h light/dark cycle). The animals had free access to activated carbon-filtered water and commercial rat chow (Nuvilab CR-1; Quimtia S.A; Paraná, Brazil). This study strictly followed the recommendations of the National Council for the Control of Animal Experimentation (CONCEA). All experimental procedures were reviewed and approved by the Ethics Committee of Animal Use of the UFV (CEUA process number 32/2023).

2.2 Experimental design

After ten days of acclimatization, the animals were randomly divided into three experimental groups (n = 10/ group). Animals from control group received 0.5 mL of distilled water, while rats from Calaris® groups received, respectively, 2 and 20 mg Kg^{-1} of the herbicide. The Calaris® solutions were prepared after dilution in saline solution (140 mM NaCl; pH 7.4), administering 0.5 mL volume through gavage daily for 80 days. The doses administered correspond to 1,000 and 100/LD50 of Calaris® (2,000 mg Kg^{-1}). The exposure period of 80 days corresponded to one cycle of the seminiferous epithelium

in rats (56 days) plus the time of epididymal sperm transit (approximately 10 days), and the analysis of fertility indexes. In this context, male rats were placed with healthy females to occur the natural mating for 72 hours starting on the 70th day of experiment. Afterward, the animals were separated and maintained for another seven days to recover the sperm reservoir in the cauda epididymis, totaling 80 days of experiment receiving Calaris® solution.

2.3 Euthanasia, organ collection, and biometry

On day 80 of experimentation, the males were weighed and fasted for 10 hours. Subsequently, the animals were euthanized using high doses of xylazine (30 mg Kg^{-1} intraperitoneal) and ketamine hydrochloride (300 mg Kg^{-1} intraperitoneal) following cardiac puncture. The latter procedure was performed to obtain blood samples, which were centrifuged for 15 min at 700 x g to recover the serum for hormonal measurement. The hypothalamus, testis, and epididymis were removed, dissected, weighed, and preserved in fixative or frozen nitrogen. The final body weight and the absolute weight of the reproductive organs were measured and further used to obtain the relative weight of male organs by normalizing their weights with the body weight.

2.4 Serum pituitary and intratesticular hormone concentration

The serum obtained was evaluated for the concentration of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) using commercial kits according to the manufacturer's instructions. Intratesticular testosterone levels were also determined using immunosorbent assay (ELISA) kits (Coimbra et al., 2023).

2.5 Antioxidant enzyme activity in the hypothalamus, testis, and epididymis

Fragments of the hypothalamus, testis, and proximal (initial segment, caput and corpus) and distal (cauda) portions of the epididymis were frozen in liquid nitrogen and stored at -80°C until processing for analysis of antioxidant enzyme activity and oxidative stress markers. Superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities were performed using the supernatant obtained after homogenizing 100 mg of frozen organs (using the Tissue Master 125 homogenizer, OMNI) in phosphate buffer (pH 7.4) and centrifuged for 10 minutes at 10,000 xg (12,000 rpm), under refrigeration at 4 °C. SOD activity was evaluated by the pyrogallol method, based on the enzyme's ability to catalyze the reaction of superoxide and hydrogen peroxide (Dieterich et al., 2000). Also, CAT activity was assessed by measuring the rate of hydrogen peroxide decomposition, following the method described by Aebi (1984). The activity of GST was estimated at 340 nm, at two intervals (the 30s and 90s), as described by Habig et al. (1974) and calculated based on the rate of formation of the 1-chloro-2,4-dinitrobenzene (CDNB) conjugate with glutathione. The molar extinction coefficient used for CDNB is $\varepsilon 340 = 9.6 mmol \times L^{-1} \times cm^{-1}$. Plate readings were performed using an ELISA microplate reader (Multiskan SkyHigh, Thermo Scientific) at 320nm. The results are expressed in units (U) per milligram of protein. Total protein quantification was performed using the Bradford method (Bradford, 1976), and the result was used to normalize the enzyme activity values. The determination of antioxidant activity was measured in the testis and epididymis by the FRAP methodology (Ferric ion reducing antioxidant power) (Benzie & Strain, 1996).

2.6 Oxidative stress markers in the hypothalamus, testis, and epididymis

The production of nitric oxide (NO) was determined using the Griess reaction, as described by Tsikas (2007). 50 µL of samples were incubated with Griess reagent (1:1) (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine, and 2.5% phosphoric acid) at room temperature for 10 min. Absorbance was measured at 570 nm using a microplate reader. NO concentrations (µmol/L) were determined from a standard curve of sodium nitrite (0–125 µM). For malondialdehyde (MDA) analyses, the supernatant was added to the TBARS solution (15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25N HCl) for 15 min. The formation of thiobarbituric acid reactive substances was monitored at 535 nm as described by Buege and Aust (1978). The concentration of protein carbonyls (PC) was measured by adding 0.5 mL of dinitrophenylhydrazine solution to the resulting homogenate pellets. Protein damage was determined in the final supernatant, based on the carbonyl groups reacting with DNPH, and measured spectrophotometrically at 370 nm (Levine et al., 1994). The analyses were performed using an ELISA microplate reader (Multiskan SkyHigh, Thermo Scientific).

2.7 Proportion of trace elements in the hypothalamus, testis, and epididymis

Modifications in the proportion of trace elements were investigated by energy-dispersive x-ray spectroscopy (EDS) using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with a coupled x-ray detector system (Tracor TN5502, Middleton, WI, USA). Frozen fragments of the hypothalamus, testis, and proximal and distal regions of the epididymis were dehydrated in ethanol, subjected to critical point drying, and coated with a carbon film. The elements analyzed were selenium (Se), sodium (Na), potassium (K), calcium (Ca), zinc (Zn), magnesium (Mg), iron (Fe),

copper (Cu), and manganese (Mn) (Couto-Santos et al., 2024). The results were expressed as a percentage.

2.8 Total, Mg^{2+}, Ca^{2+}, and Na^{+}/K^{+} ATPases activity in the testis

Frozen testis fragments (100mg) were homogenized in Tris-HCl buffer (0.1 M; pH 7.4) and centrifuged at 1,500 x g for 10 min (4°C). The supernatant was used for the determination of total ATPase (Evans, 1969), Ca^{2+} (Hjertén and Pan, 1983), Na^{+}/K^{+} (Bonting et al., 1962), and Mg^{2+} (Ohnishi et al., 1982). The pellet was used to quantify the level of total proteins by the Bradford method (Bradford 1976). ATPase activity was expressed in micrograms of released phosphorus per minute per milligram of protein (Carvalho et al., 2022).

2.9 Histopathology in the hypothalamus, testis, and epididymis

Hypothalamus, testis, and epididymis were fixed for 24 h in Karnovsky solution. Fragments from all organs were then dehydrated in increasing series of ethanol (70, 80, 90, and absolute) and immersed in historesin (Historesin®, Leica Microsystems, Nussloch, Germany). Three-µm sections were obtained using a rotating microtome (RM 2255, Leica Biosystems, Nussloch, Germany). The sections were evaluated in semi-series to avoid analyzing the same histological area, using one in every 8 sections for the hypothalamus and epididymis and one in every 13 sections for the testis. The sections were stained in toluidine blue - 1% sodium borate solution and with hematoxylin and eosin, and analyzed using an optical microscope (Olympus CX40, Tokyo, Japan) (Guimarães-Ervilha et al., 2021).

The histopathological evaluation of the organs was performed in a blind trial. The histological structure of the hypothalamus was qualitatively evaluated regarding its

tissue integrity. Nerve cells were assessed based on their cytoplasmic and nuclear characteristics. Moreover, 200 seminiferous tubules per animal were analyzed in non-serial testicular sections considering tubular (lumen and seminiferous epithelium containing Sertoli and germ cells) and intertubular components (Leydig cells, connective tissue, and blood and lymphatic vessels). Seminiferous tubules exhibiting any change in their structure, such as cellular vacuolization, desquamation, displacement, multinucleated cells, and luminal cell debris, were classified as abnormal (Guimarães-Ervilha et al., 2021; Souza et al., 2023). The epididymal regions, in turn, were qualitatively analyzed according to the integrity of the duct structure, including epithelial morphology, luminal content, and interstitial structure. The presence of germ cells in the lumen, vacuoles in the epididymal epithelium, and foci of inflammation in the interstitium were considered histological alterations (Kempinas & Klinefelter, 2014).

2.10 Testicular histomorphometry

For morphometric analyses, digital images of the testis were obtained using a photomicroscope (Olympus BX53, Tokyo, Japan) and analyzed using Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD). The diameter of the seminiferous tubule (μm) was obtained by averaging the measurement of 30 transverse sections of seminiferous tubules, with the contour as circular as possible, per animal. The diameter comprises a straight line between the two poles of the tubule, starting from the tunica propria at one end, passing through the center of the tubule, and ending with the tunica propria at the other end. Using the same cross-sections of seminiferous tubules, the height of the seminiferous epithelium (μm) was measured. A straight line was drawn in the center of the section, starting at the tunica propria and reaching the lumen of the tubule. The value found corresponded to the average of two diametrically opposite measurements. Using the tubular diameter and the height of the seminiferous epithelium, it was possible to

calculate the diameter of the lumen of the seminiferous tubule (μm), subtracting the diameter of the tubule from 2* the height of the epithelium (Souza et al., 2016; Couto-Santos et al., 2020).

The volumetric proportion of the components of the testicular parenchyma was determined using a grid, with 266 intersections, on 10 digital images of the testicular parenchyma per animal, totaling 2,660 points/animal. Points were marked on the seminiferous tubules, differentiating points on the lumen, the seminiferous epithelium, the tunica propria, and the intertubule. The volumetric proportion of these components (PVx) was determined by the formula: PVx (%) = (NT / NTT) * 100; where x = component in question, NT = number of points on the specific component and NTT is the number of total points (2,660) (Guimarães-Ervilha et al., 2023). To calculate the volumetric proportion of the intertubule components, 1,000 points were counted on the intertubular compartment, per animal. Points on Leydig cells, differentiating points on the cytoplasm and nucleus of Leydig, macrophages, blood vessels, connective tissue, and lymphatic space were counted. The same formula described in determining the volumetric proportion of the testicular parenchyma components was used to calculate the proportions of the intertubule components. Leydig nucleus diameter (μm) was determined by averaging 30 Leydig cell nuclei per animal (Guimarães-Ervilha et al., 2023).

2.11 Genotoxic assays in the testis

Two genotoxic methodologies were examined in the testis, the comet assay and flow cytometry. To perform the comet assay, cryopreserved testicular samples were individually processed to generate cell suspensions containing free nuclei. Testicular fragments were macerated using a pestle in a 1.5 mL microtube containing 300 μL of PBS solution. Following maceration, the suspension underwent a 3-min incubation at room

temperature before being filtered through membranes with pore sizes of 50 µm, 30 µm, and 20 µm. Cleaned slides were pre-coated with a layer of 1% normal melting point agarose gel at 50°C for 5 seconds and left to polymerize in a clean, ventilated area for 24 h. Subsequently, 40 µL of the cell suspension was mixed with 120 µL of 1% low melting point agarose at 37°C and placed onto the pre-coated agarose slide. After covering the slide with a microscopy coverslip (24 x 40 mm), it was cooled in a refrigeration system at 4°C for 20 min. Following this, the coverslip was removed, and the slides were incubated in a lysis solution.

The slides were then subjected to electrophoresis in an alkaline solution (250 mM Trizma, 75 mM NaOH, and 10 mM EDTA, with pH = 13) at 4°C for 20 minutes. Electrophoresis was carried out for 20 minutes at a voltage of 18 V.cm -1 with an amperage of 150 mA. Subsequent steps involved immersion in a neutralization solution (400 mM Tris-HCl, pH = 7.5) for 15 minutes at 4°C, followed by dehydration in 95% ethanol for 5 minutes. Staining was performed with 100 µL of 50 µM acridine orange in darkness for 15 minutes, followed by immersion in distilled water at 4°C for 2 minutes. Finally, the slides were examined under an Olympus BX-60 fluorescence microscope equipped with a stabilized light source. Images of the nucleoids were captured using an Olympus DP-71 camera with a 20x MPlanApo objective. Analysis of the images involved categorizing the nucleoids as either intact (showing no DNA damage) or exhibiting comet tails (indicating DNA damage) (Collins et al. 1995).

The flow cytometry was performed in testis fragments (100 mg) frozen and triturated in microtubes containing 100 µL of OTTO I nuclear extraction buffer. Following maceration, the cell suspension was incubated for 5 minutes at room temperature and supplemented with 1000 µL of OTTO I buffer (in two 500 µL additions) before being filtered through a 30 µm membrane. The microtubes were then centrifuged

at 100 x g for 5 min, and the supernatant was carefully removed. The precipitated material was resuspended in 100 µL of OTTO I buffer using a vortex mixer and incubated for 5 min at room temperature. Following this step, the cell suspension was stained with 500 µL of OTTO II buffer (containing 75 µM propidium iodide and 50 µg mL^{-1} of RNAse pH 7.8) for 30 minutes in the dark and at room temperature.

The stained nuclear suspension was then filtered through a 20 µm membrane into a specific acrylic tube for analysis in the BD Accuri™ C6 Flow Cytometer (Accuri, Belgium), equipped with a 488 nm laser source to excite emission in FL2 (615-670nm) and FL3 (>670 nm) filters. Before any analysis, the equipment was properly inspected and calibrated, and the laser source was stabilized. Analyses were conducted in both monoparametric and biparametric configurations, with statistical parameters being monitored throughout data acquisition. The resulting histograms were analyzed using BD Accuri™ C6 software, and the data were interpreted relative to control samples. The level of DNA ploidy (1C, 2C, and 4C) was then quantified.

2.12 Daily testicular sperm production and epididymal sperm number and transit time

Spermatids resistant to homogenization (stage 19 of spermatogenesis) in the testis and spermatozoa from the caput/corpus and cauda portions of the epididymis were counted according to the method described by Robb et al. (1978). Firstly, the testis were decapsulated, weighed, and homogenized in 5 mL of 0.9% NaCl containing 0.05% Triton X-100. After a 10-fold dilution, the sample was transferred to Neubauer chambers for counting mature spermatids (four fields per animal). Daily sperm production (DSP) was evaluated by dividing the number of spermatids (per testis) by 6.1 (the number of days these spermatids are present in the seminiferous tubules). Similarly, caput/corpus and cauda portions of the epididymis were cut into small fragments and homogenized in 0.9%

NaCl containing 0.05% Triton X-100 (1:20), and spermatozoa were counted as described for the testis. The transit time of sperm through the epididymis was determined by dividing the number of spermatozoa in each portion of the epididymis by the DSP (Lima et al., 2018; Guimarães-Ervilha et al., 2021).

