UNIVERSIDADE FEDERAL DO PAMPA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

DIOGO FERREIRA BICCA

AVALIAÇÃO DO EFEITO DA EXPOSIÇÃO A HERBICIDAS SOBRE A QUALIDADE E FUNÇÃO DE ESPERMATOZOIDES BOVINOS

Uruguaiana
2025

DIOGO FERREIRA BICCA

AVALIAÇÃO DO EFEITO DA EXPOSIÇÃO A HERBICIDAS SOBRE A QUALIDADE E FUNÇÃO DE ESPERMATOZOIDES BOVINOS

Trabalho apresentado ao Programa de Pós-graduação *Stricto sensu* em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutor em Bioquímica.

Orientadora: Francielli Weber Santos Cibin
Coorientadora: Daniela dos Santos Brum

Uruguaiana
2025

Ficha catalográfica elaborada automaticamente com os dados fornecidos
pelo(a) autor(a) através do Módulo de Biblioteca do
Sistema GURI (Gestão Unificada de Recursos Institucionais) .

B583a Bicca, Diogo Ferreira
AVALIAÇÃO DO EFEITO DA EXPOSIÇÃO A HERBICIDAS SOBRE A QUALIDADE E FUNÇÃO DE ESPERMATOZÓIDES BOVINOS / Diogo Ferreira Bicca.
75 p.

Tese(Doutorado)-- Universidade Federal do Pampa, DOUTORADO EM CIÊNCIA ANIMAL, 2025.
"Orientação: Francielli Weber Santos Cibin".

1. herbicidas. 2. bovinos. 3. espermatozoides. 4. toxicologia. 5. reprodução. I. Título.

DIOGO FERREIRA BICCA

AVALIAÇÃO DO EFEITO DA EXPOSIÇÃO A HERBICIDAS SOBRE A QUALIDADE E FUNÇÃO DE ESPERMATOZOIDES BOVINOS

Tese apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutor em Bioquímica.

Tese defendida e aprovada em: 29 de setembro de 2025.

Banca examinadora:

_____

Profa. Dr[a] Francielli Weber Santos Cibin
Orientadora
UNIPAMPA

_____

Profa . Dr[a]. Janislene Mach Trentin
UFPR

_____

Profa . Dr[a]. Juliana Germano Ferst
USP

_____

Prof. Dr. Robson Luiz Puntel
UNIPAMPA

AGRADECIMENTOS

Ao final desta etapa gostaria de agradecer, primeiramente, a minha orientadora Prof[a.] Dr[a] Francielli Weber Santos Cibin e a coorientadora Prof[a.] Dr[a] Daniela dos Santos Brum por todos os ensinamentos e incentivo para que esta jornada fosse possível. A elas dedico esta tese pois foram fundamentais para o meu crescimento pessoal e profissional.

Às minhas colegas de laboratório, Kimberly e Carol por todo o companheirismo e partilha de momentos leves, e por tornarem o "nosso laboratório" um local difícil de ir embora.

Às minhas colegas de BIOTECH, Rafa, Laura, Lari e demais estagiários os quais tive o prazer de conviver e realizar os experimentos do doutorado. Muito obrigado pelo apoio em todas as etapas desse trabalho.

Às minhas colegas Camila, Melina e Fabi, pelo apoio incondicional, compreensão e presença durante todo esse caminho.

Ao Prof. Dr. Fábio Leivas, pela parceria nos experimentos, assim como a paciência de ensinar os procedimentos que usamos durante as análises no laboratório.

À Monike Quirino, obrigado por me apresentar o mundo da citometria de fluxo, e também por ser um exemplo de trabalho e dedicação a qual tive a oportunidade de conhecer com o doutorado, e hoje tenho como uma amiga.

À minha namorada, noiva, companheira Luana, por todo apoio e incentivo, pela presença dos momentos alegres e de "desespero". Sou muito grato por compartilhar contigo estas conquistas, te amo.

À minha vó Inah, meu exemplo de vida e amor. Agradeço pela possibilidade de poder dar mais essa notícia boa. Sempre presente, obrigado por existir.

A todos aqueles que me auxiliaram no desenvolvimento deste trabalho e contribuíram para sua realização, assim como ao Programa de Pós Graduação em Bioquímica e a UNIPAMPA pela oportunidade, e a CAPES pelo suporte à realização deste trabalho.

Muito obrigado.

“Eu que nunca fui assim, muito de ganhar

Junto as mãos ao meu redor

Faço o melhor que sou capaz

Só pra viver em paz”.

(O vencedor, Los Hermanos)

RESUMO

Os herbicidas são a categoria de pesticidas mais utilizada no mundo. No Brasil, as formulações com glifosato (Gli), ácido diclorofenoxiacético (2,4-D) e atrazina (Atz) são intensivamente aplicadas nas lavouras e culturas, e a ocorrência de resíduos em produtos e no ambiente é um fator preocupante. Devido à proximidade dessas áreas e ao manejo, os bovinos são expostos a uma diversidade de pesticidas, e o impacto sobre suas funções reprodutivas é desconhecido. Neste contexto, avaliamos os efeitos de concentrações biologicamente relevantes dos ingredientes ativos dos herbicidas Gli, 2,4-D e Atz, sobre a qualidade e função de espermatozoides de bovinos. No primeiro estudo, um pool de sêmen de quatro touros foi incubado a 37°C por 1 e 3 horas em meio TALP-Fert e tratado da seguinte forma: grupo controle (veículo dimetilsulfóxido/DMSO); grupo Gli: 5 (G5), 36 (G36) e 50 (G50) µg/mL; grupo 2,4-D: 0,5 (D05), 1 (D1) e 5 (D5) µM; e grupo Atz: 0,05 (A005), 0,1 (A01) e 1 (A1) µM. A avaliação da cinética com o sistema CASA (*Computer Assisted Sperm Analysis*) permitiu verificar que o tempo de incubação e tratamento com os herbicidas afetaram os parâmetros cinéticos espermáticos, sem ocorrência de interação entre as variáveis. A motilidade total foi reduzida no tratamento com Atz em relação ao controle, enquanto a motilidade progressiva demonstrou redução devido ao tratamento com os três herbicidas. Os parâmetros cinéticos relativos à velocidade foram reduzidos significativamente em comparação ao controle, pelo tratamento com o Gli. Adicionalmente, este grupo também se diferenciou quanto a velocidade curvilínea (VCL) em relação aos espermatozoides tratados com 2,4-D. Embora não tenham ocorrido diferenças entre os grupos de tratamento quantos aos parâmetros de amplitude de deslocamento lateral da cabeça e batimento flagelar cruzado, a hiperatividade foi reduzida pelos três grupos de tratamento com herbicidas, em relação ao controle. A análise morfológica dos espermatozoides evidenciou o aumento de alterações significativas nos grupos G50, D5 e no Atz como um todo, representadas pelo percentual de defeitos maiores em comparação com o controle quanto a ocorrência espermatozoides com danos ou ausência do acrossoma, e com cauda fortemente enrolada. O teste hiposmótico (HOS) demonstrou que a funcionalidade da membrana plasmática foi alterada apenas pelos tratamentos A005 e A01. Quanto ao estresse oxidativo, não houveram diferenças significativas quanto a geração de espécies reativas de oxigênio ou no aumento da peroxidação lipídica nos tratamentos com herbicidas e em relação aos períodos avaliados. No entanto, a capacidade antioxidante foi reduzida em decorrência do tempo de incubação e devido ao tratamento com 2,4-D. Por fim, as taxas de fertilização de oócitos não tratados avaliadas no protocolo de fertilização *in vitro* demonstraram que a

capacidade de fertilização foi reduzida em espermatozoides tratados com 2,4-D, em comparação com o grupo controle. Apesar de as taxas de fertilização diminuírem apenas na concentração selecionada de 2,4-D, verificamos a capacidade dos três herbicidas analisados comprometerem parâmetros morfofuncionais de espermatozoides bovinos. No segundo trabalho, avaliamos o efeito *in vitro* de misturas de herbicidas sobre espermatozoides bovinos. O sêmen de quatro touros foi preparado como um pool e dividido em grupos de tratamento: Controle, DMSO, Gli (glifosato 50 µg/mL), 2,4-D (2,4-D 0,5 µM), Atz (atrazina 0,05 µM), GD (Gly+2,4-D), GA (Gli+Atz), AD (Atz+2,4-D) e GDA (Gli+2,4-D+Atz). Após 3 horas de incubação a 37ºC, as células espermáticas foram avaliadas. Os resultados dos parâmetros cinéticos medidos com CASA demonstraram efeitos antagônicos dos herbicidas isolados e em misturas. Os grupos Gli, 2,4-D e AD promoveram reduções significativas na motilidade progressiva, velocidade média do trajeto e nas frequências de batimento flagelar, em relação ao Controle. Os grupos Gli e 2,4-D reduziram as VCL e a velocidade linear, enquanto o 2,4-D também afetou a amplitude do deslocamento lateral da cabeça. Apesar de não haverem sido detectadas diferenças nos níveis de espécies reativas, uma redução na capacidade antioxidante ocorreu em todos os tratamentos. Os parâmetros de citometria de fluxo (integridade da membrana, dano ao acrossoma e potencial da membrana mitocondrial) não apresentaram diferença significativa entre os grupos. No entanto, a avaliação da fertilização *in vitro* identificou que as misturas diminuíram a capacidade de fertilização dos espermatozoides nos grupos GD, AD e GDA, em comparação com o controle. Os resultados demonstraram que mesmo com os efeitos distintos desses compostos sobre parâmetros espermáticos bovinos, as consequências na capacidade de fertilização revelam a importância de investigar a exposição a pesticidas no contexto das misturas.