2.13 Sperm motility, viability, and morphology

For sperm analysis, freshly dissected portions of the cauda epididymal were fragmented on a Petri dish containing 500 µL of BWW at 37 °C to allow sperm release. For total motility analysis, 10 µL of epididymal fluid was placed between a slide and coverslip, previously warmed, and examined under 400 x magnification. Cells were classified as motile or immotile (Morakinyo et al., 2010). A drop of sperm solution was mixed with 1% eosin Y and 10% nigrosin. The sperm-eosin-nigrosin mixture was placed on a glass slide, and a smear was prepared. After drying, 200 spermatozoa were classified as live (unstained) or dead (stained) under light microscopy (Eliasson et al., 1977). Sperm morphology was analyzed using 50 µL of fluid fixed in 100 µL of 4% formaldehyde. The preparation was stained with eosin solution and examined under light microscopy at 1000 x magnification. 200 cells were evaluated for sperm abnormalities, categorized by defects in the head, midpiece, and tail (Filler, 1993).

2.14 Mitochondrial activity assessment in spermatozoa

Spermatozoa were suspended in a solution of 1 mg/mL DAB in phosphate-buffered saline (PBS; 1:1) and incubated in a water bath at 37 °C in the dark for 1 h. Two 15 µL smears were prepared on conventional glass slides. After drying, the slides were fixed in 10% formaldehyde for 10 min at room temperature. The slides were analyzed under an optical microscope, using light microscopy. A total of 200 spermatozoa were counted at 1,000x magnification for each animal. Cells were classified as Class I (100%

of the midpiece stained - all mitochondria active), Class II (more than 50% of the midpiece stained), Class III (less than 50% of the midpiece stained), or Class IV (no staining of the midpiece – all mitochondria inactive) (Colli et al., 2019).

2.15 Fertility indexes and reproductive efficiency of Calaris®-exposed male rats

On day 70 of experimentation, male rats (*n* = 8/ group) were allocated with healthy females for natural mating (1:1). Vaginal smears were performed after 12, 24, and 72 hours to verify the presence of sperm and characterize the mount occurrence. The identification of sperm cells in the vaginal cytology exam determined the gestational day 0 (GD0) (Lima et al., 2018; Coimbra et al., 2023). The males were separated at the end of the mating period. Pregnant females were maintained until GD19 (Lima et al., 2018), when they were sedated and anesthetized with high concentrations of xylazine (30 mg Kg^{-1} intraperitoneal) and ketamine hydrochloride (300 mg Kg^{-1} intraperitoneal) for euthanasia. Later on, the females were dissected to remove the ovaries and uterus containing the fetuses. All of them were fixed in 10% buffered formalin for subsequent analysis of fertility parameters (Lima et al., 2018).

The ovaries were dissected and used to count the number of corpora lutea (indicative of the number of ovulations), recognized by their pink color and rounded shape measuring around 2 mm. The uterine horns were dissected to verify the presence of embryonic reabsorptions through direct observation. The number of implantation sites was obtained by adding fetuses and resorptions (Souza et al., 2020). Then, the fetuses were removed along with their placentas, weighed, sexed, and measured the anus-genital distance (AGD) in male and female fetuses (Coimbra et al., 2023). The fertility indexes assessed were male mating index (number of males mated/number of males x 100), male fertility index (number of males fertilizing females/number of males mated x 100),

implantation index (number of implantations/number of corpora lutea x 100), pre-implantation loss (number of corpora lutea - number of implantations/number of corpora lutea x 100) and post-implantation loss (number of implantations - number of viable fetuses/ number of implantations x 100) (Hood et al., 2012).

2.16 Statistical analyzes

All results underwent normality evaluation using the Shapiro-Wilk test. Data were subjected to One-way ANOVA followed by the post hoc Tukey test. Statistical significance was considered when $p < 0.05$. Statistical analyses were conducted using GraphPad Prism (version 8.0, GraphPad Software Inc., San Diego, CA, USA). Results were expressed as means ± standard deviation (SD).

3. Results

3.1 Body and organ biometry

The body weight and the absolute and relative weights of testis and epididymis did not alter in Calaris®-exposed rats compared to their controls ($p > 0.05$; Table 1).

Table 1. Biometric parameters of Wistar rats exposed to Calaris® at two concentrations for 80 days.

Parameters	Control	Calaris®	
		2 mg Kg^{-1}	20 mg Kg^{-1}
Final body weight (g)	395.02 ± 16.35[a]	379.03 ± 25.96[a]	391.81 ± 22.46[a]
Testis (g)	1.43 ± 0.17[a]	1.46 ± 0.09[a]	1.44 ± 0.10[a]
Testis (g/100g)	0.74 ± 0.11[a]	0.76 ± 0.09[a]	0.75 ± 0.07[a]
Epididymis (mg)	0.38 ± 0.06[a]	0.39 ± 0.04[a]	0.40 ± 0.04[a]
Epididymis (mg/100g)	0.20 ± 0.03[a]	0.20 ± 0.01[a]	0.21 ± 0.03[a]

Mean ± SD. Control group: Rats receiving distilled water (0.5 mL) by gavage; Calaris® Groups: Rats exposed to Calaris® (diluted in distilled water; 0.5 mL) by gavage. $p > 0.05$ (n = 8 rats/group).

3.2 Serum hormonal and testicular testosterone levels

The serum concentration of LH decreased in animals exposed to 20 mg Kg^{-1} of Calaris® when compared to control animals ($p < 0.05$; Fig. 1A). The concentration of serum FSH and intratesticular testosterone did not differ between control rats and those exposed to Calaris® at two concentrations ($p > 0.05$; Fig. 1B, C).

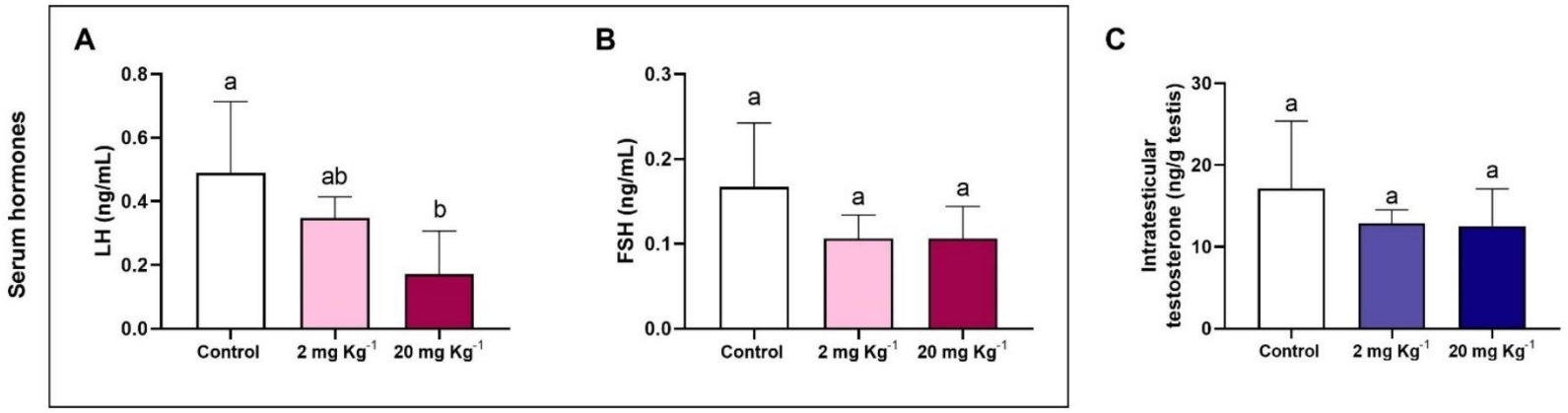


Fig. 1: The effect of Calaris® exposure on the serum concentration of (**A**) luteinizing hormone (LH), (**B**) follicle-stimulating hormone (FSH), and (**C**) intratesticular testosterone in healthy animals and rats exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days. Mean ± SD. Letters "a" and "b" indicate differences between groups ($p < 0.05$) ($n = 6$ rats/group).

3.3 Antioxidant enzyme activities in the hypothalamus, testis, and epididymis

SOD activity was higher in the hypothalamus of rats exposed to 2 and 20 mg Kg^{-1} of the herbicide than in control animals, with the highest value observed in the 20 mg Kg^{-1} group ($p < 0.05$; Fig. 2A). Rats exposed to 20 mg Kg^{-1} of Calaris® also presented the lowest activity of CAT and GST in this organ ($p < 0.05$; Fig. 2B and C). The testicular activity of SOD and CAT decreased in animals exposed to Calaris® at two concentrations compared to control animals ($p < 0.05$; Fig. 2D and E). In contrast, GST activity did not alter in the testis after herbicide exposure ($p > 0.05$; Fig. 2F). The proximal region of the epididymis presented the lowest SOD and CAT activity from rats exposed to 20 mg Kg^{-1} of the herbicide ($p < 0.05$), with no difference between groups regarding GST activity ($p > 0.05$; Fig. 2G-I). In the distal epididymis, in turn, SOD activity was higher in rats exposed to Calaris® at two concentrations than in control animals ($p < 0.05$; Fig. 2J), whereas the activity of GST was higher only in the distal epididymis of rats from the 20 mg Kg^{-1} group ($p < 0.05$; Fig. 2L). CAT enzyme activity was not altered after herbicide exposure in the distal epididymis ($p > 0.05$; Fig. 2K).

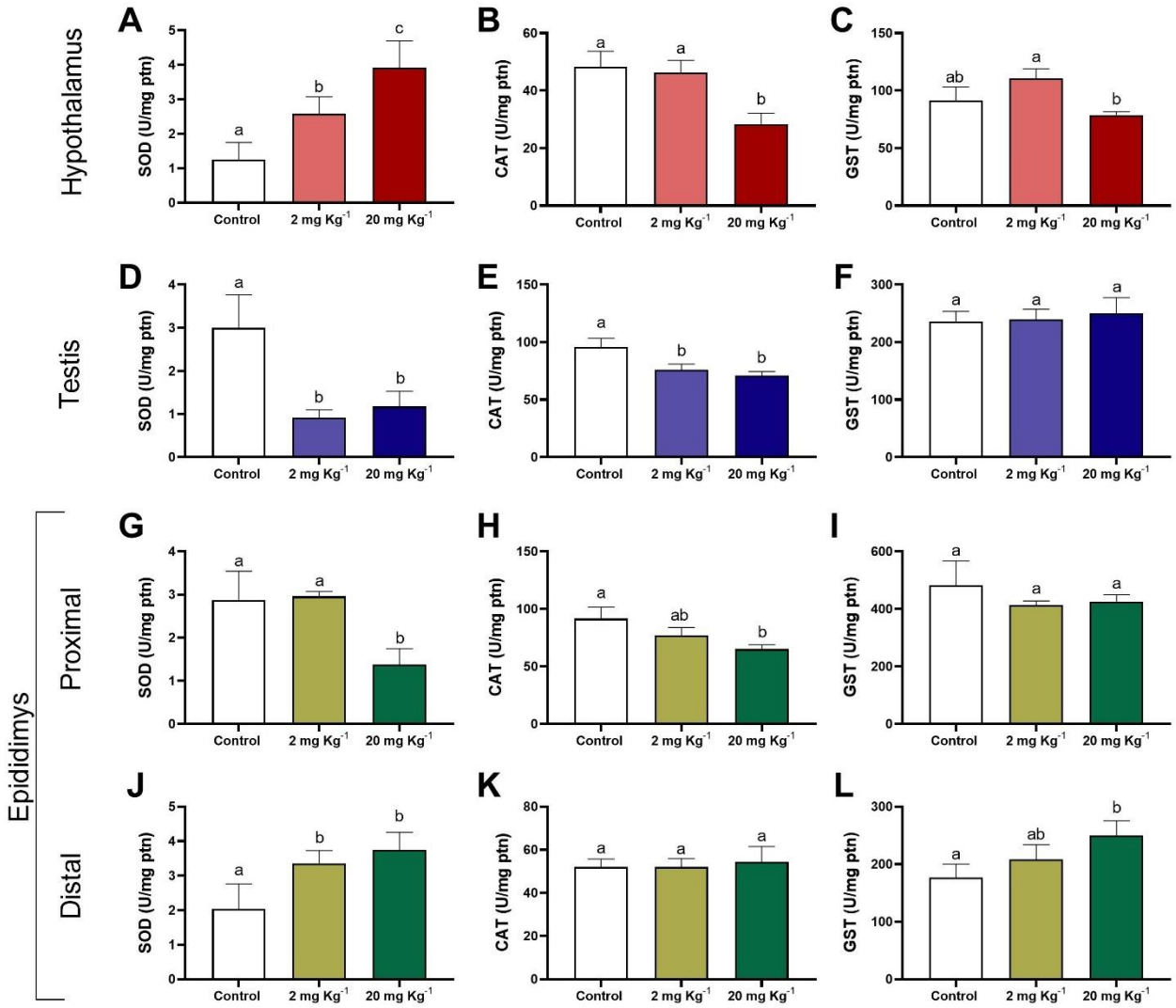


Fig. 2: The effect of Calaris® exposure on the activity of antioxidant enzymes in the hypothalamus (**A-C**), testis (**D-F**), and proximal (**G-I**) and distal (**J-L**) regions of the epididymis in healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days. SOD, superoxide dismutase; CAT, catalase; GST, glutathione S-transferase. Mean ± SD. Letters "a" and "b" indicate differences between groups ($p < 0.05$) ($n = 6$ rats/group).

3.4 Oxidative stress markers in the hypothalamus, testis, and epididymis

NO levels were higher in the hypothalamus of animals exposed to the 20 mg Kg^{-1} of Calaris® than in control animals ($p < 0.05$; Fig. 3A). However, MDA and PC levels were not altered in the hypothalamus after exposure to two concentrations of Calaris® ($p > 0.05$; Fig. 3B and C). In the testis, NO levels were higher in animals from Calaris® groups than the control group ($p < 0.05$; Fig. 3D). Testicular MDA levels increased in rats

exposed to 20 mg Kg^{-1} of Calaris® than their controls ($p < 0.05$; Fig. 3E). PC and FRAP levels did not differ between groups ($p > 0.05$; Fig. 3F and G). Moreover, in the epididymis, NO levels increased in its proximal portion after Calaris® exposure, regardless of the concentration ($p < 0.05$; Fig. 3H and L). MDA levels, in turn, increased in animals exposed to 20 mg Kg^{-1} of Calaris® compared to control animals ($p < 0.05$; Fig. 3I). PC levels were not altered after treatment with both doses ($p > 0.05$; Fig. 3J). FRAP decreased in animals receiving both concentrations of Calaris® compared to control animals ($p < 0.05$; Fig. 3K). In the distal region of the organ, NO levels increased in animals after exposure to the herbicide at two concentrations compared to control animals ($p < 0.05$; Fig. 3L). MDA, PC, and FRAP levels were not altered in exposed animals compared to control animals ($p > 0.05$; Fig. 3M, N and O).

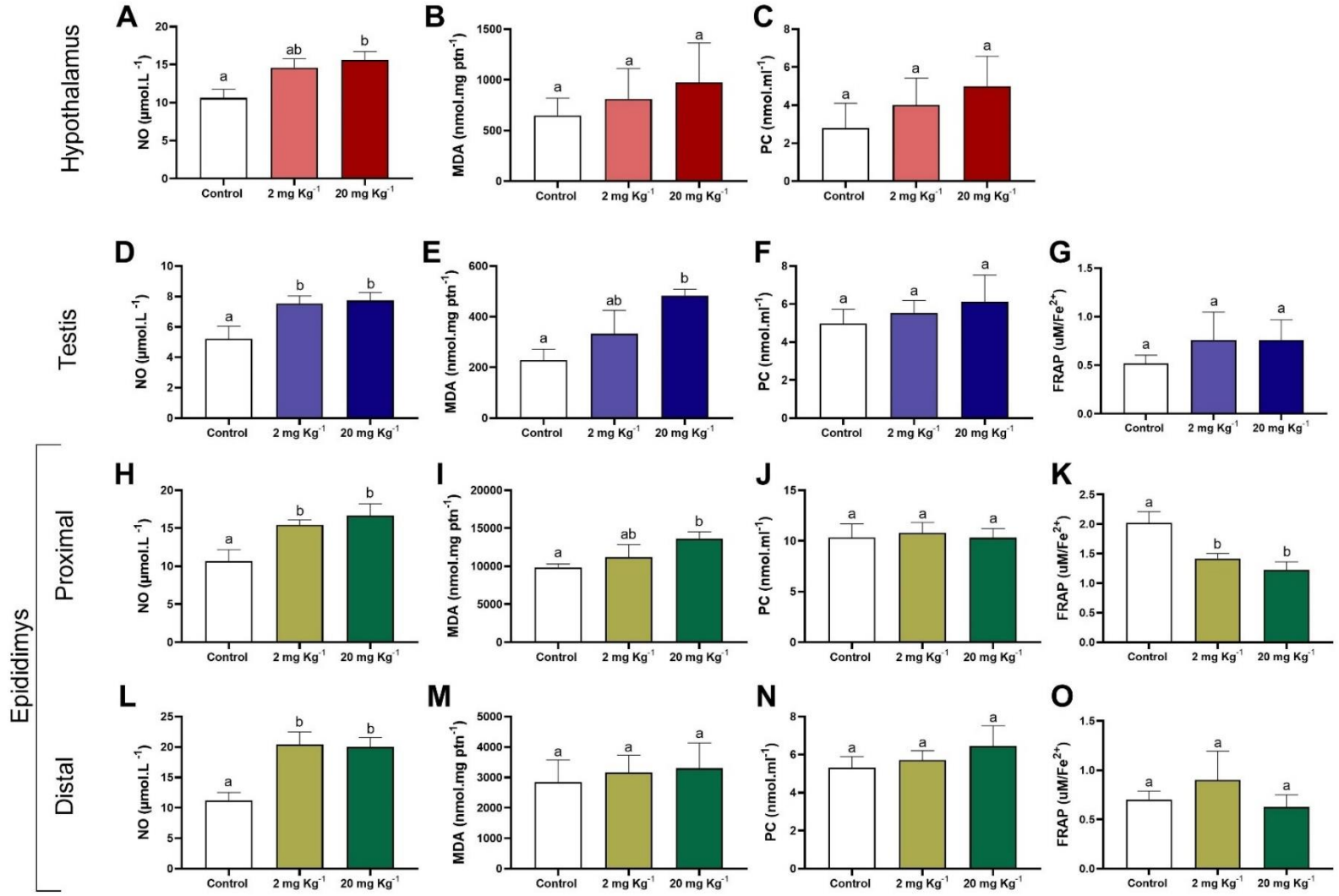


Fig. 3: The effects of Calaris® exposure on oxidative stress markers and total antioxidant capacity (FRAP) in the hypothalamus (**A-C**), testis (**D-G**), and proximal (**H-K**) and distal (**L-O**) regions in healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days. NO, nitric oxide; PC, carbonyl protein; MDA, malondialdehyde. Mean ± SD. Letters "a" and "b" indicate differences between groups ($p < 0.05$) (*n* = 6 rats/group).

3.5 Proportion of trace elements in the hypothalamus, testis, and epididymis

There was a change in the proportion of trace elements and cofactors of antioxidant enzymes in the hypothalamus, testis, and epididymis. In the first organ, the proportion of Fe and Mn was lower in animals exposed to two concentrations of Calaris® than in control animals ($p < 0.05$; Fig. 4A). Conversely, the proportion of Zn was higher in rats exposed to 2 and 20 mg Kg^{-1} of Calaris® than their controls ($p < 0.05$; Fig. 4A). In the testis, the proportion of Fe and Zn decreased in animals exposed to two concentrations of the herbicide compared to control animals ($p < 0.05$; Fig. 4B). The proportion of Mg decreased and Se increased in rats exposed to 20 mg Kg^{-1} of Calaris® compared to control animals ($p < 0.05$; Fig. 4B). Other chemical elements, such as Ca, Na, Cu and K, did not show difference in their proportion between groups ($p > 0.05$; Fig. 5A and B). In the proximal epididymis, the proportion of Fe, Zn, and Mg reduced in rats exposed to 2 and 20 mg Kg^{-1} of Calaris® compared to their controls ($p < 0.05$; Fig. 4C). Still, the proportion of Cu increased in animals exposed only to 20 mg Kg^{-1} of Calaris® ($p < 0.05$; Fig. 4C). Animals exposed to the herbicide presented high Zn and low Na proportion in their distal epididymis than control animals, regardless of its concentration ($p < 0.05$; Fig. 4D). There were no differences between groups in the proportion of the other chemicals (Ca, Se, Mn and K) analyzed in the epididymis ($p > 0.05$; Fig. 4C and D).

3.6 ATPases activity in the testis

Total ATPase activity was higher in the testis of rats exposed to 20 mg Kg^{-1} of Calaris® than in control animals ($p < 0.05$; Fig. 4E). The activity of Mg^{2+} ATPase was lower in animals exposed to 20 mg Kg^{-1} of Calaris® than in their controls ($p < 0.05$; Fig. 4G). No difference between groups were observed in the activity of Na^{+}/K^{+} ATPase and Ca^{2+} ATPase ($p > 0.05$; Fig. 4F and H).

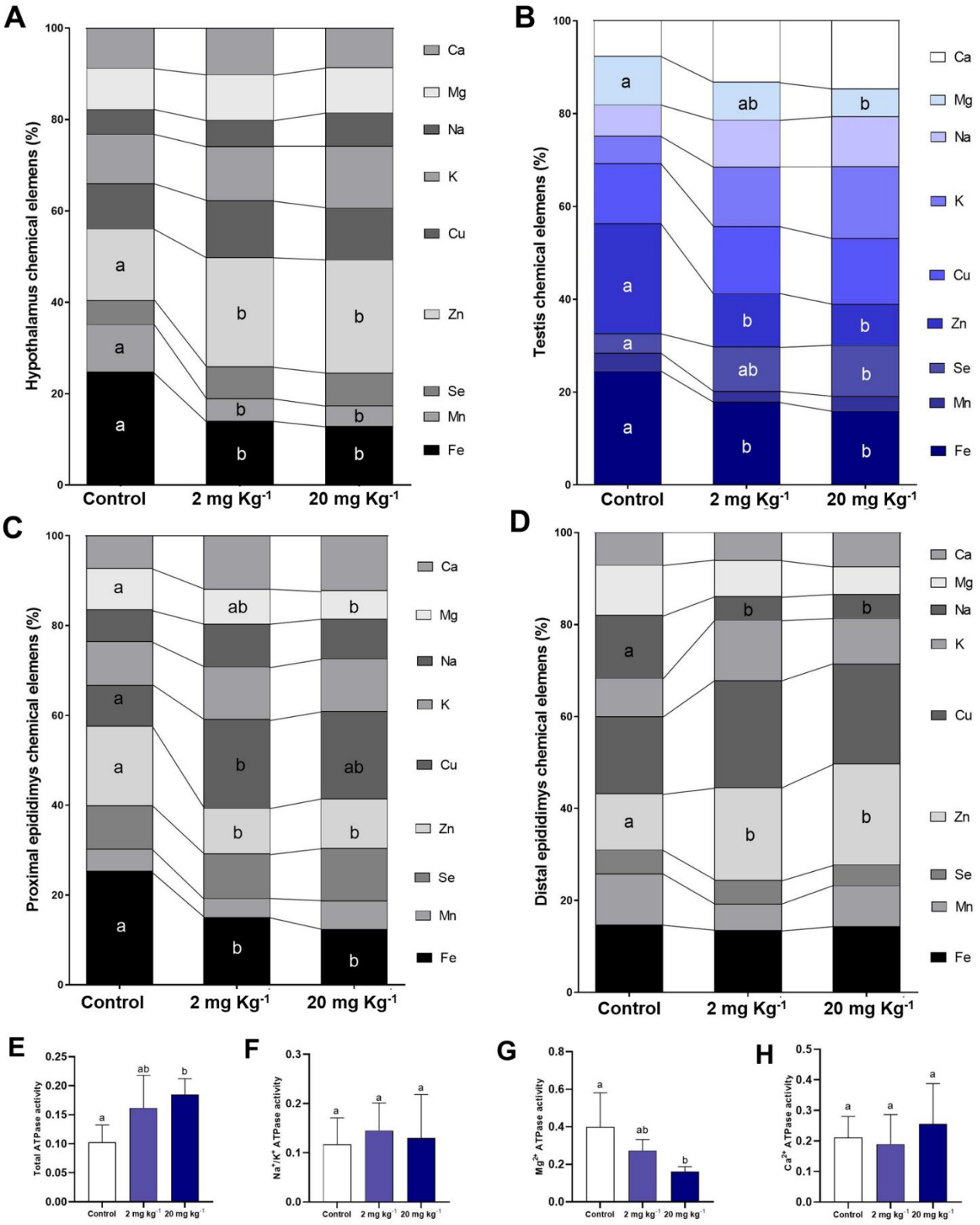


Fig. 4: The proportion of chemical elements in the (**A**) hypothalamus, (**B**) testis, and (**C**) proximal and (**D**) distal region of the epididymis in healthy Wistar rats and animals exposed to Calaris® at 2 or 20 mg Kg^{-1} daily for 80 days. Iron (Fe), manganese (Mn), selenium (Se), zinc (Zn), copper (Cu), potassium

(K), sodium (Na), magnesium (Mg) and calcium (Ca). **E-H**: total ATPase activity, and Na^+/K^+, Mg^{2+}, and Ca^{2+} ATPase activity in the testis of healthy rats and animals exposed to different concentrations of Calaris® for 80 days. Mean ± SD. Letters "a" and "b" indicate differences between groups ($p < 0.05$ ($n = 6$ rats/group).

3.7 Histopathology

In the hypothalamus, nerve cells underwent few changes following exposure to the herbicide. Animals from the control group and those receiving the lowest concentration of the herbicide presented nerve cells with normal size and shape, with complete nuclei and uniform opaque content (Fig. 5A and F). Nerve cells in the hypothalamus of animals receiving 20 mg Kg^{-1} of Calaris® showed slight cytoplasmic swelling (Fig. 5K and P). In the testis, there was a decrease in the proportion of seminiferous tubules classified as normal (Table 2) in animals exposed to 20 mg Kg^{-1} of the herbicide. Overall, control rats presented seminiferous tubules with ordinary structure, exhibiting germ cells arranged in layers following the usual pattern (spermatogonia - spermatocyte - spermatid) and Sertoli cells at the base of the seminiferous epithelium (Fig. 5B and C). There was no presence of vacuoles in the seminiferous epithelium, nor the presence of loose germ cells in the lumen. In the intertubular region, Leydig cells appeared with rounded nuclei and clear cytoplasm, with little presence of connective tissue and blood vessels showing no swelling (Fig. 5D). Rats exposed to the lower dose of Calaris® showed regular seminiferous epithelium layers but with a higher incidence of microvacuoles at the base of the seminiferous epithelium (Fig. 5G and H). Germ cells in the lumen were not found frequently. The intertubule presented normal histology with characteristic Leydig cells (Fig. 5I). Rats exposed to 20 mg Kg^{-1} of Calaris® showed higher incidence of pathologies in the seminiferous tubules. The incidence of microvacuoles increased, as well as the presence of macrovacuoles at the base of the seminiferous epithelium. Initial processes of epithelial desquamation were also found (Fig. 5L, M, and N). Despite the usual organization of germ cell layers, there were regions where germ cells were degenerating, presenting large intracellular vacuoles or incomplete cell division (Fig. 5G, R, and S).

The intertubule of these animals presented Leydig cells with little alteration, with an ordinary appearance with concentric nuclei. An increase in the size of blood vessels was evident (Fig. 5N).

Overall, all regions of the epididymis of control animals presented regular histology, with duct sections composed of regular epithelium and lumen, without alterations in the interstitium. In the initial segment, there were few modifications after exposure to the herbicide at two concentrations, with only a higher presence of mast cells in the interstitium and defense cells present in greater quantity within the blood vessels. In the caput region, animals exposed to the herbicide presented an incidence of inflammatory infiltrate foci, being higher in animals exposed to 20 mg Kg^{-1}. In the cauda region of the epididymis, animals exposed to the higher concentration of Calaris® showed a higher incidence of mast cells in the interstitium with an increase in the incidence of inflammatory infiltrate foci. There was also an increase in defense cells in the blood vessels (Fig. 5E, J, O, and T).