Palavras-chave: herbicidas, glifosato, 2,4-D, atrazina, espermatozoides, bovinos, fertilidade.

ABSTRACT

Herbicides are the most widely used category of pesticides worldwide. In Brazil, formulations containing glyphosate (Gly), 2,4-dichlorophenoxyacetic acid (2,4-D), and atrazine (Atz) are intensively applied to crops and plantations, and the presence of residues in products and the environment is a concerning factor. Due to the proximity of these areas and management practices, cattle are exposed to a variety of pesticides, and the impact on their reproductive functions remains unknown. In this context, we evaluated the effects of biologically relevant concentrations of the active ingredients of the herbicides Gly, 2,4-D, and Atz on the quality and function of bovine sperm. In the first study, a semen pool from four bulls was incubated at 37°C for 1 and 3 hours in TALP-Fert medium and treated as follows: control group (vehicle dimethyl sulfoxide/DMSO); Gly group: 5 (G5), 36 (G36), and 50 (G50) µg/mL; 2,4-D group: 0.5 (D05), 1 (D1), and 5 (D5) µM; and Atz group: 0.05 (A005), 0.1 (A01), and 1 (A1) µM. Kinetic evaluation using the CASA (Computer Assisted Sperm Analysis) system revealed that incubation time and herbicide treatment affected sperm kinetic parameters, without interaction effects. Total motility was reduced in the Atz treatment compared to the control, while progressive motility showed reductions due to all three herbicide treatments. Velocity-related kinetic parameters were significantly reduced by Gly treatment compared to the control. Additionally, this group also differed in curvilinear velocity (VCL) compared to sperm treated with 2,4-D. Although no differences were observed among treatment groups regarding lateral head displacement amplitude and cross-flagellar beat parameters, hyperactivity was reduced in all herbicide-treated groups compared to the control. Morphological analysis of sperm revealed a significant increase in abnormalities in the G50, D5, and Atz groups, represented by a higher percentage of major defects compared to the control, including sperm with damaged or absent acrosomes and tightly coiled tails. The hypo-osmotic swelling (HOS) test showed that plasma membrane integrity was altered only by A005 and A01 treatments. Regarding oxidative stress, no significant differences were found in reactive oxygen species generation or increased lipid peroxidation across herbicide treatments or incubation periods. However, antioxidant capacity was reduced due to incubation time and 2,4-D treatment. Finally, fertilization rates of untreated oocytes assessed via in vitro fertilization protocol showed that fertilization capacity was reduced in sperm treated with 2,4-D compared to the control

group. Although fertilization rates decreased only at the selected 2,4-D concentration, we observed that all three herbicides compromised morphofunctional parameters of bovine sperm. In the second study, we evaluated the in vitro effect of herbicide mixtures on bovine sperm. Semen from four bulls was pooled and divided into treatment groups: Control, DMSO, Gly (glyphosate 50 µg/mL), 2,4-D (0.5 µM), Atz (atrazine 0.05 µM), GD (Gly+2,4-D), GA (Gly+Atz), AD (Atz+2,4-D), and GDA (Gly+2,4-D+Atz). After 3 hours of incubation at 37°C, sperm cells were evaluated. CASA-measured kinetic parameters showed antagonistic effects of isolated and mixed herbicides. Gli, 2,4-D, and AD groups significantly reduced progressive motility, average path velocity, and flagellar beat frequency compared to the control. Gly and 2,4-D reduced VCL and linear velocity, while 2,4-D also affected lateral head displacement amplitude. Although no differences were detected in reactive species levels, antioxidant capacity was reduced in all treatments. Flow cytometry parameters (membrane integrity, acrosome damage, and mitochondrial membrane potential) showed no significant differences among groups. However, in vitro fertilization assessment revealed that mixtures reduced sperm fertilization capacity in GD, AD, and GDA groups compared to the control. These results demonstrate that despite the distinct effects of these compounds on bovine sperm parameters, the consequences for fertilization capacity highlight the importance of investigating pesticide exposure in the context of mixtures.

Keywords: herbicides, glyphosate, 2,4-D, atrazine, spermatozoa, cattle, fertility.

LISTA DE FIGURAS

REFERENCIAL TEÓRICO

ARTIGO 1 - Herbicide Exposure Impairs The Morphofunctional Parameters Of Bovine Sperm

ARTIGO 2 - Effects of Herbicide Mixtures On The Fertilizing Capacity Of Bovine Sperm

LISTA DE QUADROS

REFERENCIAL TEÓRICO

LISTA DE TABELAS

ARTIGO 1 - Herbicide Exposure Impairs The Morphofunctional Parameters Of Bovine Sperm

ARTIGO 2 - Effects of Herbicide Mixtures On The Fertilizing Capacity Of Bovine Sperm

LISTA DE SIGLAS

2,4-D – ácido 2,4 diclorofenoxiacético

ALC – amplitude do deslocamento lateral da cabeça

Atz – atrazina

BFC – batimento flagelar cruzado

CASA – C*omputer-Assisted Semen Analysis*

COC – complexo cumulus-oócito

DMSO – dimetilsulfóxido

DNA – ácido desoxirribonucléico

EPSP – 5-enolpiruvilchiquimato-3-fostato sintetase

EROS – espécies reativas de oxigênio

FAO – *Food and Agriculture Organization*

FIV – fertilização *in vitro*

Gli – glifosato

IARC - *International Agency for Research on Cancer*

OGM – organismo geneticamente modificado

OMS -Organização Mundial da Saúde

SCA – *Sperm Class Analyzer*

VCL – velocidade curvilínea

VSL – velocidade linear

VAP – velocidade média do trajeto

SUMÁRIO

1) INTRODUÇÃO

Atualmente, os herbicidas consistem na categoria de pesticidas agrícolas mais utilizada no mundo em decorrência da crescente demanda por alimentos. A necessidade de um uso cada vez mais intenso desses compostos vem de encontro com a preocupação gerada pelo grau de exposição e associação desse fato com a ocorrência de problemas de saúde e impactos no meio ambiente como um todo (MESNAGE et al., 2021).

O Brasil é consumidor significativo de grande número e variedade de pesticidas, inclusive os denominados "extremamente perigosos" (*Highly Hazardous Pesticides* - HHP), banidos em diversos países (FERNANDES et al., 2020). Entre os pesticidas mais utilizados no país citam-se os herbicidas a base de glifosato, seguidos pelas formulações com ácido diclorofenoxiacético (2,4-D) e de atrazina (IBAMA, 2024), cujos níveis em águas superficiais já representam médio a alto risco ambiental em mais da metade dos estados brasileiros (BROVINI et al.,2021). A aquisição de resistência a pesticidas por espécies vegetais indesejáveis na produção agrícola, bem como o aumento da utilização de organismos geneticamente modificados (OGM), tem estimulado o manejo através da aplicação de um volume cada vez maior de herbicidas, os quais podem ser utilizados de forma isolada ou em conjunto para garantia de melhores resultados (GAZZIERO, 2015; ALMEIDA et al., 2017).

Contudo, a ação destes compostos não se restringe aos efeitos no controle de espécies vegetais não desejáveis, visto que o aumento da exposição tem sido correlacionado ao comprometimento da sanidade em organismos não-alvo (SENGUPTA; BANERJEE, 2014). A reprodução é um dos processos biológicos mais afetados pela exposição aos pesticidas, os quais podem atuar como perturbadores endócrinos, mas também como promotores de danos diretos em órgãos do sistema reprodutivo mediante mecanismos de toxicidade (VURAL et al., 2016; SADEGHNIA et al., 2022; STONE et al., 2025). Devido aos efeitos negativos a reprodução, a exposição a pesticidas como os herbicidas tem sido elencada como uma das possíveis causas para o aumento da infertilidade global, da ocorrência de abortos espontâneos e da frequência de doenças nas gerações seguintes no que diz respeito a espécie humana (FUCIC et al., 2021).

No que se refere às consequências sobre o sistema reprodutor masculino, a exposição a compostos a base de glifosato, 2,4-D e atrazina foram associadas a níveis reduzidos do hormônio testosterona (PANUWET et al., 2018; SADEGHNIA et al., 2022;

BRANCO et al., 2025). Além da perturbação hormonal, estudos com roedores permitiram verificar a ocorrência de danos em órgãos reprodutores, como testículo e epidídimo, bem como no comprometimento da estrutura e função das células de Sertoli, Leydig e da linhagem germinativa, decorrentes de tratamentos com estes herbicidas (CLAIR et al., 2012; SONG et al., 2014; MAROUANI et al., 2017). Consequentemente, poluentes ambientais como os herbicidas, tem o potencial de impactar a espermatogênese de forma direta e indireta, afetando negativamente a qualidade espermática (SELVARAJU et al., 2021).