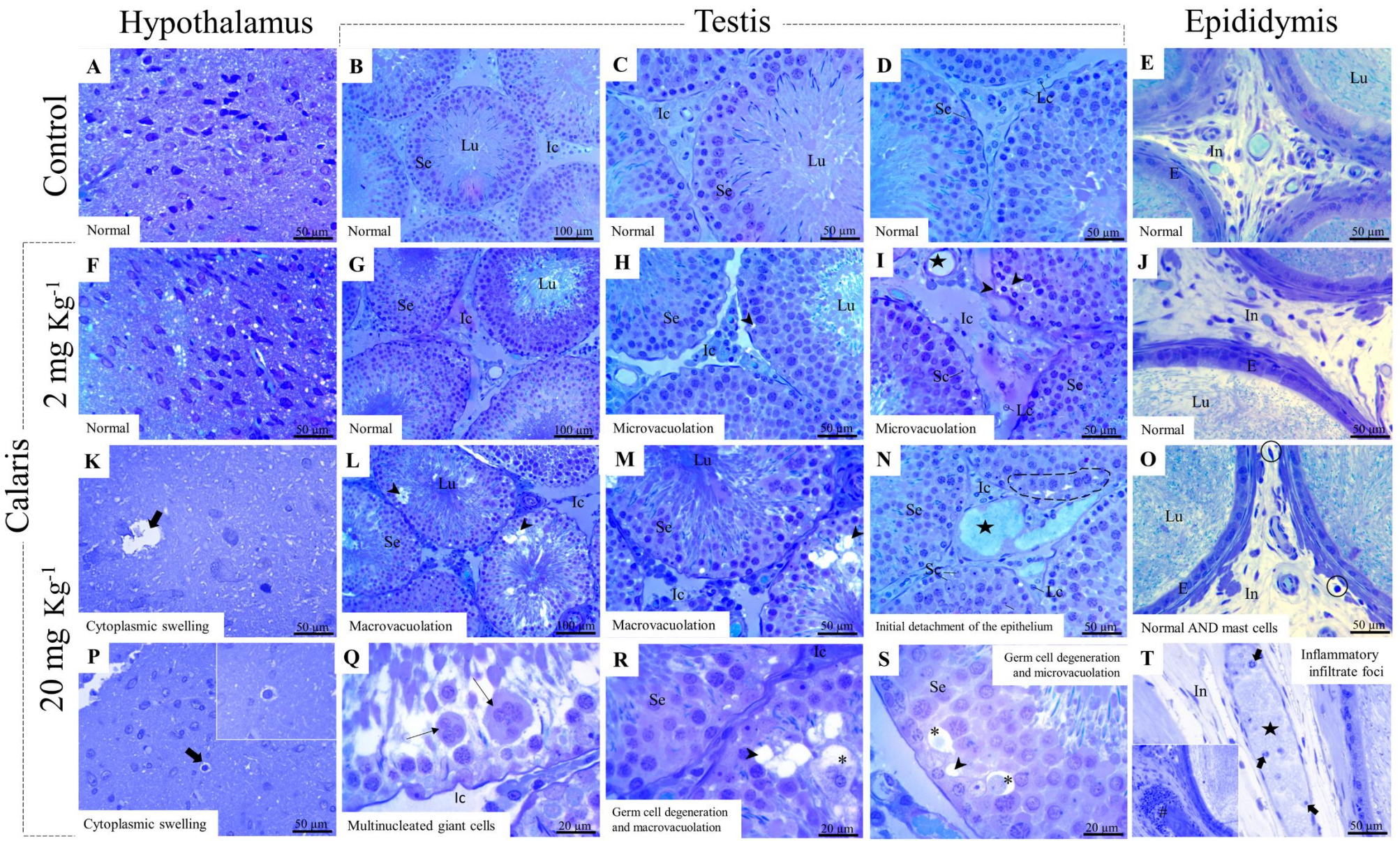

Hypothalamus
Testis
Epididymis
Calaris
Control
2 mg Kg-1
20 mg Kg-1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
Normal
Normal
Normal
Normal
Normal
Normal
Normal
Microvacuolation
Microvacuolation
Normal
Cytoplasmic swelling
Macrovacuolation
Macrovacuolation
Initial detachment of the epithelium
Normal AND mast cells
Cytoplasmic swelling
Multinucleated giant cells
Germ cell degeneration and macrovacuolation
Germ cell degeneration and microvacuolation
Inflammatory infiltrate foci
50 µm
100 µm
20 µm
Lu
Se
Ic
Sc
Lc
In
E

Fig. 5: Histological sections of the hypothalamus, testis, and epididymis of healthy animals (Control Group) exposed to 2 or 20 mg Kg^{-1} of Calaris® for 80 days. Panels **A**, **F**, **K**, and **P** display sections of the hypothalamus. Animals in the control groups and those that received a lower concentration of Calaris® (2 mg Kg^{-1}) exhibited normal histoarchitecture of the organ, with nerve cells displaying normal shape and size (**A** and **F**). Animals exposed to 20 mg Kg^{-1} of Calaris® showed nerve cells with cytoplasmic swelling (**K** and **P**). Wide black arrow; cytoplasmic swelling. In the testes, animals in the control group displayed normal histoarchitecture with germ cells arranged in layers supported by Sertoli cells. The intertubule comprised Leydig cells with spherical nuclei (**B**, **C**, and **D**). Animals from the group exposed to 2 mg Kg^{-1} of Calaris® also exhibited seminiferous epithelium composed of cells arranged in usual layers; however, there was a higher incidence of microvacuoles at the base of the seminiferous epithelium (**G** and **H**). The intertubule showed normal histology with characteristic Leydig cells (**I**). Animals from the group exposed to 20 mg Kg^{-1} of Calaris® showed a higher incidence of pathologies in the seminiferous tubules. The incidence of microvacuoles increased, along with the presence of macrovacuoles at the base of the seminiferous epithelium (**L**, **M**). Initial processes of epithelial desquamation were also observed (**N**). Despite the usual organization of the layers of germ cells, there were regions where germ cells were degenerating, presenting large intracellular vacuoles or incomplete cell division (**Q**, **R**, and **S**). Blood vessels were evident (**S**). Lumen; Se, seminiferous epithelium; *****, degenerating cell; Punctate, epithelial desquamation. In the epididymis of animals from the control group and those exposed to the lowest concentration of Calaris® (2 mg Kg^{-1}), regular histology was observed, with ducts composed of regular epithelium and lumen, without alterations in the interstitium (**E** and **J**). Animals exposed to 20 mg Kg^{-1} of Calaris® showed an increase in the incidence of mast cells in the interstitium, marginalization of inflammatory cells in the blood vessels, and an increase in the incidence of foci of inflammatory infiltrate in the interstitium (**O** and **T**). Lu, lumen; E, epithelium; In, interstitium; Circle, mast cells; #, inflammatory infiltrate; Broad arrows; defense cells.

3.8 Testicular morphometry and stereology

Animals exposed to 20 mg Kg^{-1} of the herbicide showed lower epithelium height than rats from 2 mg Kg^{-1} and control groups ($p < 0.05$; Table 2). The luminal diameter was higher in rats exposed to 20 mg Kg^{-1} of Calaris® than in control animals ($p < 0.05$; Table 2). The tubular diameter and the percentage of tubular compartment did not differ between groups ($p > 0.05$; Table 2). Moreover, rats exposed to 20 mg Kg^{-1} of Calaris® showed a lower percentage of seminiferous epithelium and a higher percentage of lumen than control rats and animals receiving 2 mg Kg^{-1} of Calaris® ($p < 0.05$; Table 2). The proportion of the intertubular compartment increased in rats from the 20 mg Kg^{-1} group compared to animals from the other two groups ($p < 0.05$; Table 2). In this compartment,

rats exposed to two herbicide concentrations also presented a higher proportion and volume of connective tissue, blood vessels, and macrophages than in control rats ($p < 0.05$; Table 3). No difference was observed between groups for the proportion and volume of lymphatic space ($p > 0.05$; Table 3). The nuclear diameter and nuclei percentage of Leydig cells were lower in animals exposed to 20 mg Kg^{-1} than in control animals ($p < 0.05$; Tables 3-4). The percentual of Leydig cell cytoplasm increased in animals exposed to the two concentrations of Calaris® as compared to control rats ($p < 0.05$; Table 4). The number of Leydig cells per testis did not differ between groups ($p > 0.05$; Table 4).

Table 2. Histopathology, histomorphometry, and stereology of the testis of healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days

		Calaris®	
Parameters	Control	2 mg Kg^{-1}	20 mg Kg^{-1}
Testicular pathology			
Normal seminiferous tubules (%)	97.33 ± 1.83[a]	95.58 ± 1.71[ab]	93.92 ± 2.06[b]
Abnormal seminiferous tubules (%)	2.66 ± 1.83[a]	4.41 ± 1.71[ab]	6.08 ± 2.06[b]
Testicular morphometry			
Tubular diameter (μm)	377.31 ± 20.44[a]	388.74 ± 18.80[a]	379.96 ± 11.33[a]
Epithelium height (μm)	139.22 ± 11.10[a]	140.95 ± 10.58[a]	124.36 ± 9.73[b]
Luminal diameter (μm)	119.74 ± 4.12[a]	123.94 ± 4.90[ab]	129.23 ± 3.48[b]
Volumetric proportion			
Tubular compartment (%)	84.98 ± 4.57[a]	83.92 ± 1.54[a]	85.58 ± 2.10[a]
Intertubular compartment (%)	15.01 ± 1.86[a]	16.40 ± 1.61[a]	17.57 ± 2.14[a]
Seminiferous epithelium (%)	70.19 ± 3.07[a]	65.20 ± 1.34[ab]	61.36 ± 2.90[b]
Tunica propria (%)	4.89 ± 1.36[a]	5.69 ± 0.92[a]	5.31 ± 1.45[a]
Lumen (%)	10.04 ± 2.91[a]	12.69 ± 1.78[ab]	15.75 ± 2.94[b]

Mean ± SD. Control group: Rats receiving distilled water (0.5 mL) by gavage; Calaris® Groups: Rats exposed to Calaris® (diluted in distilled water; 0.5 mL) by gavage. Different superscript letters ([a, b]) on the same roll indicate differences between groups ($p < 0.05$) (n = 6 rats/group).

Table 3. Histomorphometry and stereology of the intertubular compartment of the testis of healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days.

Parameters	Control	Calaris®	
		2 mg Kg^{-1}	20 mg Kg^{-1}
Volumetric proportion			
Connective tissue (%)	0.26 ± 0.15[a]	0.56 ± 0.14[b]	0.54 ± 0.15[b]
Lymphatic space (%)	4.76 ± 1.40[a]	4.65 ± 1.02[a]	5.20 ± 1.02[a]
Blood vessel (%)	0.92 ± 0.30[a]	1.95 ± 0.46[b]	1.79 ± 0.35[b]
Macrophages (%)	0.04 ± 0.03[a]	0.16 ± 0.06[b]	0.19 ± 0.065[b]
Leydig cell (%)	9.04 ± 2.93[a]	9.06 ± 1.14[a]	9.83 ± 1.33[a]
Volume			
Connective tissue (mL)	0.007 ± 0.004[a]	0.016 ± 0.003[b]	0.015 ± 0.004[b]
Lymphatic space (mL)	0.13 ± 0.03[a]	0.13 ± 0.03[a]	0.14 ± 0.03[a]
Blood vessel (mL)	0.02 ± 0.009[a]	0.05 ± 0.011[b]	0.05 ± 0.007[b]
Macrophages (mL)	0.001 ± 0.001[a]	0.004 ± 0.001[b]	0.005 ± 0.001[b]
Leydig cell (mL)	0.25 ± 0.08[a]	0.26 ± 0.03[a]	0.28 ± 0.03[a]

Mean ± SD. Control group: Rats receiving distilled water (0.5 mL) by gavage; Calaris® Groups: Rats exposed to Calaris® (diluted in distilled water; 0.5 mL) by gavage. Different superscript letters ($^{a, b}$) on the same roll indicate differences between groups ($p < 0.05$) (*n* = 6 rats/group).

Table 4. Histomorphometry and stereology of Leydig cells in the testis of healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days.

Parameters	Control	Calaris®	
		2 mg Kg^{-1}	20 mg Kg^{-1}
Nuclear diameter (μm)	8.32 ± 0.29[a]	7.92 ± 0.22[ab]	7.74 ± 0.22[b]
Nuclear percentage (%)	1.63 ± 0.28[a]	1.34 ± 0.14[ab]	1.30 ± 0.18[b]
Cytoplasm percentage (%)	7.41 ± 2.48[a]	7.72 ± 1.02[b]	8.52 ± 1.07[b]
Nuclear volume (μm^3)	303.01 ± 29.00[a]	260.41 ± 19.88[b]	243.64 ± 18.57[b]
Cytoplasmatic volume (μm^3)	1363.41 ± 240.54[a]	1496.11 ± 173.15[a]	1708.56 ± 245.84[a]
Cell volume (μm^3)	1666.45 ± 108.54[a]	1757.23 ± 75.71[a]	1952.44 ± 249.66[a]
Number of cells/testis ($\times 10^6$)	150.88 ± 34.05[a]	150.82 ± 23.39[a]	155.40 ± 43.36[a]

Mean ± SD. Control group: Rats receiving distilled water (0.5 mL) by gavage; Calaris® Groups: Rats exposed to Calaris® (diluted in distilled water; 0.5 mL) by gavage. Different superscript letters ([a, b]) on the same roll indicate differences between groups ($p < 0.05$) ($n = 6$ rats/group).

3.9 Comet assay and flow cytometry in the testis

The testis of rats exposed to 20 mg Kg^{-1} of Calaris® showed a higher percentage of fragmented DNA than animals from the other groups ($p < 0.05$; Fig. 6A and B). No difference between groups was observed regarding the total cell population in the testis and the DNA content with 4C and 2C cells ($p > 0.05$; Fig. 6C and D). However, rats exposed to 20 mg Kg^{-1} of Calaris® decreased the 1C DNA content compared to their controls ($p < 0.05$; Fig. 6D).

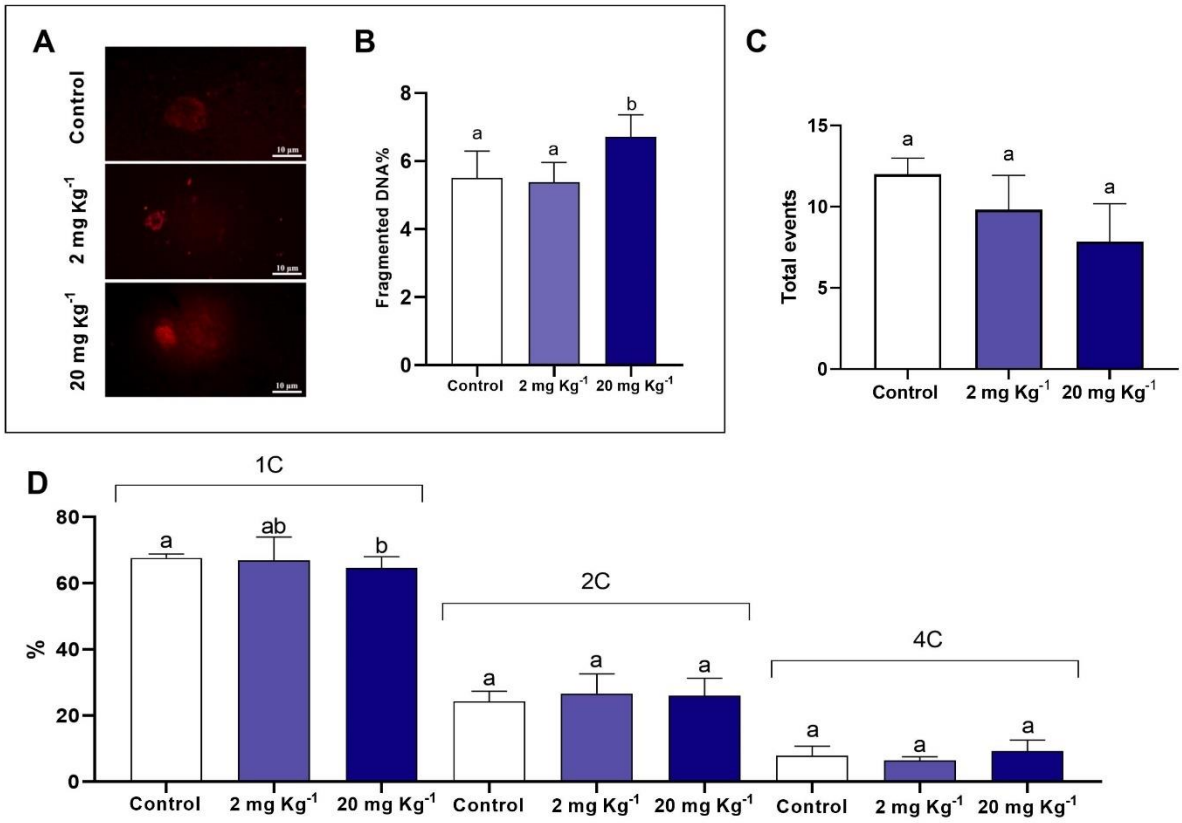


Fig. 6: The effects of Calaris® exposure on testis DNA damage and cellular content in healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg kg^{-1}daily for 80 days. **A,** representative comet image of testicular cells; **B**, the proportion of testicular cells with fragmented DNA by comet analysis; **C**, total testicular events quantified by flow cytometry; **D**, the proportion of 1C, 2C, and 4C testicular events. Mean ± SD. Letters "a" and "b" indicate differences between groups ($p < 0.05$) (*n* = 6 rats/group).

3.10 Daily testicular sperm production and epididymal sperm number and transit time

The number of spermatids in the testis and DSP decreased after exposure to 20 mg Kg^{-1} of Calaris® as compared to controls ($p < 0.05$; Table 5). Sperm transit time in the caput/corpus regions of the epididymis did not differ after exposure to Calaris® ($p > 0.05$; Table 5), differently to the observed in the cauda epididymis. Rats exposed to 20 mg Kg^{-1} of the herbicide presented longer transit time than control animals and those exposed to 2 mg Kg^{-1} of Calaris® ($p < 0.05$; Table 5).

Table 5. Sperm count in testis and epididymis, and transit time in the epididymis of healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days.

Parameters	Control	Calaris®	
		2 mg Kg^{-1}	20 mg Kg^{-1}
Spermatid number (x10^6/testis)	284.60 ± 40.23^{a}	214.91 ± 19.44^{ab}	181.33 ± 50.90^{b}
Spermatid number (x10^6/g testis)	182.56 ± 30.11^{a}	134.34 ± 14.96^{a}	181.36 ± 25.45^{a}
Dailly sperm production (x10^6/organ)	46.66 ± 6.59^{a}	35.23 ± 3.17^{ab}	29.73 ± 8.34^{b}
Caput/corpus epididymis sperm number (x10^6/organ)	149.04 ± 22.17^{a}	127.56 ± 18.31^{a}	122.34 ± 13.32^{a}
Caput/corpus epididymis sperm number (x10^6/g organ)	91.88 ± 12.20^{a}	75.94 ± 8.90^{a}	67.06 ± 8.17^{a}
Sperm transit time in the caput/corpus (days)	3.20 ± 0.36^{a}	3.62 ± 0.59^{a}	3.97 ± 0.96^{a}
Cauda epididymis sperm number (x10^6/organ)	273.64 ± 22.39^{a}	205.74 ± 19.05^{b}	242.56 ± 16.58^{ab}
Cauda epididymis sperm number (x10^6/g organ)	91.88 ± 5.11^{a}	82.06 ± 7.36^{a}	87.00 ± 3.83^{a}
Sperm transit time in the cauda epididymis (days)	5.87 ± 0.74^{a}	5.89 ± 1.00^{a}	7.61 ± 0.19^{b}

Mean ± SD. Control group: Rats receiving distilled water (0.5 mL) by gavage; Calaris® Groups: Rats exposed to Calaris® (diluted in distilled water; 0.5 mL) by gavage. Different superscript letters ($^{a, b}$) on the same roll indicate differences between groups ($p < 0.05$) (n = 6 rats/group).

3.11 Sperm parameters

Sperm motility was lower in rats exposed to 20 mg Kg^{-1} of Calaris® than in control rats and animals receiving 2 mg Kg^{-1} of the herbicide ($p < 0.05$; Fig 7A). Sperm viability was lower in herbicide-exposed groups than in control group ($p < 0.05$; Fig. 7B). Sperm morphology was not altered after exposure to Calaris® ($p > 0.05$; Fig. 7C). Total mitochondrial activity (Class I) decreased in spermatozoa from herbicide-exposed rats compared to their controls ($p < 0.05$; Fig. 7D). Meanwhile, lagging mitochondrial activity (Class II-IV) was higher in in sperm cells of rats from the two herbicide groups than the control group ($p < 0.05$; Fig. 7D).

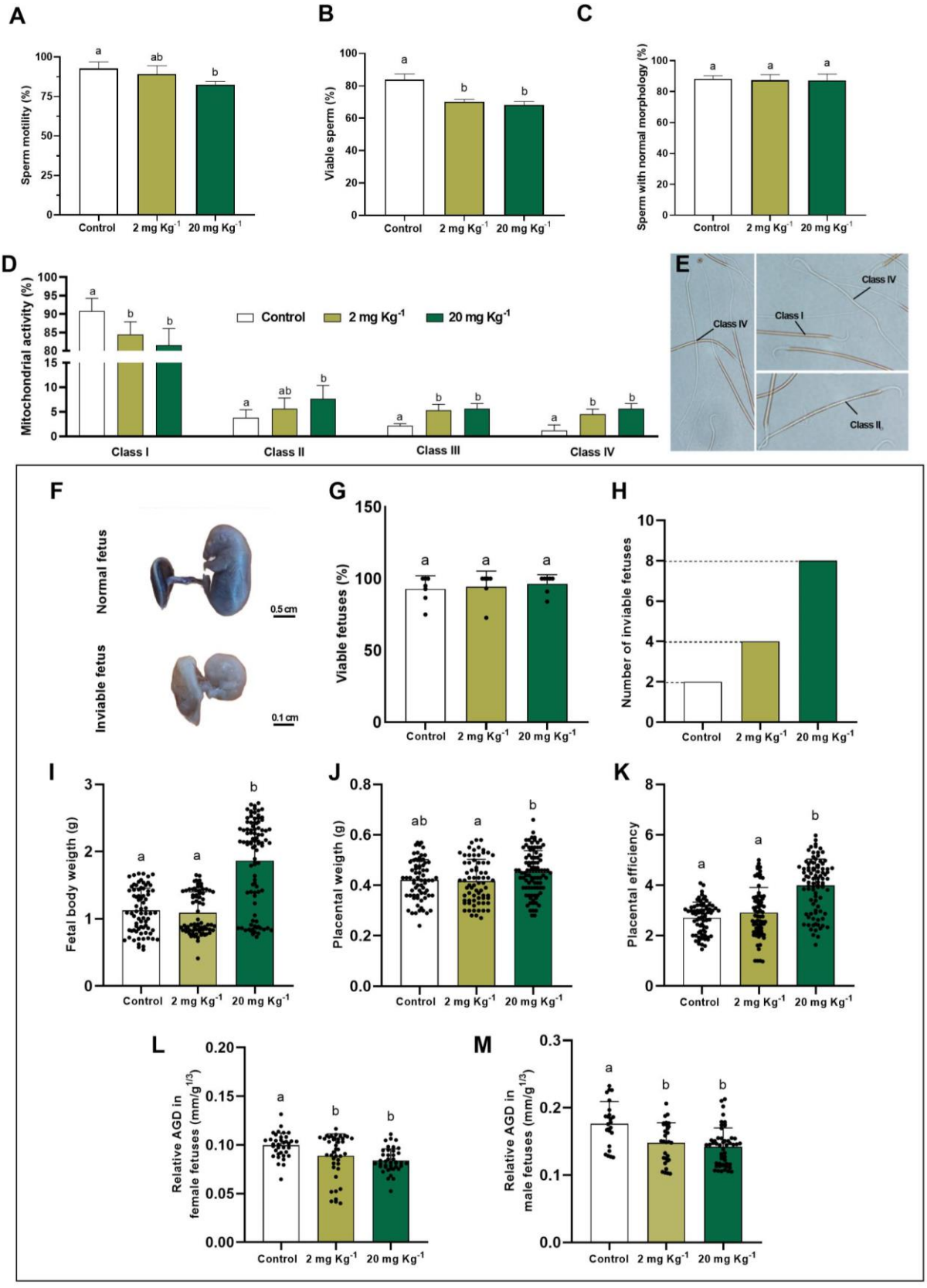

A
Sperm motility (%)
Control
2 mg Kg-1
20 mg Kg-1
B
Viable sperm (%)
C
Sperm with normal morphology (%)
D
Mitochondrial activity (%)
Class I
Class II
Class III
Class IV
E
Class IV
Class I
Class II
F
Normal fetus
Inviable fetus
0.5 cm
0.1 cm
G
Viable fetuses (%)
H
Number of inviable fetuses
I
Fetal body weigth (g)
J
Placental weigth (g)
K
Placental efficiency
L
Relative AGD in female fetuses (mm/g1/3)
M
Relative AGD in male fetuses (mm/g1/3)

Fig. 7: The effects of Calaris® on sperm and fertility parameters in healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg kg^{-1} daily for 80 days. Representation of the proportion of spermatozoa that are motile (**A**), viable (**B**), and with normal morphology (**C**). **D**, percentage of sperm according to the classification of mitochondrial activity: class I - 100% mitochondrial activity; class II - more than 50%; class III - less than 50%; class IV - absence of mitochondrial activity. **E**, representative images of mitochondria in the sperm midpiece stained based on the oxidation, polymerization, and deposition of 3,3′-diaminobenzidine (DAB) by mitochondrial cytochrome c-oxidase. (**F**) representative images of viable and non-viable fetuses and (**G**) proportion of viable fetuses. (**I**) Fetal weight, (**J**) placental weight, and (**K**) placental efficiency. Anus-genital distance (AGD) of (**L**) female and (**M**) male fetuses. Mean ± SD. The letters "a" and "b" indicate differences between groups ($p < 0.05$) ($n = 6$ or 8 rats/group).

3.12 Fertility indexes and reproductive efficiency

The results indicate that the herbicide did not affect the mating capacity of the animals (Table 6). The number of uterine implants, viable fetuses, and female parameters (number of corpora lutea and ovary weight) did not differ between groups, nor did the male mounting index and male fertility index ($p > 0.05$; Table 6). However, the implantation index was reduced in animals exposed to 20 mg Kg^{-1} of Calaris® compared to control rats and animals exposed to 2 mg Kg^{-1} ($p < 0.05$; Table 6). Pre-implantation loss was higher in rats exposed to20 mg Kg^{-1} of Calaris® than control rats and those exposed to 2 mg Kg^{-1} ($p < 0.05$; Table 6). The percentage of viable fetuses did not differ between groups ($p > 0.05$; Fig. 7F and G). There was an increase in fetal and placental weights, accompanied by high placental efficiency in rats exposed to 20 mg Kg^{-1} compared to the other groups ($p < 0.05$; Fig. 7I-K). The anogenital distance of female fetuses was shorter in animals exposed to both concentrations of the herbicide than in control animals, whereas the male anogenital distance decreased only in rats exposed to 20 mg Kg^{-1} of the herbicide ($p < 0.05$; Fig. 7L, M).

Table 6. Fertility index and reproductive efficiency of female rats mated with healthy Wistar rats and animals exposed to Calaris® at 2 and 20 mg Kg^{-1} daily for 80 days.

Parameters	Control	Calaris®	
		2 mg Kg^{-1}	20 mg Kg^{-1}
Number of females	8	8	8
Number of females mated	7	7	8
Number of males mated	7	7	8
Number of males impregnating females	7	6	8
Number of females pregnant	7	6	8
Number of corpora lutea	13.43 ± 1.27[a]	14.00 ± 1.81[a]	14.13 ± 1.28[a]
Number of implants	12.29 ± 1.70[a]	13.00 ± 1.09[a]	12.50 ± 1.06[a]
Number of viable fetuses	12.00 ± 1.15[a]	12.33 ± 1.33[a]	11.13 ± 1.08[a]
Ovary weight (g)	0.08 ± 0.01[a]	0.10 ± 0.01[a]	0.10 ± 0.02[a]
Male mating index (%)	87.5	87.5	100
Male fertility index (%)	100	87.5	100
Implantation index (%)	91.54 ± 2.23[a]	92.73 ± 1.10[a]	87.56 ± 2.06[b]
Preimplantation loss (%)	8.06 ± 1.14[a]	7.27 ± 2.62[a]	12.44 ± 0.72[b]
Postimplantation loss (%)	2.31 ± 1.51[a]	5.55 ± 3.18[a]	5.49 ± 3.64[a]

Mean ± SD. Control group: Rats receiving distilled water (0.5 mL) by gavage; Calaris® Groups: Rats exposed to Calaris® (diluted in distilled water; 0.5 mL) by gavage. Different superscript letters ([a, b]) on the same line indicate differences between groups ($p < 0.05$) (*n* = 8 rats/group).

4. Discussion

This is the first study to evaluate the effects of the herbicide Calaris®, a mixture based on the herbicides atrazine and mesotrione, on the male reproductive system of mammals. Our results support the hypothesis of the toxic effects of relevant concentrations of Calaris® on testicular and epididymal parameters of Wistar rats. The herbicide caused oxidative and nitrosative stress in the organs of the HPG axis, resulting from mineral imbalance. In this scenario, these results promotea decrease in the LH hormone, remodeling of the testicular parenchyma, damage to the DNA of testicular cells

and losses in the quantity and quality of sperm, which presented mitochondrial dysfunction. Despite the maintenance of testicular testosterone levels, the fertility of animals exposed to 20 mg Kg^{-1} of Calaris® for 80 days was reduced, with an increase in preimplantation losses. Furthermore, male and female fetuses, from males in the group exposed to 20 mg Kg^{-1} of the herbicide, showed a reduction in the AGD. Calaris® is an herbicide marketed as the evolution of atrazine, an already widely used agrochemical that recently confirmed its strong potential as an endocrine disruptor in the HPG axis (Chapter 2). The tendency to increase the use of Calaris®, due to its better efficiency compared to the isolated use of mesotrione or atrazine, opens the door for toxicology studies through the interaction of these compounds.