Contudo, devido ao aumento da exposição, a detecção de herbicidas em fluidos corporais como o sêmen é um achado recorrente, o que demonstra que os espermatozoides permanecem expostos a estes agentes, mesmo após a espermatogênese (ARBUCKLE et al., 1999; RODRÍGUEZ-ROBLEDO et al., 2022; VASSEUR et al., 2024). Estudos *in vitro* com espermatozoides maduros de diferentes espécies de animais reportaram que a cinética espermática é afetada pela ação dos herbicidas a base de glifosato (ANIFANDIS et al., 2018; NEROZZI et al., 2020), 2,4-D (TAN et al., 2016) e atrazina (KOMSKY-ELBAZ & ROTH, 2017). No entanto, indicadores de viabilidade, potencial de membrana mitocondrial e integridade do acrossoma, bem como os processos de capacitação, reação acrossômica também podem ser comprometidos pela exposição a herbicidas (TAN et al., 2016; KOMSKY-ELBAZ & ROTH, 2017; NEROZZI et al., 2020). Dessa forma se evidencia que, além do sêmen constituir um biomarcador de exposição, os espermatozoides possuem o potencial de servir como uma ferramenta de avaliação toxicológica de compostos devido as suas características morfofuncionais (MARCU et al., 2023).

Apesar da correlação negativa entre os herbicidas e a reprodução demonstrada em estudos anteriores, tornam-se necessárias investigações que avaliem concentrações ambientalmente relevantes, oriundas de dados de detecção no ambiente e em indivíduos expostos (PERRY 2008; MOREIRA et al., 2021). Ao mesmo tempo, é pertinente mencionar a necessidade de considerar as consequências dos efeitos dos pesticidas no contexto das misturas, visto que a exposição a um número diverso de químicos reflete o cenário atual com maior veracidade (ALEHASHEM et al., 2024; GAO et al., 2024).

Nesse sentido, os bovinos consistem em uma espécie animal peculiarmente exposta a diversos tipos de pesticidas, devido ao manejo e pela utilização de recursos (alimentares, hídricos, área de pastagem, etc.) contaminados por resíduos destes

compostos (SORENSEN et al.,2021; BRUINENBERG et al.,2023). Contudo, as consequências sobre os aspectos reprodutivos são pouco estudadas e tendo vista que os rebanhos de bovinos brasileiros possuem importância econômica significativa (IBGE, 2023), avaliar os impactos dos herbicidas sobre a saúde destes animais adquire maior importância juntamente com a preocupação ambiental.

Por essa razão, neste estudo avaliamos o potencial de diferentes concentrações dos ingredientes ativos dos herbicidas glifosato, 2,4-D e atrazina, de comprometer parâmetros morfofuncionais de espermatozoides bovinos, bem como seus efeitos sobre a capacidade de fecundação destes gametas. Ao mesmo tempo, consideramos a exposição aos compostos de forma isolada e em associação a fim de averiguar os impactos das misturas sobre a qualidade e função dos espermatozoides bovinos.

2) REFERENCIAL TEÓRICO

2.1) Conceito de pesticidas

De acordo com o conceito da *Food and Agriculture Organization* (FAO,1986a), pesticidas consistem na substância ou mistura de substâncias com finalidade de prevenir, destruir, ou controlar qualquer organismo com potencial para interferir na produção, processamento, armazenamento, transporte e comercialização de alimentos, *commodities* e produtos de origem vegetal. O termo se estende aos compostos utilizados na prevenção e controle de infestações biológicas em animais domésticos e de produção, bem como àqueles destinados à regulação do crescimento vegetal e auxiliares nos processos pré e pós colheita. A Organização Mundial da Saúde (OMS) complementa o conceito de pesticidas conforme o critério de origem, podendo ser de ordem natural ou sintética (WHO, 1990).

Conforme AKASHE; PAWADE; NIKAM (2018), os pesticidas podem ser classificados conforme o grau de toxicidade, a finalidade (organismo alvo de eliminação), a composição química, a aplicação, o modo de ação, o período de uso, as formulações e as fontes de origem.

Em se tratando de toxicidade, a OMS classifica os pesticidas conforme o quadro abaixo:

Quadro 1: Classificação dos pesticidas com base na toxicidade (WHO, 2019)

Classe		**LD50 para ratos (mg/kg peso corporal)**	
		Oral	Dermal
Ia	Extremamente perigoso	< 5	< 50
Ib	Altamente perigoso	5 – 50	50 – 200
II	Moderadamente perigoso	50 – 2000	200 – 2000
III	Levemente perigoso	> 2000	> 2000
U	Improvável efeito perigoso agudo	≥ 5000	≥ 5000

O critério da finalidade permite o enquadramento dos pesticidas conforme o organismo alvo, como no caso dos inseticidas, herbicidas, fungicidas, rodenticidas, bactericidas, larvicidas, etc. A diversidade de compostos aumenta significativamente quando considerados os princípios ativos e composições químicas presentes nas formulações, o que origina um leque vasto de substâncias químicas utilizadas com a

mesma finalidade. As composições químicas dos pesticidas permitem criar subgrupos nas categorias fim e constitui um dos principais meios pelos quais esses químicos são comumente conhecidos (AKASHE; PAWADE; NIKAM, 2018).

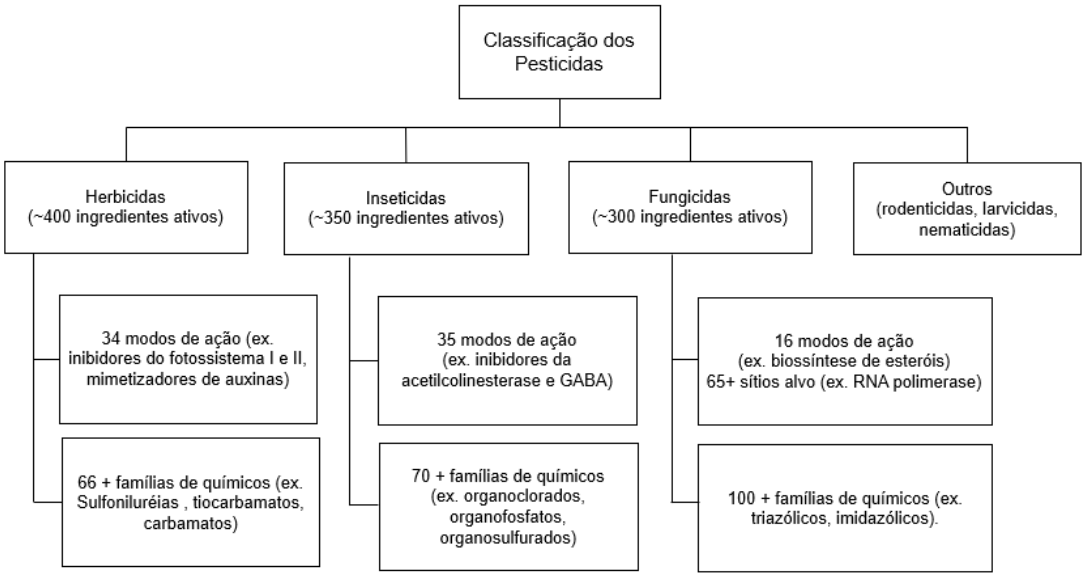


Figura 1: Classificação dos pesticidas quanto a finalidade, adaptado de SOUZA et al. (2023).

2.2) Panorama do uso de pesticidas no Brasil

Devido a sua extensão territorial de natureza continental, o Brasil é um dos maiores produtores de alimentos do mundo juntamente com os Estados Unidos, China, Índia e Indonésia (FAO, 2025a). Em 2025, o Brasil consolidou sua posição como um dos maiores produtores de grãos do planeta, com uma produção estimada em 333,3 milhões de toneladas, segundo dados da Companhia Nacional de Abastecimento (Conab, 2025). Esse volume representa aproximadamente 14% da produção mundial de grãos, estimada em cerca de 2,375 bilhões de toneladas de acordo com projeções da FAO (FAO, 2025b). A soja e o milho representam mais de 90% da produção nacional de grãos (Conab, 2025). Além da liderança global na produção de soja, o Brasil também se destaca na produção de arroz, algodão, café e cana-de-açúcar. Em 2024, o Brasil manteve sua posição de destaque na pecuária mundial, com um rebanho bovino de corte estimado em 186,66 milhões de cabeças, segundo dados do Departamento de Agricultura dos Estados Unidos (USDA, 2025) representando aproximadamente 20,2% do rebanho mundial, tendo

também participação significativa na produção global de galináceos (15%) e suínos (3,6%) (MARCENOVICZ, 2025; EMBRAPA, 2025).

Em decorrência destes indicadores, o país também detém a posição de destaque no consumo de compostos voltados a produção agrícola, como os pesticidas. Conforme o relatório da FAO (2025c) o Brasil ocupa a 1ª posição na aplicação de pesticidas agrícolas, com impressionantes 801.000 toneladas destes compostos, o que corresponde a 21% do volume global, ultrapassando os Estados Unidos (468.000 toneladas) e a China (244.000 toneladas). Em termos de uso de pesticidas por hectare, o país também lidera junto com o Vietnã, com a utilização de 10 kg por hectare (FAO, 2025c). De acordo com o Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis/IBAMA (2024), entre os ingredientes ativos mais comercializados em território brasileiro em 2023, o glifosato, atualmente o herbicida mais utilizado no mundo, ocupa a 1ª posição em toneladas, seguido pelo fungicida mancozeb e o ácido diclorofenoxiacético (2,4-D), também com finalidade herbicida (Quadro 2).

Quadro 2: Pesticidas mais comercializados no Brasil no ano de 2023 (Fonte: IBAMA, 2024)

OS 10 INGREDIENTES ATIVOS MAIS VENDIDOS - AGROTÓXICOS QUÍMICOS*		
2023		
Unidade de medida: tonelada de Ingrediente Ativo (IA) de Produto Formulado		
Ingrediente Ativo	**Quantidade**	**Posição**
Glifosato e seus sais	253.301,95	1º
Mancozebe	52.316,65	2º
2,4-D	51.872,24	3º
Acefato	49.557,98	4º
Clorotalonil	45.533,10	5º
Atrazina	26.804,98	6º
S-metolacloro	15.776,35	7º
Glufosinato - sal de amônio	13.027,38	8º
Malationa	12.731,67	9º
Dibrometo de Diquate	10.561,29	10º

Somado ao aumento crescente do uso de pesticidas para atender a demanda produtiva está o fato de o país ter adotado políticas mais permissivas na liberação e no uso de compostos desta natureza (SOUZA et al., 2023). Apenas no ano de 2022 ocorreu o registro de 652 novos ingredientes ativos conforme o relatório da Agência Nacional de Vigilância Sanitária do Brasil/ANVISA (2023). Além disso, de acordo com FRIEDRICH et al. (2021), mesmo em comparação com os maiores produtores mundiais, o Brasil utiliza pesticidas já proibidos em alguns países, com regularização mais rígida ou em processo de substituição, como são exemplos o 2,4-D e a atrazina.

Um outro aspecto importante que influencia no manejo de pesticidas no Brasil reside no fato da utilização maciça de organismos geneticamente modificados (OGMs), manipulados estrategicamente para resistirem à exposição de agrotóxicos. Esta característica das cultivares brasileiras permite o emprego de volumes maiores de pesticidas com o intuito de garantir a eficácia dos produtos, sem prejuízo da produção (ALMEIDA et al., 2017). Ao mesmo tempo, a adoção de protocolos de manejo que utilizam mais de um pesticida com a mesma finalidade, dão a origem a misturas de pesticidas, formadas por compostos de composições químicas e mecanismos de ação distintos (PEDLOWSKI et al., 2012; GAZZIERO, 2015).

Tendo em vista que o aumento da utilização de pesticidas está associado a exposição mais frequente a esses compostos, origina-se o questionamento quanto a extensão dos impactos do uso demasiado de agrotóxicos sobre a saúde humana e animal, perda da biodiversidade e da qualidade dos produtos brasileiros (PANIS et al., 2021). Esta também é razão pela qual estudos com estes químicos são imprescindíveis para avaliação dos níveis seguros de exposição aos mesmos, os quais podem servir de embasamento para políticas e atualização de mecanismos regulatórios do país (FRIEDRICH et al., 2021).

2.3) Impactos da exposição a pesticidas sobre a reprodução

O potencial de pesticidas de promover efeitos em espécies não alvo é um fator que gera preocupação quanto a segurança da utilização destes compostos. Entre as principais consequências da exposição, estão aquelas atreladas aos efeitos sobre o sistema reprodutivo, os quais inclusive podem ser utilizados como critério para registro ou não de um pesticida, como no caso da União Europeia (FRIEDRICH et al., 2021).

A exposição a pesticidas tem sido associada ao aumento da infertilidade devido a capacidade destes químicos em promover alterações importantes a nível de sistema reprodutivo de ambos os sexos, considerando a espécie humana e diversas espécies animais (JARRELL; AHAMMAD; BENSON, 2020; FUCIC et al., 2021; SADEGHNIA et al., 2022). Os pesticidas podem atuar como perturbadores endócrinos, alterando a homeostase hormonal quanto a síntese, secreção, transporte, metabolismo, capacidade de ligação (*binding action*), comprometendo significativamente a fertilidade (SENGUPTA; BANERJEE, 2014; ROTH; KOMSKY-ELBAZ; KALO, 2020). No entanto, também podem causar efeitos diretos nas células, por ação do princípio ativo ou formas derivadas da metabolização, interferindo no metabolismo energético, promovendo danos em membranas, fragmentação do DNA e apoptose celular (MARCU et al., 2023).

Neste sentido, a qualidade e viabilidade dos gametas pode ser comprometida resultante da ação de pesticidas sobre o sistema reprodutor (MERVIEL et al., 2017; GIULIONI et al., 2021). A presença de anomalias como granulações centrais citoplasmáticas em oócitos, teve como consequência a redução das taxas de clivagem em embriões menores percentuais de sucesso de fertilização intracitoplasmática em mulheres expostas a pesticidas (MERVIEL et al., 2017). Alterações morfológicas de fuso e na atividade mitocondrial, bem como na integridade do DNA, foram correlacionados com o impacto de pesticidas sobre a maturação dos oócitos, com reflexos sobre a competência destas células (CABRY et al., 2020). A redução da qualidade oocitária pode ser associada negativamente com o desenvolvimento embrionário (CABRY et al., 2020) e com o aumento de abortos espontâneos até os três meses (MERVIEL et al., 2018), permitindo correlacionar a exposição aos pesticidas a menores taxas de natalidade (FUCIC et al., 2021).

De forma semelhante, a fertilidade masculina pode ser prejudicada pelo impacto na qualidade espermática, expressa pela diminuição do volume ejaculado, menor concentração de espermatozoides, aumento da frequência de alterações morfológicas, redução da motilidade e ocorrência de fragmentação do DNA, fatores os quais podem comprometer significativamente a capacidade de fertilização destas células (GIULIONI et al., 2022).

Consequentemente, os efeitos da toxicidade de pesticidas sobre o sistema reprodutor masculino têm sido demonstrados pela baixa qualidade do sêmen de indivíduos expostos, através da análise de parâmetros espermáticos (SENGUPTA;

BANERJEE, 2014). Assim, averiguar a extensão dos danos de pesticidas sobre as células espermáticas ganha maior importância tendo em vista o contexto atual de exposição contínua, devido à presença ubíqua decorrente do uso crescente dos agrotóxicos (MARCU et al., 2023).

2.4) Herbicidas mais utilizados no Brasil e consequências ao sistema reprodutor masculino

Os compostos com a finalidade herbicida consistem em químicos de natureza fitotóxica, utilizados para prevenir e controlar o crescimento de espécies vegetais indesejáveis na produção agrícola. Podem ser classificados como seletivos ou não seletivos, mas também por meio da sua estrutura química e mecanismo de ação (SOUZA et al., 2023). Os herbicidas constituem a categoria de pesticida mais utilizada no Brasil e atualmente as formulações mais comercializadas utilizam o glifosato, o 2,4-D e a atrazina como ingredientes ativos (BROVINI et al., 2021).

2.4.1) Herbicida glifosato

Os herbicidas à base de glifosato referem-se ao grupo de pesticidas mais utilizado no Brasil (Quadro 2) e no mundo. A comercialização do glifosato teve início a partir do ano de 1974 pela empresa Monsanto e a sua utilização vem crescendo desde então, tendo como maiores impulsionadores a inserção de OGMs nas produções agrícolas, e a perda da exclusividade da patente pela empresa nos anos 2000, permitindo a criação de novas formulações com o princípio ativo (DUKE, 2018).

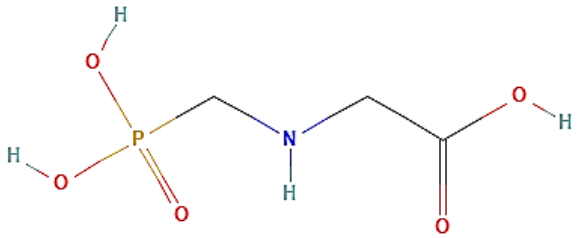


Figura 2: Estrutura química do glifosato (Fonte: Site PubChem)

O glifosato (N-fosfonometil glicina) (Figura 2) é um composto herbicida sistêmico, não seletivo, cujo o mecanismo de ação se dá a partir da inibição da enzima EPSP (5-enolpiruvilchiquimato-3-fostato sintetase), assim interrompendo a via do ácido chiquímico que é necessária para a síntese de aminoácidos aromáticos (fenilalanina, tirosina e triptofano) no metabolismo vegetal (WILLIAMS; KROES; MUNRO, 2000). Apesar desta rota não estar presente em espécies animais, o uso extensivo de glifosato tem gerado preocupação pelo aumento da exposição decorrente da presença deste composto em diferentes compartimentos ambientais, fontes alimentares e amostras biológicas (sangue, urina, leite, mecônio e esperma) de indivíduos expostos (BENBROOK, 2016; VASSEUR et al., 2024; Wei et al., 2024). Em 2015, o IARC (*International Agency for Research on Cancer*) classificou o glifosato no Grupo 2A – compostos prováveis cancerígenos (FRIEDRICH et al., 2021). Além disso, a literatura tem demonstrado que a exposição ao glifosato também resulta em alterações de mecanismos fisiológicos, entre os quais está incluso o sistema reprodutor masculino (JARRELL; AHAMMAD; BENSON, 2020).

A partir de estudos com roedores, o potencial do glifosato em atuar como um perturbador endócrino foi descrito como multifatorial (BRANCO et al., 2025), interferindo no balanço de hormônios esteroidais pela capacidade de desregular a enzima aromatase (CASSAULT-MEYER et al., 2014), e ao promover a redução da expressão das proteínas esteroidogênicas StAR (proteína reguladora aguda esteroidogênica) e CYP17A1 nas células de Leydig (ZHAO et al., 2021). CLAIR et al. (2012) verificaram a redução da testosterona nos tratamentos *in vitro* com glifosato e com a formulação comercial Roundup®, nos quais a formulação promoveu dano por necrose a partir de 48h em todas as células testiculares avaliadas (Leydig, Sertoli e germinativas) enquanto o glifosato sozinho induziu apoptose de células germinativas, demonstrando maior sensibilidade ao ingrediente ativo. Conforme a revisão de CAI et al. (2017), a oligooszoospermia ou diminuição na concentração espermatozoides é um achado recorrente em estudos com roedores, e pode ser associada com a perda da qualidade espermática, contribuindo para o aumento da infertilidade.