Exposure to the herbicide impaired the mineral balance in the hypothalamus, testis, and different regions of the epididymis. In particular, the elements Zn and Fe were the most affected, with variations in all the organs mentioned after exposure to Calaris®. These two elements are cofactors of important antioxidant enzymes, such as SOD and CAT (Jomova et al., 2022), which are fundamental in the processes of steroidogenesis, spermatogenesis, and sperm maturation (Harchegani et al., 2020; Morabbi and Karimian, 2024). The increase in the proportion of Zn in the hypothalamus and the distal portion of the epididymis, as well as the reduction in the proportion of Zn in the testis and proximal region of the epididymis, may be related to the organs' antioxidant defense. The activity of the SOD enzyme followed the variation in Zn levels, with Zn being a key cofactor in the enzyme's activity (Jomova et al., 2022). The need/efficiency of the SOD enzyme may be related to mitochondrial problems arising from the cellular interaction of atrazine and/or mesotrione. For example, exposure to atrazine resulted in low mitochondrial membrane potential and high mitochondrial DNA release in the mouse cerebellum (Liu et al., 2024). There is no data on the isolated action of mesotrione on mitochondrial

function. Thus, mitochondrial problems impair the electron transport chain, resulting in the accumulation of ROS and free radicals, mainly superoxide (O^{2-}), requiring greater SOD action (Indo et al., 2014). The imbalance in Zn availability may be related to problems in the enzyme's activity. A recent study revealed positive effects of Zn on the toxicity of atrazine in the liver of rats (Mohamed et al., 2023). Mitochondrial dysfunction and problems in SOD activity result in the accumulation of O^{2-}, which is naturally converted into hydrogen peroxide (H_2O_2) by the CAT enzyme (Anwar et al., 2024). The reduction in Fe proportion appears to precede a decline in CAT enzymatic activity in all the mentioned organs. It remains to be seen whether this occurs due to competition between atrazine and/or mesotrione for Fe or the action of herbicides on the structure/expression of the CAT enzyme. The activity of the GST enzyme did not undergo significant changes in the organs in question, except in the distal region of the epididymis where there was an increase in the enzyme's activity. The proportion of Se, its cofactor, also did not change. GST is an important enzyme involved in the xenobiotic detoxification process (Zhang et al., 2024), and the maintenance of its activity mirrors the ongoing process of cells neutralizing the most toxic molecules present in Calaris®. A recent study revealed the harmful action of atrazine on the nuclear factor erythroid-2-related factor 2 (Nrf2) pathway, which is linked to the expression of genes involved in antioxidant defense, indicating damage to the redox balance as a result of the herbicide (Edwards et al., 2024).

Exposure to both concentrations of the herbicide Calaris® caused nitrosative stress in organs, culminating in lipid damage. Problems in the activity of antioxidant enzymes lead to the accumulation of ROS and free radicals in the organs, such as H_2O_2 and O^{2-}. Low levels of NO in organs, associated with redox balance dysfunction, reveal the possible conversion of the molecule into reactive nitrogen species (RNS). These

species, like peroxynitrite, are highly reactive in the cell (Prolo et al., 2024). Furthermore, NO plays an important role in vasodilation (Soloviev and Sydorenko, 2024). Initially, we expected a decrease in the proportion of vessels in the testis, however, there was an increase in the proportion and volume of vascular components in the testis. Other pathways may have compensated for the absence of the free NO molecule, such as the vascular endothelial growth factor (VEGF) pathway positively stimulated by atrazine in epithelial ovarian cancer cells (Chen et al., 2021) and in porcine granulosa cells (Basini et al., 2012). Interestingly, isolated exposure to the herbicide atrazine results in an increase in NO (Liu et al., 2014; Ikeji et al., 2022), while a recent study that evaluated the action of Calaris® in bees also found a reduction in NO levels (Araújo et al., 2023). There are no reports of the individual action of mesotrione on NO levels. Initially, ROS and RNS caused damage to lipids, with an increase in MDA levels in the testis and the initial portion of the epididymis. The oxidative damage caused by Calaris® appears not to have affected the protein structure. Modifications in lipids can affect the structure of membranes in cells (Nicolson and Mattos, 2022). In the testis, the reduction in Mg^{2+} ATPase activity in animals exposed to 20 mg Kg^{-1} of Calaris® may be the result of damage to lipids caused by nitrosative stress, which disrupted membrane functionality.

The loss in LH production in animals exposed to 20 mg Kg^{-1} of Calaris® may have been due to mineral imbalance and oxidative damage to cells. The functionality of gonadotropic cells in the pituitary appears to have been impaired by exposure to Calaris®. Despite the maintenance of serum FSH levels, reduced LH levels are indicators of the function of the hypothalamus in the release of the gonadotropin-releasing hormone GnRH (Maggi et al., 2016). The decrease in LH also indicates problems in the pulsatile release of GnRH from the hypothalamus. Atrazine may affect Kisspeptin neurons that, in turn, compromises the GnRH neurons stimulus (Kimura et al., 2019). Despite the reduction in

serum LH levels and alteration in Leydig nuclear diameter and cytoplasm proportion, intratesticular testosterone levels were not altered after exposure to Calaris®. We recently showed that hormonal levels are affected mainly by high doses of atrazine (> 100 mg Kg^{-1}) (Chapter 2), which did not occur in our work, where the animals were exposed to 10 mg Kg^{-1} of atrazine in the group that received 20 mg Kg^{-1} of Calaris®. However, due to changes in LH levels and nuclear Leydig morphology, the tendency is that the continuation of the exposure period will result in a visible failure of steroidogenesis, with a reduction in testosterone levels.

The parenchyma of the organs of animals exposed to the herbicide showed disorganization, with an increase in histopathology. Despite the maintenance of testosterone levels, mineral imbalance and the accumulation of free radicals resulted in damage to the structure of the organs. In the hypothalamus, only 20 mg Kg^{-1} of Calaris® caused changes, at first there was slight swelling in the neurons that may indicate impairment of the cell membrane (Rungta et al., 2015). The herbicide atrazine compromises the neurological system (Ricci et al., 2024), and there is no data on the action of mesotrione on the nervous system. In the testis, morphometric analyses revealed changes in the composition of the parenchyma after exposure to Calaris®, with a reduction in the seminiferous epithelium, without compromising the proportion between the intertubular space and the tubular space. Despite maintaining the proportion, the components of the intertubular space were altered, with an increase in the proportion of macrophages. Specifically, the increase in these defense cells may indicate an inflammatory process in the organ, while they positively stimulate the production of androgens by Leydig cells (Heinrich and DeFalco, 2019).

Furthermore, the increase in macrophages may be associated with the proliferation of fibroblasts that contribute to the increase in connective tissue, which may

hinder communication between the intertubular and tubular compartments (Hedger, 2002). In addition to structural changes, the testicular parenchyma was affected by several histopathologies. The presence of inter and intracellular microvacuoles in the seminiferous epithelium of animals in the group exposed to 2 mg Kg^{-1}, accompanied by macrovacuoles in the epithelium of animals exposed to 20 mg Kg^{-1} of Calaris®, corroborates the damage to lipids indicated by the increase in MDA and implications for membrane problems, in addition to indicating possible problems in the structure of the Sertoli cell (Creasy et al., 2012). The presence of multinucleated giant cells in the seminiferous epithelium of animals exposed to 20 mg Kg^{-1} of Calaris® increases evidence of impaired Sertoli function, which is inefficient in the process of closing cytoplasmic bridges between germ cells (Wu et al., 2023). Taken together, these pathologies indicate an initial tubular degeneration (Creasy et al., 2012), implying a reduction in sperm production. Interestingly, no detached germ cells were found in the lumen of the testis or the epididymis. Atrazine disorganizes the seminiferous tubules (Martins-Santos et al., 2017; Ikeji et al., 2022; Arulanandu et al., 2024; Rotimi et al., 2024), with the individual effects of mesotrione unknown. In the epididymis, the increase in mast cells in the interstitium corroborates a possible inflammation process affecting the organs.

Taken together, the challenges posed by exposure to the herbicide resulted in DNA damage of the animals' testicular cells. ROS and RNS interacted with the DNA of testicular, somatic, and germ cells, resulting in problems in the molecule structure, especially in animals exposed to 20 mg Kg^{-1} of Calaris®. DNA damage can lead to genetic problems that affect sperm viability and function (Ziouziou et al., 2024). The herbicide atrazine is capable of causing damage to the DNA of somatic and germ cells in different organisms (Ahmed et al., 2022; Gao et al., 2022; Hadeed et al., 2022; Arulanandu et al., 2024), while it has been DNA damage has been demonstrated in fish after exposure to the

herbicide mesotrione (Piancini et al., 2015; Wang et al., 2018). DNA fragmentation associated with the presence of degenerating cells resulted in a reduction in the seminiferous epithelium with losses in DSP. As we showed, the proportion and height of the seminiferous epithelium decreased in animals exposed to the herbicide Calaris®, although the total population of testicular cells was not altered. However, the content of haploid cells (spermatids and spermatozoa) was reduced after exposure to the herbicide. Atrazine reduces the meiosis process (Gely-Pernot et al., 2015), compromising gamete production. Thus, the germ cell population decreased, which we confirmed with lower DSP. Overall, the process of spermatogenesis depends on Fe, especially in successive cell divisions that require Fe for both DNA synthesis and cell growth (Tvrda et al., 2014). The low levels of the element in the testis may have resulted in protein changes that resulted in a reduction in DSP.

In addition to the quantitative damage, the quality of sperm was also harmed by Calaris®. Sperm motility decreased in treated animals, as well as mitochondrial activity in the midpiece. Mitochondrial activity depends on Fe (Morabbi and Karimian, 2024). The increase in non-viable sperm also indicates problems in gamete formation. This may be associated with DNA damage found in testicular cells, the precursor to sperm. Furthermore, H_2O_2, which we concluded to have increased after failure in CAT activity in the testis, is capable of generating toxic effects on sperm function under in vitro conditions and inducing the expression of apoptotic markers, such as caspase-3, and DNA fragmentation (Bravo et al., 2024). Interestingly, sperm morphology was not altered. The process of spermiation, closely linked to sperm morphology, involves a complex network of cytoskeletal filaments and adequate Sertoli function (O'Donnell et al., 2022). The combination of atrazine and mesotrione, in relevant doses, does not compromise this process. Another hypothesis is that the phagocytic action of the Sertoli cell can neutralize

sperm with abnormal morphology with an increased spermiophagy process. Other studies associate isolated exposure to high concentrations of atrazine (120 mg Kg^{-1}) with abnormal sperm morphology (Aziz et al., 2018; Rotimi et al., 2024). The increase in epididymal transit time in the cauda region is another factor that may have contributed to the impairment of sperm quality after exposure to Calaris®. To our knowledge, this is the first study to evaluate changes in epididymal transit time in triazine-based products. The increase in transit time can delay sperm maturation, compromising sperm quality (Sullivan et al., 2005), in addition to keeping them in a pro-oxidant environment and possibly with an initial inflammatory process.

Finally, given all the damage caused to the structure of the organs, the fertility of the animals was reduced. Corroborating the data presented so far, spermatozoa produced in an environment compromised by the herbicide Calaris® have reduced efficiency. Oxidative stress may be related to the production of these defective sperm, as it is widely recognized as a contributor to male infertility (Bravo et al., 2024). The animals' libido appears not to have been affected, as there was no difference in the number of females mounted by the treated males. Libido is closely related to hormone levels (Wang et al., 2024), which were not affected by the herbicide. The reduced implantation rate and the increased pre-implantation loss rate in animals exposed to 20 mg Kg^{-1} of Calaris® confirm a reduced sperm efficiency. Probably, defects in the sperm membrane decreased the fertilization capacity of the oocyte in the female, while damage to sperm DNA compromised the initial development of the embryo. The embryos that survived the implantation process generated larger fetuses than those generated by males in the control group. Sperm epigenetic changes may be related to this increase in fetal weight, as well as a greater maternal nutritional intake as compensation for some embryonic failure (Belkacemi et al., 2010). Finally, the AGD was smaller in the fetuses of animals treated

with Calaris®. It is possible that sperm influenced embryonic sexual development in both females and males. AGD is associated with adverse effects on male reproductive health, such as low sperm quality and reduced fertility in adulthood (Thankamony et al., 2016; Docea et al., 2023). Additional studies of the F1 generation may better elucidate the epigenetic mechanisms involved in sperm DNA and how this may compromise the reproductive development of offspring.

5. Conclusion

This study presents a comprehensive view of the toxic effects of relevant concentrations of the herbicide Calaris® on the male reproductive organs of Wistar rats. Our results indicated that exposure to Calaris®, particularly at 20 mg Kg^{-1}, caused oxidative and nitrosative stress, possibly due to mineral imbalance, culminating in damage to the DNA of testicular cells. The findings resulted in a decrease in the population of germ cells with a reduction in DSP. Despite normal testicular testosterone levels, the fertility of exposed animals was reduced, due to low gamete quality, with increased pre-implantation losses and decreased AGD in fetuses. These findings highlight the importance of additional toxicological studies to understand better the risks associated with the increasing use of herbicide mixtures such as Calaris® in agriculture. This is a first step in understanding the epigenetics behind the harmful effects of herbicides on gamete functionality.