Contudo, a exposição direta de espermatozoides maduros ao glifosato tem demonstrado consequências como a astenozoospermia, descrita como a redução da motilidade espermática, um parâmetro funcional relevante para desempenho reprodutivo (CHAKRABORTY; SAHA, 2022). A astenozoospermia foi descrita em diversas espécies

inclusive a humana (ANIFANDIS et al., 2018), mas também suínos (NEROZZI et al., 2020; TORRES-BADIA et al., 2021), equinos (SPINACI et al., 2022), galináceos (SERRA et al., 2021) e peixes (GONÇALVES et al., 2018). Entre os mecanismos que podem ser associados ao prejuízo da motilidade espermática cita-se o metabolismo energético, o qual apresentou-se afetado já em concentrações baixas de glifosato como descrito por FERRAMOSCA et al. (2021).

2.4.2) Herbicida 2,4-D

O ácido diclorofenoxiacético (Figura 3) conhecido popularmente como 2,4-D corresponde ao ingrediente ativo de herbicidas utilizados na eliminação ervas daninhas do grupo das dicotiledôneas, razão pela qual é considerado um pesticida seletivo. O 2,4-D atua mimetizando o hormônio auxina, alterando o padrão de desenvolvimento a nível molecular e promovendo uma proliferação celular desordenada que culmina com a morte da espécie vegetal (SONG, 2014). O 2,4-D foi o primeiro herbicida com este princípio ativo, criado nos anos 1945 e desde então é amplamente utilizado em decorrência do seu baixo custo, eficiência e amplo espectro de atuação.

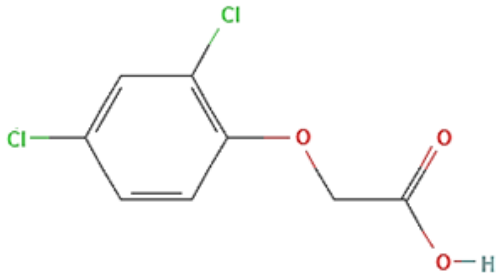


Figura 3: Estrutura química do 2,4-D (Fonte: Site PubChem).

As características químicas do 2,4-D condicionam para que possua uma alta solubilidade em água, ao mesmo tempo em que é um composto moderadamente persistente, tornando sua presença frequente em recursos hídricos, e assim adquirindo importância ambiental. O IARC classificou o 2,4-D no Grupo 2B – compostos possíveis carcinogênicos, e a Comunidade Europeia o incluiu na lista dos químicos com potencial perturbador endócrino com base em estudos *in vitro* (FRIEDRICH et al., 2021). Apesar disso, o uso do 2,4-D tem apresentado crescimento significativo na produção agrícola, o que resulta em uma maior exposição de indivíduos em razão da atividade, ou de forma

não ocupacional pela contaminação de alimentos, água, ar e animais expostos (ALCALA et al., 2022; FREISTHLER et al., 2022). A quantificação de 2,4-D na urina de indivíduos expostos vem demonstrando o aumento das concentrações deste composto e seus metabólitos e tem sido utilizada como um marcador de exposição e de monitoramento (FREISTHLER et al., 2022). ARBUCKLE et al. (1999) verificaram a presença de 2,4-D no sêmen de fazendeiros canadenses, ressaltando que o efeito tóxico às células espermáticas pode influenciar em sua qualidade.

Neste contexto, a influência do 2,4-D sobre o sistema reprodutor masculino foi descrita por ZHANG et al. (2017), que a partir do protocolo de exposição de camundongos machos a altas concentrações do pesticida (100 e 200 mg/kg) constatou alterações estruturais nos testículos, como atrofia e desorganização do epitélio dos túbulos seminíferos, ausência de espermatozoides, degeneração e redução de células germinativas, e disfunção nas células de Leydig, os quais foram relacionados ao estresse oxidativo induzido pelo herbicida. No entanto, uma redução significativa do hormônio testosterona foi observada inclusive na dose mais baixa (50 mg/kg). Em ratos, MAROUANI et al. (2017) verificaram a redução do hormônio testosterona e o aumento do hormônio folículo estimulante (FSH) e luteinizante (LH), assim como as alterações histológicas nos túbulos seminíferos e diminuição na concentração de espermatozoides, os quais apresentaram maior incidência de anormalidades morfológicas e motilidade reduzida em indivíduos tratados com 2,4-D.

O efeito direto do 2,4-D sobre células espermáticas foi avaliado por TAN et al. (2016) a partir da submissão de sêmen humano a diferentes concentrações do herbicida, verificando um efeito dose-dependente e o comprometimento de parâmetros como a motilidade total e progressiva em concentrações maiores. No entanto, a capacidade do 2,4-D acumular-se no plasma seminal e líquido folicular pode resultar em um aumento do risco a fertilidade.

2.4.3) Herbicida atrazina

A atrazina (Figura 4) é um composto pertencente ao grupo das triazinas, lançado em 1958 como um herbicida seletivo, pré e pós emergente, utilizado em diferentes culturas com o intuito de erradicar gramíneas e espécies herbáceas invasoras em produções agrícolas. Estes herbicidas estão inclusos na categoria dos inibidores do

fotossistema II, pois possuem como mecanismo de ação a capacidade de interromper a produção energética oriunda do processo fotossintético ao ligarem-se ao complexo proteico D1 presente no cloroplasto, impedindo a fixação de CO_2 e a produção de ATP e NADPH (SASS; COLANGELO, 2006).

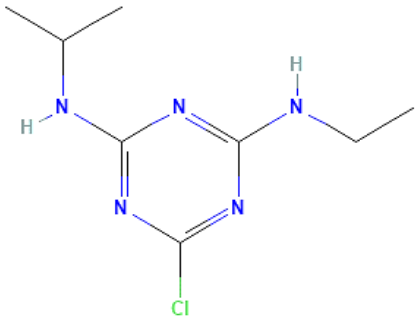


Figura 4: Estrutura química da atrazina (Fonte: Site PubChem).

Apesar de os herbicidas a base de atrazina constituírem uma das categorias mais utilizadas no mundo, as consequências da utilização sobre a saúde e meio ambiente são preocupantes. Isto se deve ao fato de que a atrazina possui baixa absorção do solo, o que permite ser facilmente lixiviada, contaminando águas superficiais e lençóis freáticos, onde podem permanecer por longos períodos (DE ALBUQUERQUE et al., 2020). A ameaça à biodiversidade e à saúde humana tem sido demonstradas por estudos realizados com a atrazina, os quais permitiram seu enquadramento como um potencial perturbador endócrino, razão pela qual o uso foi banido na União Europeia a partir do ano 2003. No entanto, grandes produtores mundiais como o Brasil e os Estados Unidos permanecem utilizando formulações com esse princípio em suas produções agrícolas (SASS; COLANGELO, 2006; FRIEDRICH et al., 2021).

O uso disseminado e o potencial da atrazina como perturbador endócrino motivam as investigações sobre os impactos da exposição aos sistemas biológicos, como o reprodutivo, e mais especificamente, à fertilidade masculina. Neste sentido, autores como SONG et al. (2014), descreveram os efeitos da exposição oral da atrazina sobre o sistema reprodutivo de ratos machos verificando a alteração do padrão hormonal pelo aumento nos níveis de hormônios FSH e LH e redução da testosterona. Danos histológicos em túbulos seminíferos, menor concentração espermática e maior frequência de anormalidades morfológicas nos espermatozoides, ocorrência de estresse oxidativo,

também estiveram associados às concentrações de atrazina testadas. COOK et al. (2019) verificaram em camundongos um aumento no percentual de espermatozoides mortos ou com a motilidade reduzida no epidídimo de grupos tratados, enquanto HARPER; FINGER; GREEN (2020), constataram o efeito da exposição pré natal sobre a redução da concentração de espermatozoides, e aumento da expressão de genes reguladores da aromatase em indivíduos jovens.

A expressão de genes relacionados à espermatogênese, defesas antioxidantes e reparo do DNA foi significativamente reduzida nos testículos de *zebrafish*, assim como a redução da motilidade espermática, potencial de membrana mitocondrial e integridade de membrana nestas células (BAUTISTA et al., 2018). Fragmentação de DNA e alterações de parâmetros de motilidade foram detectados na carpa comum (ÖZGÜR, 2019). A diminuição da motilidade também foi verificada por BETANCOURT et al. (2006) em espermatozoides suínos, assim como a redução da capacitação induzida ou não pela progesterona após a exposição a atrazina, descritas por MARAVILLA-GALVAN et al. (2009). O efeito da atrazina e do metabólito diaminoclorotriazina (DACT) sobre espermatozoides bovinos foi demonstrado pelo comprometimento da viabilidade e funcionalidade espermática, com prejuízos à membrana plasmática, redução da reação acrossômica e do potencial de membrana mitocondrial (KOMSKY-ELBAZ; ROTH, 2017).

2.5) Uso simultâneo de pesticidas

A necessidade de garantir maior eficácia dos pesticidas não resulta apenas no aumento de volume utilizado destes compostos, mas também na aplicação concomitante de diferentes produtos com finalidades distintas ou não, o que dá origem às misturas de pesticidas (PEDLOWSKI et al., 2012). Tanto a aplicação finalística destas misturas, quanto a sua ocorrência e permanência no meio ambiente são fatores importantes a serem considerados nas avaliações de risco de efeitos de exposição aos pesticidas, tendo em vista que a maioria dos estudos científicos são conduzidos com os ingredientes ativos ou formulações comerciais, de forma isolada (BROVINI et al., 2023).