6. References

Aebi, H., 1984. [13] Catalase in Vitro. Methods Enzymol 105, 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Ahmed, Y.H., AbuBakr, H.O., Ahmad, I.M., Ahmed, Z.S.O., 2022. Histopathological, Immunohistochemical, And Molecular Alterations In Brain Tissue And Submandibular Salivary Gland Of Atrazine-Induced Toxicity In Male Rats. Environmental Science and Pollution Research 29, 30697–30711. https://doi.org/10.1007/s11356-021-18399-x

Akinola, O.B., Biliaminu, S.A., Adedeji, O.G., Oluwaseun, B.S., Olawoyin, O.M., Adelabu, T.A., 2016. Combined effects of chronic hyperglycaemia and oral aluminium intoxication on testicular tissue and some male reproductive parameters in Wistar rats. Andrologia 48, 779–786. https://doi.org/10.1111/and.12512

Anwar, S., Alrumaihi, F., Sarwar, T., Babiker, A.Y., Khan, A.A., Prabhu, S.V., Rahmani, A.H., 2024. Exploring Therapeutic Potential of Catalase: Strategies in Disease Prevention and Management. Biomolecules 14. https://doi.org/10.3390/biom14060697

Araújo, R. dos S., Viana, T.A., Botina, L.L., Bastos, D.S.S., da Silva Alves, B.C., Machado-Neves, M., Bernardes, R.C., Martins, G.F., 2023. Investigating the effects of mesotrione/atrazine-based herbicide on honey bee foragers. Science of the Total Environment 898, 165526. https://doi.org/10.1016/j.scitotenv.2023.165526

Arulanandu, A.M., Kalimuthu, V., Manimegalai, S.C., Venkatesan, R., Krishnamoorthy, S.P., Abdulkader, A.M., Balamuthu, K., 2024. Association of Atrazine-Induced Overexpression of Aldo–Keto-Reductase 1C2 (AKR1C2) with Hypoandrogenism and Infertility: An Experimental Study in Male Wistar Rat. Reproductive Sciences 2. https://doi.org/10.1007/s43032-024-01627-3

Aziz, A.R.L., Abdel-Wahab, A., Abo El-Ela, F.I., Hassan, N.E.H.Y., El-Nahass, E.S., Ibrahim, M.A., Khalil, A.T.A.Y., 2018. Dose- dependent ameliorative effects of quercetin and L-Carnitine against atrazine- induced reproductive toxicity in adult

male Albino rats. Biomedicine and Pharmacotherapy 102, 855–864. https://doi.org/10.1016/j.biopha.2018.03.136

Basini, G., Bianchi, Francesco, Bussolati, S., Baioni, L., Ramoni, R., Grolli, S., Conti, V., Bianchi, Federica, Grasselli, F., 2012. Atrazine disrupts steroidogenesis, VEGF and NO production in swine granulosa cells. Ecotoxicol Environ Saf 85, 59–63. https://doi.org/10.1016/j.ecoenv.2012.08.027

Belkacemi, L., Michael Nelson, D., Desai, M., Ross, M.G., 2010. Maternal undernutrition influences placental-fetal development. Biol Reprod 83, 325–331. https://doi.org/10.1095/biolreprod.110.084517

Benzie, I.F.F., Strain, J.J., 1996. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Analytical Biochemistry, 239(1), 70–76. doi:10.1006/abio.1996.0292

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

Bravo, A., Sánchez, R., Zambrano, F., Uribe, P., 2024. Exogenous Oxidative Stress in Human Spermatozoa Induces Opening of the Mitochondrial Permeability Transition Pore: Effect on Mitochondrial Function, Sperm Motility and Induction of Cell Death. Antioxidants 13, 1–18. https://doi.org/10.3390/antiox13060739

Bonting, S.L., Caravaggio, L.L., Hawkins, N.M., 1962. Studies on sodium-potassium activated adenosinetriphosphatase. IV. Correlation with cation transport sensitive to cardiac glycosides. Arch. Biochem. Biophys. 98 413–419, https://doi. org/10.1016/0003-9861(62)90206-0.

Buege, J.A., Aust, S.D., 1978. Biomembranes - Part C: Biological Oxidations. Methods Enzymol 52, 302–310.

Cai, W., Ji, Y., Song, X., Guo, H., Han, L., Zhang, F., Liu, X., Zhang, H., Zhu, B., Xu, M., 2017. Effects of glyphosate exposure on sperm concentration in rodents: A systematic review and meta-analysis. Environ Toxicol Pharmacol 55, 148–155. https://doi.org/10.1016/j.etap.2017.07.015

Carvalho, R.P.R., Ribeiro, F.C.D., Lima, T.I., Ervilha, L.O.G., de Oliveira, E.L., Faustino, A. de O., Lima, G.D. de A., Machado-Neves, M., 2022. High doses of eugenol cause structural and functional damage to the rat liver. Life Sci 304. https://doi.org/10.1016/j.lfs.2022.120696

Chen, J., Liu, J., Wu, S., Liu, W., Xia, Y., Zhao, J., Yang, Y., Wang, Y., Peng, Y., Zhao, S., 2021. Atrazine promoted epithelial ovarian cancer cells proliferation and metastasis by inducing low dose reactive oxygen species (ROS). Iran J Biotechnol 19, 89–100. https://doi.org/10.30498/IJB.2021.2623

Coimbra, J.L.P., Dantas, G. de P.F., de Andrade, L.M., Brener, M.R.G., Viana, P.I.M., Lopes, R.A., O.G Gontijo, D., Ervilha, L.O.G., Assis, M.Q., Barcelos, L.S., E. Szawka, R., Damasceno, D.C., Machado-Neves, M., Mota, A.P., Costa, G.M.J., 2023. Gold nanoparticle intratesticular injections as a potential animal sterilization tool: Long-term reproductive and toxicological implications. Toxicology 492. https://doi.org/10.1016/j.tox.2023.153543

Collins, A.R., Ma A-g, Duthie, S.J., 1995. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res DNA Repair 336:69–77.

Colli, L.G., Belardin, L.B., Echem, C., Akamine, E.H., Antoniassi, M.P., Andretta, R.R., Mathias, L.S., Rodrigues, S.F. de P., Bertolla, R.P., de Carvalho, M.H.C., de Paula Rodrigues, S.F., Bertolla, R.P., de Carvalho, M.H.C., 2019. Systemic arterial hypertension leads to decreased semen quality and alterations in the testicular

microcirculation in rats. Sci Rep 9, 11047. https://doi.org/10.1038/s41598-019-47157-w

Costas-Ferreira, C., Durán, R., Faro, L.R.F., 2022. Toxic Effects of Glyphosate on the Nervous System: A Systematic Review. Int J Mol Sci 23. https://doi.org/10.3390/ijms23094605

Couto-Santos, F., Guimarães-Ervilha, L.O., Carvalho, R.P.R., Bastos, D.S.S., Souza, A.C.F., da Silva, R.C., de Oliveira, L.L., de Oliveira, J.A., Machado-Neves, M., 2024. Impact of Early Arsenic Exposure on the Mineral Content and Oxidative Status of the Liver and Kidney of Pubescent and Adult Rats. Biol Trace Elem Res 202, 1644–1655. https://doi.org/10.1007/s12011-023-03787-w

Couto-Santos, F., Souza, A.C.F., Bastos, D.S.S., Ervilha, L.O.G., Dias, F.C.R., Araújo, L. de S., Guimarães, S.E.F., Oliveira, L.L. de, Machado-Neves, M., 2020. Prepubertal exposure to arsenic alters male reproductive parameters in pubertal and adult rats. Toxicol Appl Pharmacol 409. https://doi.org/10.1016/j.taap.2020.115304

Creasy, D., Bube, A., de Rijk, E., Kandori, H., Kuwahara, M., Masson, R., Nolte, T., Reams, R., Regan, K., Rehm, S., Rogerson, P., Whitney, K., 2012. Proliferative and nonproliferative lesions of the rat and mouse male reproductive system. Toxicol Pathol 40, 40–121. https://doi.org/10.1177/0192623312454337

Dieterich, S., Bieligk, U., Beulich, K., Hasenfuss, G., Prestle, J., 2000. Gene Expression of Antioxidative Enzymes in the Human Heart. Circulation 101, 33–39. https://doi.org/10.1161/01.CIR.101.1.33

Docea, A.O., Cirstea, A.E., Cercelaru, L., Drocas, A.I., Dinca, V., Mesnage, R., Marginean, C., Radu, A., Popa, D.G., Rogoveanu, O., Mitrut, R., Antoniou, M.N., Tsatsakis, A., Hernández, A.F., Calina, D., 2023. Effect of perinatal exposure to glyphosate and its mixture with 2,4-D and dicamba on rat dam kidney and thyroid

function and offspring's health. Environ Res 237. https://doi.org/10.1016/j.envres.2023.116908

Edwards, H., Javed, K., Yadev, K., Ara, C., Omer, A.M., 2024. Therapeutic potential of salvigenin to combat atrazine induced liver toxicity in rats via regulating Nrf-2/Keap-1 and NF-κB pathway. Pestic Biochem Physiol 202, 105966. https://doi.org/10.1016/j.pestbp.2024.105966

Eliasson, R., 1977. Supravital staining of human spermatozoa. Fertil Steril 28, 1257. https://doi.org/10.1016/s0015-0282(16)42927-4

Evans, D.J., 1969. Membrane adenosine triphosphatase of Escherichia coli: activation by calcium ion and inhibition by monovalent cations. J. Bacteriol. 100 914–922, https://doi.org/10.1128/jb.100.2.914-922.1969.

Filler, R., 1993. Methods for Evaluation of Rat Epididymal Sperm Morphology, Male Reproductive Toxicology. ACADEMIC PRESS, INC. https://doi.org/10.1016/b978-0-12-461207-5.50025-0

Gao, M., Yang, N., Lei, Y., Zhang, W., Liu, H., Lin, H., 2022. Tannic acid antagonizes atrazine exposure-induced autophagy and DNA damage crosstalk in grass carp hepatocytes via NO/iNOS/NF-κB signaling pathway to maintain stable immune function. Fish Shellfish Immunol 131, 1075–1084. https://doi.org/10.1016/j.fsi.2022.11.024

Gely-Pernot, A., Hao, C., Becker, E., Stuparevic, I., Kervarrec, C., Chalmel, F., Primig, M., Jégou, B., Smagulova, F., 2015. The epigenetic processes of meiosis in male mice are broadly affected by the widely used herbicide atrazine. BMC Genomics 16, 1–22. https://doi.org/10.1186/s12864-015-2095-y

Gianessi, L.P., 2013. The increasing importance of herbicides in worldwide crop production. Pest Manag Sci 69, 1099–1105. https://doi.org/10.1002/ps.3598

Giglio, A., Vommaro, M.L., 2022. Dinitroaniline herbicides: a comprehensive review of toxicity and side effects on animal non-target organisms. Environmental Science and Pollution Research 29, 76687–76711. https://doi.org/10.1007/s11356-022-23169-4

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr Rev 36, 1–150. https://doi.org/10.1210/er.2015-1010

Guimarães-Ervilha, L.O., Ladeira, L.C.M., Carvalho, R.P.R., Bento, I.P.D.S., Bastos, D.S.S., Souza, A.C.F., Santos, E.C., De Oliveira, L.L., Maldonado, I.R.D.S.C., MacHado-Neves, M., 2021. Green Tea Infusion Ameliorates Histological Damages in Testis and Epididymis of Diabetic Rats. Microscopy and Microanalysis 27, 1133–1145. https://doi.org/10.1017/S1431927621012071

Guimarães-Ervilha, L.O., Soares, L.L., Bento, I. da S.P., Carvalho, R.P.R., Lima, T.I., Dias, F.C.R., Bastos, D.S.S., Cossoin, J.F.S., Souza, A.C.F., Reis, E.C.C., Serrão, J.E., Natali, A.J., Machado-Neves, M., 2023. Effect of Induced Pulmonary Arterial Hypertension on Testicular Parameters of Wistar Rats Subjected to Resistance Exercise Training. Microscopy and Microanalysis 29, 635–648. https://doi.org/10.1093/micmic/ozac037

Guvvala, P.R., Ravindra, J.P., Selvaraju, S., 2020. Impact of environmental contaminants on reproductive health of male domestic ruminants: a review. Environmental Science and Pollution Research 27, 3819–3836. https://doi.org/10.1007/s11356-019-06980-4

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249, 7130–9.

Hadeed, M.N., Castiglione, C.L., Saleem, S., Chammout, D.H., Muskovac, M.D., Crile, K.G., Abdulelah, S.A., Maalhagh-Fard, A., Rampuri, E.Y., Grabowski, G.M., Belanger, R.M., 2022. Environmentally relevant atrazine exposure leads to increases in DNA damage and changes in morphology in the hepatopancreas of crayfish (*Faxonius virilis*). Environmental Advances 10. https://doi.org/10.1016/j.envadv.2022.100320

Harchegani, B.A., Dahan, H., Tahmasbpour, E., Bakhtiari kaboutaraki, H., Shahriary, A., 2020. Effects of zinc deficiency on impaired spermatogenesis and male infertility: the role of oxidative stress, inflammation and apoptosis. Hum Fertil 23, 5–16. https://doi.org/10.1080/14647273.2018.1494390

Hedger, M.P., 2002. Macrophages and the immune responsiveness of the testis. J Reprod Immunol 57, 19–34. https://doi.org/10.1016/S0165-0378(02)00016-5

Heinrich, A., DeFalco, T., 2020. Essential roles of interstitial cells in testicular development and function. Andrology 8, 903–914. https://doi.org/10.1111/andr.12703

Hjertén, S., Pan, H., 1983. Purification and characterization of two forms of a low-affinity Ca^{2+}-ATPase from erythrocyte membranes. Biochim. Biophys. ActaBiomembr. 728 281–288, https://doi.org/10.1016/0005-2736(83)90480-7.

Hood, R.D., 2012. Developmental and Reproductive Toxicology: A Practical Approach, 3rd ed., CRC Press, pp. 1168.

Ikeji, C.N., Adedara, I.A., Farombi, E.O., 2023. Dietary myricetin assuages atrazine-mediated hypothalamic-pituitary–testicular axis dysfunction in rats. Environmental Science and Pollution Research 30, 15655–15670. https://doi.org/10.1007/s11356-022-23033-5

Indo, H.P., Yen, H.C., Nakanishi, I., Matsumoto, K.I., Tamura, M., Nagano, Y., Matsui, H., Gusev, O., Cornette, R., Okuda, T., Minamiyama, Y., Ichikawa, H., Suenaga, S., Oki, M., Sato, T., Ozawa, T., Clair, D.K.S., Majima, H.J., 2015. A mitochondrial superoxide theory for oxidative stress diseases and aging. J Clin Biochem Nutr 56, 1–7. https://doi.org/10.3164/jcbn.14-42

Jomova, K., Makova, M., Alomar, S.Y., Alwasel, S.H., Nepovimova, E., Kuca, K., Rhodes, C.J., Valko, M., 2022. Essential metals in health and disease. Chem Biol Interact 367, 110173. https://doi.org/10.1016/j.cbi.2022.110173

Kempinas, W.G., Klinefelter, G.R., 2014. Interpreting histopathology in the epididymis. Spermatogenesis, v. 4, n. 2, p. e979114.

Kimura, M., Ishii, M.N., Seki, N., Sakai, Y., Yamashita, T., Awatsuji, H., Kanda, K., Matsumoto, K., Matsui, H., 2019. Reduction of Kiss1 expression in the anteroventral periventricular nucleus is associated with atrazine-induced attenuation of the luteinizing hormone surge in female rats. Biol Reprod 100, 41–48. https://doi.org/10.1093/biolre/ioy159

Kumar, V., Upadhyay, N., Singh, S., Singh, J., Kaur, P., 2013. Thin-Layer Chromatography: Comparative Estimation of Soil's Atrazine. Current World Environment Journal 8, 469–472. https://doi.org/10.12944/cwe.8.3.17

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 233, 346–357. https://doi.org/10.1016/S0076-6879(94)33040-9

Lima, G.D. de A., Sertorio, M.N., Souza, A.C.F., Menezes, T.P., Mouro, V.G.S., Gonçalves, N.M., Oliveira, J.M. de, Henry, M., Machado-Neves, M., 2018. Fertility in male rats: Disentangling adverse effects of arsenic compounds. Reproductive Toxicology 78, 130–140. https://doi.org/10.1016/j.reprotox.2018.04.015

Liu, L., Li, M.Z., Yao, M.H., Yang, T.N., Tang, Y.X., Li, J.L., 2024. Melatonin inhibits atrazine-induced mitochondrial impairment in cerebellum of mice: Modulation of cGAS-STING-NLRP3 axis-dependent cell pyroptosis. Science of the Total Environment 912. https://doi.org/10.1016/j.scitotenv.2023.168924

Liu, W., Du, Y., Liu, J., Wang, H., Sun, D., Liang, D., Zhao, L., Shang, J., 2014. Effects of atrazine on the oxidative damage of kidney in Wister rats. Int J Clin Exp Med 7, 3235–3243.