A ocorrência de misturas de pesticidas foi detectada em diferentes compartimentos ambientais em todo o território brasileiro (OLIVEIRA et al., 2023). Embora a constituição das misturas demonstrou ter relação direta com a atividade agrícola

realizada na região avaliada, o glifosato foi um dos compostos mais frequentes encontrados em recursos hídricos, contribuindo para o risco ambiental (BROVINI et al., 2021). No contexto das misturas, a atrazina juntamente com os inseticidas clorpirifós e o não autorizado DDT, configuraram maior risco, abrangendo 69% das cidades brasileiras (BROVINI et al., 2023)

Por razões como esta, considerar o contexto das misturas é necessário para o conhecimento das consequências da interação destes químicos visto que os mesmos podem apresentar efeitos aditivos, antagônicos ou sinérgicos, dependendo do ingrediente ativo, concentrações utilizadas e condições ambientais (HERNANDEZ; GIL; LACASANA, 2017). Em ensaios *in vitro* utilizando o glifosato, atrazina e os seus metabólitos, ácido aminometilfosfônico (AMPA) e desetilatrazina (DEA), ROUSTAN et al. (2014) demonstraram a citotoxicidade 20 vezes maior da mistura em células CHO (*Chinese Hamster Ovarian*), a qual foi intensificada mais de 100 vezes através de fotoativação. A genotoxicidade do glifosato, 2,4-D e do dicamba foi avaliada por MESNAGE et al. (2021) a partir da metodologia ToxTracker, constatando a indução ao estresse oxidativo no contexto de mistura, a qual se deu predominantemente pela ação do 2,4-D. Ao avaliar o efeito individual e da mistura de 5 pesticidas (deltametrina, fenitrotion, fipronil, lambda cialotrina e teflubenzuron), ILBOUDO et al. (2014) sugeriram a atividade pró oxidante em conjunto como um elemento chave para intensificação dos efeitos em células CaCo-2.

Em relação aos efeitos sobre o sistema reprodutivo, OLAYINKA et al. (2022) avaliaram a toxicidade da exposição à mistura atrazina-metacloro em ratos, constatando danos reprodutivos expressos na redução da viabilidade, concentração e motilidade espermática, e indução de estresse oxidativo nos testículos. No entanto, a literatura sobre o impacto dos pesticidas em conjunto ainda é escassa, assim como referencias abordando as consequências de misturas complexas sobre os aspectos reprodutivos (FUCIC et al., 2021).

2.6) Impacto da exposição de pesticidas sobre a espécie bovina

Como exposto anteriormente, a presença ubíqua de pesticidas consiste em uma fonte de exposição contínua. De maneira particular, os animais de produção como os bovinos encontram-se predominantemente expostos a pesticidas devido à proximidade

com áreas de lavouras e cultivos agrícolas, à presença de resíduos de pesticidas na alimentação, e decorrente do manejo habitual por aplicação de inseticidas, acaricidas, entre outros produtos (BRUINENBERG et al., 2023) (Figura 5). Considerando que a alimentação destinada a estes animais deriva de produtos agrícolas geneticamente modificados e suscetíveis a uma maior utilização de pesticidas, o contato com resíduos de agrotóxicos é substancialmente maior (SORENSEN et al., 2021).

A exposição destes animais a pesticidas pode ser demonstrada por KRÜGER et al. (2014) pelas concentrações de glifosato encontradas na urina em vacas leiteiras com valores médios de 35 mg/kg, quando comparados a espécie humana, em média 5,4 mg/kg. Em outro estudo, os autores verificaram que as vacas expostas apresentaram alterações em marcadores sanguíneos de toxicidade, colesterol e ureia elevados, assim como distúrbios no metabolismo de minerais (KRÜGER et al., 2013).

URSELER et al. (2022) verificaram em vacas leiteiras a contaminação com atrazina a partir da água disponível em recursos hídricos da região. A capacidade de bioacumulação do pesticida permitiu a constatação de níveis mais elevados de atrazina no leite, em comparação com a água. No entanto, ambas concentrações se encontravam acima dos limites legais máximos para o consumo humano.

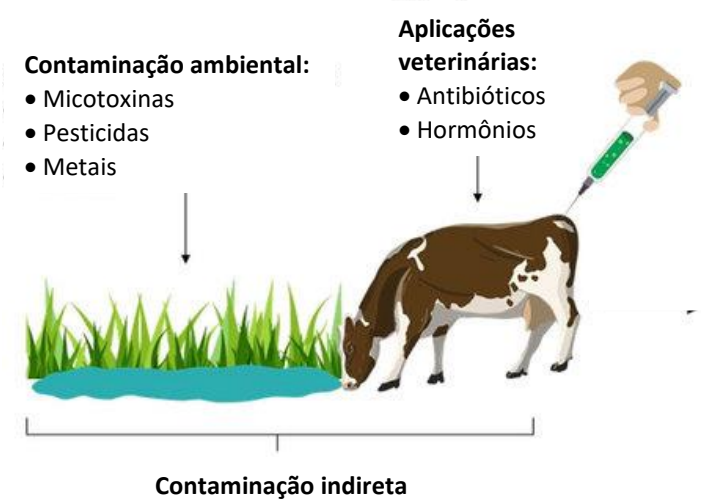


Figura 5: Fontes de contaminação de bovinos com químicos
(Adaptado de CALAHORRANO-MORENO et al., 2022)

No que se refere à qualidade espermática, SNOJ et al. (2013) reportou o efeito da criação de rebanhos bovinos próximos a regiões com utilização expressiva de pesticidas.

Como resultado, foi possível estabelecer uma correlação positiva entre a quantidade de pesticidas utilizada com a redução dos volumes dos ejaculados e da concentração de espermatozoides nos animais.

Quanto aos efeitos dos herbicidas sobre a qualidade do sêmen bovino foi verificado a capacidade da atrazina de provocar danos nas membranas espermáticas, reduzindo sua viabilidade e potencial para fertilização (KOMSKY-ELBAZ; ROTH, 2017). Além disso, alterações provocadas pela exposição de espermatozoides bovinos à atrazina e ao DACT foram extensivas aos oócitos não tratados a partir da fertilização *in vitro*, resultando em mudanças no transcriptoma do embrião (KOMSKY-ELBAZ; KALO; ROTH, 2021).

DOVOLOU et al. (2024), avaliou os efeitos da exposição ao Roundup®, formulação comercial a base de glifosato, sobre gametas bovinos, constatando a toxicidade do herbicida, apesar da concentração baixa utilizada (1 ppm). Espermatozoides expostos apresentaram redução na motilidade total após 1 hora de incubação e, apesar das taxas de fertilização e clivagem não serem afetadas, o número de embriões formados foi reduzido no grupo de espermatozoides tratado com Roundup®. O efeito do herbicida sobre oócitos, no entanto, ocorreu no decréscimo das taxas de clivagem e no número de blastocistos nos três períodos avaliados (dias 7, 8 e 9 após a fertilização).

Dessa forma, o conhecimento sobre os impactos dos pesticidas sobre a saúde reprodutiva de bovinos é de grande importância a fim de averiguar sobre os possíveis danos à fisiologia da espécie. Somado a isto está o fato de que a bovinocultura constitui umas das atividades econômicas mais relevantes do Brasil (IBGE, 2023b), a qual pode ser afetada pelos impactos na genética e sanidade dos bovinos, bem como na qualidade dos produtos de origem animal produzidos, decorrentes da exposição aos agrotóxicos (KUMAR et al., 2013). Dessa forma, avaliar as consequências da exposição aos pesticidas sobre a sanidade dos rebanhos bovinos é essencial, e adquire relevância nas esferas da economia, da saúde e do meio ambiente.

2.7) A célula espermática como modelo de avaliação toxicológica

A exposição de indivíduos aos pesticidas pode ser detectada devido à presença destes compostos em amostras biológicas como o sêmen. Os ingredientes ativos glifosato, 2,4-D e atrazina já foram reportados no sêmen de humanos, associados com a exposição

ocupacional e ambiental (ARBUCKLE et al., 1999; RODRÍGUEZ-ROBLEDO et al., 2022; VASSEUR et al., 2024). No caso do glifosato, inclusive, foi verificado que as concentrações do ingrediente ativo no sêmen dos indivíduos foram superiores às encontradas na urina, amostra biológica comumente utilizada para mensurar o grau de exposição (VASSEUR et al., 2024).

Como exposto anteriormente entre os efeitos provocados pela ação destes compostos está a perda qualidade espermática, resultante de mecanismos que afetam a espermatogênese de forma indireta ou direta (SELVARAJU et al., 2021). Contudo, a presença destes herbicidas no sêmen subentende que mesmo após os processos de espermatogênese e espermiogênese, os espermatozoides permanecem expostos a estes agentes.