Maggi, R., Cariboni, A.M., Marelli, M.M., Moretti, R.M., Andrè, V., Marzagalli, M., Limonta, P., 2016. GnRH and GnRH receptors in the pathophysiology of the human female reproductive system. Hum Reprod Update 22, 358–381. https://doi.org/10.1093/humupd/dmv059

Martins-Santos, E., Pimenta, C.G., Campos, P.R.N., Franco, M.B., Gomes, D.A., Mahecha, G.A.B., Oliveira, C.A., 2017. Persistent testicular structural and functional alterations after exposure of adult rats to atrazine. Reproductive Toxicology 73, 201–213. https://doi.org/10.1016/j.reprotox.2017.08.010

Mendaš, G., Milićević, T., Fingler, S., Drevenkar, V., Romanić, S.H., Popović, A., Relić, D., 2023. Human health risk assessment based on direct and indirect exposure to endocrine disrupting herbicides in drinking, ground, and surface water in Croatia. Environmental Science and Pollution Research 30, 106330–106341. https://doi.org/10.1007/s11356-023-29561-y

Mesnage, R., Biserni, M., Wozniak, E., Xenakis, T., Mein, C.A., Antoniou, M.N., 2018. Comparison of transcriptome responses to glyphosate, isoxaflutole, quizalofop-p-ethyl and mesotrione in the HepaRG cell line. Toxicol Rep 5, 819–826. https://doi.org/10.1016/j.toxrep.2018.08.005

Mohammed, E.T., Safwat, G.M., Bahnasawy, E.A., Abdel-Razik, A.R.H., Mohamed, D.S., 2023. Zinc Oxide Nanoparticles and Vitamin C Ameliorate Atrazine-Induced Hepatic Apoptosis in Rat via CYP450s/ROS Pathway and Immunomodulation. Biol Trace Elem Res 201, 5257–5271. https://doi.org/10.1007/s12011-023-03587-2

Moin, N., Thakur, R.S., Singh, S., Patel, D.K., Satish, A., 2023. β-triketone herbicide exposure cause tyrosine and fat accumulation in Caenorhabditis elegans. Chemosphere 326. https://doi.org/10.1016/j.chemosphere.2023.138353

Morakinyo, A.O., Achema, P.U., Adegoke, O.A., 2010. Effect of Zingiber officinale (ginger) on sodium arsenite-induced reproductive toxicity in male rats. African Journal of Biomedical Research 13, 39–45.

Morabbi, A., Karimian, M., 2024. Trace and essential elements as vital components to improve the performance of the male reproductive system: Implications in cell signaling pathways. Journal of Trace Elements in Medicine and Biology 83, 127403. https://doi.org/10.1016/j.jtemb.2024.127403

Mostafalou, S., Abdollahi, M., 2017. Pesticides: an update of human exposure and toxicity. Arch Toxicol 91, 549–599. https://doi.org/10.1007/s00204-016-1849-x

Năstăsescu, V., Mititelu, M., Goumenou, M., Docea, A.O., Renieri, E., Udeanu, D.I., Oprea, E., Arsene, A.L., Dinu-Pîrvu, C.E., Ghica, M., 2020. Heavy metal and pesticide levels in dairy products: Evaluation of human health risk. Food and Chemical Toxicology 146, 111844. https://doi.org/10.1016/j.fct.2020.111844

Nicolson, G.L., Ferreira de Mattos, G., 2022. Fifty Years of the Fluid–Mosaic Model of Biomembrane Structure and Organization and Its Importance in Biomedicine with Particular Emphasis on Membrane Lipid Replacement. Biomedicines 10. https://doi.org/10.3390/biomedicines10071711

O'Donnell, L., Smith, L.B., Rebourcet, D., 2022. Sertoli cells as key drivers of testis function. Semin Cell Dev Biol 121, 2–9. https://doi.org/10.1016/j.semcdb.2021.06.016

Ohnishi, T., Suzuki, T., Suzuki, Y., Ozawa, K., 1982. A comparative study of plasma membrane Mg^{2+}-ATPase activities in normal, regenerating and malignant cells, Biochim. Biophys. ActaBiomembr. 684 67–74, https://doi.org/10.1016/ 0005-2736(82)90050-5.

Piancini, L.D.S., Guiloski, I.C., de Assis, H.C.S., Cestari, M.M., 2015. Mesotrione herbicide promotes biochemical changes and DNA damage in two fish species. Toxicol Rep 2, 1157–1163. https://doi.org/10.1016/j.toxrep.2015.08.007

Prolo, C., Piacenza, L., Radi, R., 2024. Peroxynitrite: a multifaceted oxidizing and nitrating metabolite. Curr Opin Chem Biol 80, 102459. https://doi.org/10.1016/j.cbpa.2024.102459

Qu, R.Y., He, B., Yang, J.F., Lin, H.Y., Yang, W.C., Wu, Q.Y., Li, Q.X., Yang, G.F., 2021. Where are the new herbicides? Pest Manag Sci 77, 2620–2625. https://doi.org/10.1002/ps.6285

Ricci, E.L., Zaccarelli-Magalhães, J., Pantaleon, L.P., De-Paula, L.R., Ribeiro, G.M., Abreu, G.R., Muñoz, J.W.P., Mendonça, M., Fukushima, A.R., Spinosa, H.S., 2024. Peripubertal exposure of atrazine cause decrease in exploratory activity, deficits in sociability and few alterations on brain monoaminergic systems of rats. Toxicol Appl Pharmacol 483. https://doi.org/10.1016/j.taap.2024.116819

Robb, G.W., Amann, R.P., Killian, G.J., 1978. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil 54, 103–107. https://doi.org/10.1530/jrf.0.0540103

Rotimi, D.E., Ojo, O.A., Adeyemi, O.S., 2023. Atrazine exposure caused oxidative stress in male rats and inhibited brain-pituitary-testicular functions. J Biochem Mol Toxicol 38, 1–11. https://doi.org/10.1002/jbt.23579

Rungta, R.L., Choi, H.B., Tyson, J.R., Malik, A., Dissing-Olesen, L., Lin, P.J.C., Cain, S.M., Cullis, P.R., Snutch, T.P., Macvicar, B.A., 2015. The cellular mechanisms of neuronal swelling underlying cytotoxic edema. Cell 161, 610–621. https://doi.org/10.1016/j.cell.2015.03.029

Smith, P.N., Armbrust, K.L., Brain, R.A., Chen, W., Galic, N., Ghebremichael, L., Giddings, J.M., Hanson, M.L., Maul, J., Van Der Kraak, G., Solomon, K.R., 2021. Assessment of risks to listed species from the use of atrazine in the USA: a perspective. J Toxicol Environ Health B Crit Rev 24, 223–306. https://doi.org/10.1080/10937404.2021.1902890

Solomon, R.D.J., Kumar, A., Satheeja Santhi, V., 2013. Atrazine biodegradation efficiency, metabolite detection, and trzD gene expression by enrichment bacterial cultures from agricultural soil. J Zhejiang Univ Sci B 14, 1162–1172. https://doi.org/10.1631/jzus.B1300001

Soloviev, A., Sydorenko, V., 2024. Oxidative and Nitrous Stress Underlies Vascular Malfunction Induced by Ionizing Radiation and Diabetes. Cardiovasc Toxicol. https://doi.org/10.1007/s12012-024-09878-x

Souza, A.C.F., Bastos, D.S.S., Couto-Santos, F., Guimarães-Ervilha, L.O., Araújo, L.S., Souza, P.H.C.A., Coimbra, J.L.P., Oliveira, L.L., Guimarães, S.E.F., Machado-Neves, M., 2023. Long-term reproductive effects in male rats prenatally exposed to sodium arsenite. Environ Toxicol 38, 1162–1173. https://doi.org/10.1002/tox.23756

Souza, A.C.F., Ervilha, L.O.G., Coimbra, J.L.P., Bastos, D.S.S., Guimarães, S.E.F., Machado-Neves, M., 2020. Reproductive disorders in female rats after prenatal

exposure to sodium arsenite. Journal of Applied Toxicology 40, 214–223. https://doi.org/10.1002/jat.3897

Souza A.C.F., Marchesi S.C., Lima G.D.A.L., Ferraz R.P., Santos F.C., da Matta S.L.P., Machado-Neves M., 2016. Effects of sodium arsenite and arsenate in testicular histomorphometry and antioxidants enzymes activities in rats. Biol. Trace Elem. Res. 171, 354-62. doi:10.1007/s12011-01505230

Stradtman, S.C., Freeman, J.L., 2021. Mechanisms of neurotoxicity associated with exposure to the herbicide atrazine. Toxics 9. https://doi.org/10.3390/toxics9090207

Sullivan, R., Saez, F., Girouard, J., Frenette, G., 2005. Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells Mol Dis 35, 1–10. https://doi.org/10.1016/j.bcmd.2005.03.005

Thankamony, A., Pasterski, V., Ong, K.K., Acerini, C.L., Hughes, I.A., 2018. Europe PMC Funders Group Anogenital distance as a marker of androgen exposure in humans 4, 616–625. https://doi.org/10.1111/andr.12156.Anogenital

Tsikas, D., 2007. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the l-arginine/nitric oxide area of research. J Chromatogr B Analyt Technol Biomed Life Sci 851, 51–70. https://doi.org/10.1016/j.jchromb.2006.07.054

Tvrda, E., Peer, R., Sikka, S.C., Agarwal, A., 2015. Iron and copper in male reproduction: a double-edged sword. J Assist Reprod Genet 32, 3–16. https://doi.org/10.1007/s10815-014-0344-7

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K.C., Finckh, M.R., Morris, J.G., 2018. Environmental and health effects of the herbicide glyphosate. Science of the Total Environment 616–617, 255–268. https://doi.org/10.1016/j.scitotenv.2017.10.309

Victor-Costa, A.B., Bandeira, S.M.C., Oliveira, A.G., Mahecha, G.A.B., Oliveira, C.A., 2010. Changes in testicular morphology and steroidogenesis in adult rats exposed to Atrazine. Reproductive Toxicology 29, 323–331. https://doi.org/10.1016/j.reprotox.2009.12.006

Wang, C., Harwood, J.D., Zhang, Q., 2018. Oxidative stress and DNA damage in common carp (Cyprinus carpio) exposed to the herbicide mesotrione. Chemosphere 193, 1080–1086. https://doi.org/10.1016/j.chemosphere.2017.11.148

Wu, D., Pandupuspitasari, N.S., Zhang, K., Tang, Y., Khan, F.A., Li, H., Huang, C., Sun, F., 2023. Cytoskeletal orchestration of glucose uptake in Sertoli cell to support efferocytosis of apoptotic germ cells. Biochim Biophys Acta Mol Cell Res 1870. https://doi.org/10.1016/j.bbamcr.2023.119434

Zhang, C., Liu, Y., Wang, P., Wang, B., Zhang, S., Hua, Z., Li, Y., Wang, X., Yang, X., 2024. Effects of Se-enriched yeast on the amelioration of atrazine-induced meat quality degradation. Food Chem. Oct 1;454:139737. doi: 10.1016/j.foodchem.2024.139737. Epub 2024 May 20. PMID: 38795622.

Zhang, L., Rana, I., Shaffer, R.M., Taioli, E., Sheppard, L., 2019. Exposure to glyphosate-based herbicides and risk for non-Hodgkin lymphoma: A meta-analysis and supporting evidence. Mutat Res Rev Mutat Res 781, 186–206. https://doi.org/10.1016/j.mrrev.2019.02.001

Ziouziou, I., Rambhatla, A., Shah, R., Agarwal, A., 2024. Sperm DNA fragmentation and infertility: a narrative review. World J Urol 42, 1–8. https://doi.org/10.1007/s00345-024-05090-2

CONCLUSÕES GERAIS

De forma abrangente, avaliamos os efeitos dos herbicidas à base de triazina, com foco na atrazina e sua combinação com a mesotriona, sobre a saúde reprodutiva masculina. Através de uma revisão sistemática e meta-análise, foi possível evidenciar que a atrazina atua como um desregulador endócrino significativo, provocando alterações hormonais no eixo hipotálamo-hipófise-gônada (HPG). Esses efeitos incluem reduções nos níveis de hormônios essenciais para a reprodução, como o hormônio folículo estimulante, hormônio luteinizante e testosterona, além de elevações de estradiol e progesterona, o que pode causar prejuízos à fertilidade. É importante ressaltar o foco toxicológico dos estudos incluídos na revisão. As concentrações de atrazina administradas são bem superiores às permitidas por agências de controle da qualidade da água. Assim, os trabalhos trazem uma visão mais toxicológica do que ecotoxicológica, distanciando de um entendimento de riscos reais em áreas contaminadas com o herbicida. Por exemplo, como mostramos, concentrações > 100 mg Kg^{-1} de atrazina são as responsáveis pelos efeitos tóxicos mais evidentes do herbicida, valor muito acima do permitido e encontrado em áreas contaminadas. Embora novos estudos sobre a ação da atrazina estejam sendo publicados, ainda não há relatos se o herbicida é capaz de prejudicar a fertilidade de machos, uma lacuna ainda em aberto.

Nosso estudo experimental sobre a toxicidade subcrônica do herbicida atrazina combinado com o herbicida mesotriona, comercialmente conhecido como Calaris®, confirma que a exposição prolongada a esses compostos combinados pode resultar em danos ao sistema reprodutivo, como estresse oxidativo, desorganização do parênquima testicular, danos ao DNA nas células testiculares, baixa produção espermática e disfunção mitocondrial dos espermatozoides. O Calaris® é vendido como a evolução da atrazina. Estes efeitos, observados mesmo na ausência de alterações significativas nos níveis de

testosterona, sugerem que os mecanismos de toxicidade são complexos, para além de alterações nos níveis do andrógeno, mesmo considerando os efeitos em órgãos andrógeno-dependentes. Esses resultados corroboram com outros estudos sobre a necessidade de reavaliar as normas de segurança relativas ao uso de herbicidas. Por exemplo, não há regulação para os níveis permitidos de mesotriona na água. Além de evidenciar a importância de práticas agrícolas conscientes. A continuidade da pesquisa sobre os efeitos dos agroquímicos é essencial para garantir que as tecnologias agrícolas evoluam em harmonia com as necessidades de preservação da saúde e do meio ambiente.