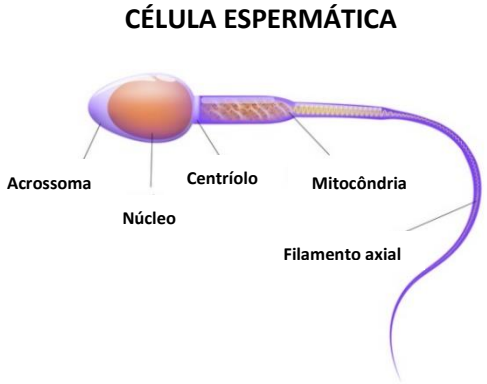


Figura 6: Estrutura da célula espermática (Fonte: Site Medline Plus)

A célula espermática possui estrutura e fisiologia bastante peculiares, e cada região do gameta (Figura 6) possui papel fundamental para o desempenho da função espermática (CHAKRABORTY, S.; SAHA, S, 2022). A sintonia entre estes componentes deve ser satisfatória para que os espermatozoides tenham o suporte adequado para realizarem a capacitação e a reação acrossômica, que permitirá sua fusão com o oócito (PINTO et al., 2023). Atualmente, diversas propriedades dos espermatozoides são passíveis de avaliação por meio de protocolos específicos, alguns rotineiramente aplicados para avaliação da qualidade espermática, como a cinética espermática, e outros focados em mecanismos específicos do metabolismo espermático, como o potencial de membrana mitocondrial e o estresse oxidativo, como exemplos (KEYSER et al. 2025).

Devido ao fato de que os espermatozoides mesmo maduros possuem certa sensibilidade a contaminantes ambientais como os pesticidas, a investigação dos efeitos

da exposição sobre espermatozoides pode constituir uma metodologia importante de avaliação (MARCU et al., 2023; KEYSER et al. 2025). Além de estudos toxicológicos com espermatozoides se enquadrarem em uma abordagem 4R's (*reduction, refinement, replacement, responsability*) (LIU et al., 2025), avaliações *in vitro* com estas células possuem o potencial de análises mais pontuais no que se refere a testes com diferentes concentrações de compostos com relevância biológica (VICENTE-CARRILLO, 2018; MORETTI et al., 2023). Da mesma forma, permitem a investigação da combinação de substâncias (misturas), a qual é necessária para avaliar os riscos da exposição a compostos como os herbicidas de forma mais condizente com a realidade (ALEHASHEM et al., 2024; GAO et al., 2024).

3) JUSTIFICATIVA

Com o aumento no uso de insumos agrícolas como os herbicidas e seus efeitos sobre espécies não alvo, torna-se relevante avaliar os impactos sobre a sanidade dos indivíduos expostos, como no caso da espécie bovina. Apesar da literatura abordar a temática das consequências ao sistema reprodutivo decorrente da exposição aos herbicidas glifosato, 2,4-D e atrazina sobre diversas espécies, são escassos os trabalhos que adotem avaliações da toxicidade em concentrações mais próximas dos níveis de detecção reportados. Da mesma forma, são necessárias as investigações tendo em vista o contexto das misturas de pesticidas, visto que a exposição a estes compostos não ocorre de forma isolada.

Assim, a partir de um modelo *in vitro* com espermatozoides bovinos, investigamos os efeitos provocados sobre a qualidade espermática através da exposição aos ingredientes ativos dos herbicidas, de forma isolada e em misturas, visando esclarecer quanto ao comprometimento da estrutura e função, bem como os impactos sobre a capacidade de fertilização destas células.

4) OBJETIVOS

4.1) Objetivo Geral

Avaliar os efeitos da exposição a concentrações dos herbicidas glifosato, 2,4-D e atrazina, de forma isolada e conjunta (misturas), sobre a qualidade e função de espermatozoides bovinos.

4.2) Objetivos específicos

Investigar se os herbicidas glifosato, 2,4-D e atrazina, utilizados de forma isolada ou em conjunto (misturas), alteram a viabilidade e funcionalidade de espermatozoides bovinos, por meio das seguintes avaliações:

- Avaliação da cinética espermática, utilizando equipamento de avaliação de parâmetros espermáticos semi-computadorizado (CASA);
- Análise morfológica da célula espermática;
- Avaliação de biomarcadores de estresse oxidativo (espécies reativas de oxigênio, peroxidação lipídica, capacidade antioxidante total);
- Avaliação da célula espermática quanto à integridade da membrana plasmática, ocorrência de danos no acrossoma e o potencial de membrana mitocondrial, utilizando a citometria de fluxo;
- Avaliação da capacidade fecundante do sêmen em protocolo de fertilização in vitro (FIV).

5) ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de dois artigos científicos. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se nos respectivos artigos.

O primeiro artigo intitulado “Herbicide Exposure Impairs The Morphofunctional Parameters Of Bovine Sperm” foi publicado no periódico “*Animal Reproduction Science*”, fator de impacto 3.3, classificada como A2 no QUALIS-CAPES.

O segundo artigo está apresentado como foi publicado sob o título “Effects of Herbicide Mixtures On The Fertilizing Capacity Of Bovine Sperm”, na revista “*Reproductive Toxicology*”, fator de impacto 2.8, classificada como A3 no QUALIS-CAPES.

Animal Reproduction Science 281 (2025) 107993

Contents lists available Science Direct

Animal Reproduction Science

journal homepage:www.elsevier.com/locate/anireprosci

Herbicide exposure impairs the morphofunctional parameters of bovine sperm

Diogo Ferreira Bicca [a,b], Rafaela Dalmolin Menezes [a], Luiza Gazeta Passos [b], Clara de Carvalho Silva [b], Mariana Gimenez dos Santos [b], Laura Rohde Brondani [a], Rogério Ferreira[c], Fabio Gallas Leivas[a], Daniela dos Santos Brum [a], Francielli Weber Santos Cibin [a,b,*]

[a]*Laboratório de Biotecnologia da Reprodução (Biotech), Campus Uruguaiana, Universidade Federal do Pampa (UNIPAMPA), CEP 97500-970, Uruguaiana, RS, Brazil*

[b]*Laboratorio de Estresse Oxidativo, Campus Uruguaiana, Universidade Federal do Pampa (UNIPAMPA), CEP 97500-970, Uruguaiana, RS, Brazil*

[c] *Departamento de Ci^encia Animal, Universidade Estadual de Santa Catarina (UDESC), CEP 89815-630, Chapeco, SC, Brazil*

ARTICLEINFO

Keywords:
Herbicides Bovine
Spermatozoa
Glyphosate
2,4-D
Atrazine

ABSTRACT

Glyphosate (GLP), 2,4-dichlorophenoxyacetic acid (2,4-D), and atrazine (ATZ) are the most commercialized herbicides in Brazil. Despite the damage to male fertility caused by pesticides, information on cattle remains limited. We evaluated the effects of exposing bovine sperm to concentrations of GLP, 2, 4-D, and ATZ. A semen pool from four bulls was incubated at 37°C for 1 and 3 h in TALP-Fert medium, and treated as follows: Control group (vehicle dimethyl sulfoxide); GLP at 5 (G5), 36 (G36), and 50 (G50) µg/mL; 2,4-D, at 0.5 (D05), 1 (D1), and 5 (D5) µM; and ATZ, at 0.05 (A005), 0.1 (A01), and 1 (A1) µM. Herbicide groups affected various kinematic parameters. Total motility was reduced by ATZ, while progressive motility decreased in all treatments compared to the control. Velocity-related kinematic parameters were significantly impaired by GLP treatment, and hyperactivity was negatively influenced by all three herbicide groups. Sperm morphology was altered in the G50, D5, and ATZ groups, with a higher incidence of major defects compared to the control. The hypo-osmotic swelling test revealed that plasma membrane integrity was compromised only in the A005 and A01 treatment groups. Regarding oxidative stress markers, although no differences were observed in reactive oxygen species generation or lipid peroxidation, total antioxidant capacity was significantly reduced by the 2,4-D treatment. Moreover, fertilization rates declined in the D05 group, which corresponded to the lowest 2,4-D concentration. These findings demonstrate that herbicide concentrations can adversely affect bovine spermatozoa by impairing critical quality parameters, ultimately compromising sperm function.

1. Introduction

Global population growth has increased the demand for food. To ensure greater efficiency and productivity, agricultural chemicals known as pesticides are used to control pests, weeds, and diseases that may affect crop yield (Nicolopoulou-Stamati et al., 2016). With a territory of continental dimensions, Brazil is one of the largest agricultural producers in the world, and consequently, has been consolidating itself as an avid consumer of pesticide products. A conflict of interest between the economy, health, and the environment has, however, arisen owing to the excessive use of these compounds (Oliveira et al., 2023).

* Correspondence to: Campus Uruguaiana, Universidade Federal do Pampa (UNIPAMPA), Uruguaiana, RS 97500-970, Brazil.
E-mail address: franciellicibin@unipampa.edu.br (F.W.S. Cibin).

https://doi.org/10.1016/j.anireprosci.2025.107993
Received 16 May 2025; Received in revised form 11 September 2025; Accepted 12 September 2025
Available online 13 September 2025

Herbicides are the most commonly used pesticides in Brazil. Among these, glyphosate (GLP; N-(phosphonomethyl) glycine) occupied the top spot as the most commercialized compound in 2021 (IBAMA 2023). Considering the low toxicity reported by the Brazilian Health Surveillance Agency (ANVISA, 2018), GLP has been classified as probable carcinogen by the International Agency for Research on Cancer (IARC) (Friedrich et al., 2021). The second spot was occupied by 2,4-dichlorophenoxyacetic acid (2,4-D), which despite the high toxicological classification provided by ANVISA (2016) and IARC as a possible carcinogen, is widely applied in corn, soybean, rice, bean, cotton, and sugarcane crops (Friedrich et al., 2021; IBAMA 2023). Finally, atrazine (ATZ) constitutes the third most used herbicide in Brazil in 2021 according to IBAMA (2023). It is a persistent compound that easily leaches after application. It is classified as moderately toxic by ANVISA (2016) and was banned in 2003 from use in the European Community (Sass and Colangelo, 2006; De Albuquerque et al., 2020; Friedrich et al., 2021).

A negative relationship between reproductive health and pesticide exposure has been described, and male fertility is affected by hormonal disruption and loss of sperm quality (SenGupta and Banerjee, 2014; Marcu et al., 2023). Endocrine disruption owing to the deregulation of the aromatase enzyme and diminished testosterone levels has been demonstrated in rodents after GLP treatment (Clair et al., 2012; Cai et al., 2017), as well as 2,4-D and ATZ exposure, by increasing FSH and LH levels and modifying the hormone profile in rodents (Song et al., 2014; Marouani et al., 2017; Roth et al., 2020). The cytotoxicity of these three herbicides in the male reproductive system damages somatic and germ cells with histological and morphological effects (Clair et al., 2012; Marouani et al., 2017; Zhang et al., 2017), which can negatively influence sperm production and quality (Cai et al., 2017; Cook et al., 2019; Harper et al., 2020). In addition, the post-spermatogenic effects of pesticides have been demonstrated in several species with the effects of direct exposure on spermatozoa being observed as the impairment of the structural and functional parameters of the cells, which could affect their fertilization capacity (Tan et al., 2016; Komsky-Elbaz and Roth, 2017; Nerozzi et al., 2020). Owing to the presence of these compounds in the seminal plasma resulting from continuous exposure to pesticides, evaluation of the direct effects of low doses on spermatozoa has gained importance in current practice (Perry, 2008; Moreira et al., 2021; Chhillar et al., 2023).

In this context, cattle are peculiarly exposed to a wide variety of chemicals used in agricultural activities because these animals are frequently bred close to crops areas with contaminated air and water resources, but also through routine handling and pesticides residues in their feeding (Sørensen et al., 2021; Bruinenberg et al., 2023). The consequences of bovine exposure to pesticides and their effects on reproductive health, however, remain unclear. Considering that cattle breeding in Brazil is one of the most relevant sources of animal production (IBGE 2023), evaluating the reproductive effects of pesticide exposure on herds is essential and economically important, in addition to health and environmental concerns. The analysis of sperm parameters is a relevant toxicological tool owing to the measurable structural and functional features of cells, which allows the risk assessment of exposure to different concentrations of chemicals and the detection of species-specific sensibilities to pesticides, reducing the use of *in vivo* models.

In the present study, we used frozen bovine semen as a model to investigate the effects of exposure to the pesticides, GLP, 2,4-D and ATZ on the quality and function of sperm markers using low doses and data of exposure to the compounds from previous toxicological studies.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. GLP (n° 45521, CAS: 1071–83–6), 2,4-D (n° 31518, CAS: 94–75–7), and ATZ (n° 45330, CAS: 1912–24–9) were used to prepare gamete exposure solutions in dimethyl sulfoxide (DMSO).

2.2. Sperm preparation and selection

Frozen bovine semen straws from same batch/collection of four selected bulls (*Bos taurus*) with proven fertility were acquired from commercial establishments and used during the experiment. For each experiment repetition, one sperm pool was prepared for subsequent analyses. To prepare the sperm pool, semen straws of each bull (four straws) were thawed at 37 °C for 30 s, homogenized and mixed to form the pool. After homogenization, an aliquot from the sperm pool was first placed on a pre warmed slide and evaluated with an optical microscope for motility and vigor, which was confirmed using a computer-assisted semen analysis (CASA) system with Sperm Class Analyzer (SCA) software (version 5.1; Microptotic, Barcelona, Spain). The sperm from the pool were then subjected to a selection protocol using a discontinuous Percoll gradient solution, according to Gonçalves et al. (2018). The semen pool (350 µL) was layered on top of the gradient solution and centrifuged at 4000 × *g* for 5 min. Next, 100 µL of the resulting pellet was resuspended in pre-warmed 300 µL of modified TALP-Fert medium (pH 7,2–7,4, 270–290 mOsm, with 87 mM NaCl, 3.10 mM KCl, 2 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 0.4 $MgCl_2$, 10 mM $NaHCO^-_3$, 40 mM HEPES, 1 mM pyruvic acid, 21.6 mM lactic acid, and 6 mM BSA) according to Parrish et al. (1986), and centrifuged for 1 min at 4000 × *g*. Finally, 100 µL of the pellet containing selected spermatozoa was reevaluated for motility and vigor as described in the first evaluation, to ensure the use of cells in optimal conditions for the subsequent analysis. To adjust the concentration, 5 µL of the pooled sample was fixed in 95 µL formaldehyde and counted using a Neubauer chamber, The volume of sample to be used was calculated and adjusted to achieve the concentration of 4×10^6 sperm/mL in 400 µL of modified TALP-Fert medium.

2.3. Exposure to pesticides

Spermatozoa selected during the preparation phase were exposed to pesticides. Solutions were prepared in DMSO (10 µL) and added to the TALP-Fert medium (400 µL), according to the following groups: a Control group (C) that only received DMSO, GLP groups (G5, G36, and G50), 2,4-D groups (D05, D1, and D5), and ATZ groups (A005, A01, and A1) (Fig. 1). Based on previous data from cattle (Krüger et al., 2014; Urseler et al., 2022) and human exposure (Arbuckle et al., 1999) and low doses tested in *in vitro* studies, we selected and prepared the pesticides

solutions at of 5, 36, and 50 µg/mL for GLP (Nerozzi et al., 2020); 0.5 µM, 1 µM, and 5 µM for 2,4-D (Tan et al., 2016) respectively equivalent to 0.11, 0.22 and 1.10 µg/mL; and 0.05 µM, 0.1 µM, and 1 µM for ATZ (Komsky-Elbaz and Roth, 2017), respectively equivalent to 0.0107, 0.0215 and 0.215 µg/mL. After selection, the spermatozoa were added according to the treatment groups to pre warmed medium and incubated in a laboratory oven at 37°C, for 1 and 3 h (Fig. 1). Sperm pool preparation, spermatozoa selection, and the herbicide exposure protocol were conducted for each experiment repetition. The experiment was repeated three times, as well as the subsequent analyses, except for the *in vitro* fertilization (IVF) procedure, for which four repetitions were performed.

2.4. Sperm kinematics

The effects of treatments on kinematic parameters were evaluated after 1 and 3 h of incubation, using a CASA system with SCA software (version 5.1; Microptotic, Barcelona, Spain). An aliquot of semen (5 µL) from each treatment group was placed on a pre warmed (37°C) slide and covered with a pre warmed 20 × 20 mm coverslip. The slides were placed on the warmed stage of a microscope and analyzed at 100 × magnification. The sperm kinematic parameters were evaluated in five different fields, with an average of 200 cells per treatment per replicate. The fields were analyzed by capturing 25 frames/field at a rate of 25 frames/s. The specific parameters assessed were as follows: head area: 25–70 µm^2; velocity limit for slow spermatozoa: 10 µm/s, velocity limit for medium spermatozoa: 25 µm/s, velocity limit for fast spermatozoa: 50 µm/s, minimal straightness for progressive spermatozoa: 70 %, and the maximal percentage of linearity: 50 %. Different fields of each slide were registered and subjected to evaluation of total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), mean path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), and the percentage of spermatozoa with rapid movement (hyperactivity/Hyp, %, spermatozoa with VCL >35 µm/s, ALH >2.5 µm and STR >85 %), as proposed by Mortimer (2000).

2.5. Sperm morphology

After 3 h of incubation, an aliquot of semen (5 µL) was fixed in 4 % formaldehyde, and the morphology of a minimum of 200 spermatozoa per slide was determined by examining a thin coverslip preparation of semen stained with Rose Bengal dye using an optical microscope (oil immersion objective at 1.000 × magnification). For the classification of morphological changes, the methodology proposed by Barth and Oko (1989) was adopted, categorizing total defects into major (e.g., detached or missing acrosomes; severely coiled tails) and minor (e.g., detached normal heads; distal midpiece reflex; bent or coiled tails) defects, with only one defect recorded per abnormal spermatozoon.

2.6. Hypo-osmotic swelling (HOS) test

The HOS test aims to evaluate the integrity of the sperm plasma membrane by detecting the reaction of cells in contact with a hypotonic medium. Semen aliquots (10 µL) from different treatment groups were added to 50 µL of hypotonic solution (735 mg of citrate, 135.1 mg of fructose in 100 mL of ultrapure water at 100 mOsm), incubated at 37°C for 45 min, following a protocol described by Bittencourt et al. (2005), with modifications. Next, 200 cells per group were examined under an optical microscope at 400 × magnification to quantify the number of coiled spermatozoa. Calculations were performed based on the following formula, to adjust the HOS results discounting the morphological findings: HOS = (% change from tail region after the HOS test) – (% change in tail region before the HOS test). Spermatozoa with changes were denoted as HOS positive (HOS+).

2.7. Reactive oxygen species (ROS)

The ROS levels were determined using 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Loetchutinat et al., 2005). Samples (50 µL) were incubated in the dark with 5 µL of DCF-DA (1 mM) in 10 mM Tris HCl buffer (pH 7.4). Oxidation of DCF-DA to fluorescent dichlorofluorescein (DCF) by reactive species was monitored. The fluorescence emission intensity was measured at 520 nm (with excitation at 488 nm) 120 min after the addition of DCF-DA using a Shimadzu spectrofluorometer (model RF5301PC; Kyoto, Japan). The results are expressed in fluorescence units (FU).

2.8. Lipid peroxidation

Lipid peroxidation was determined based on the formation of thiobarbituric acid reactive species (TBARS) (Ohkawa et al., 1979), in which malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) to form a colored complex, which was determined

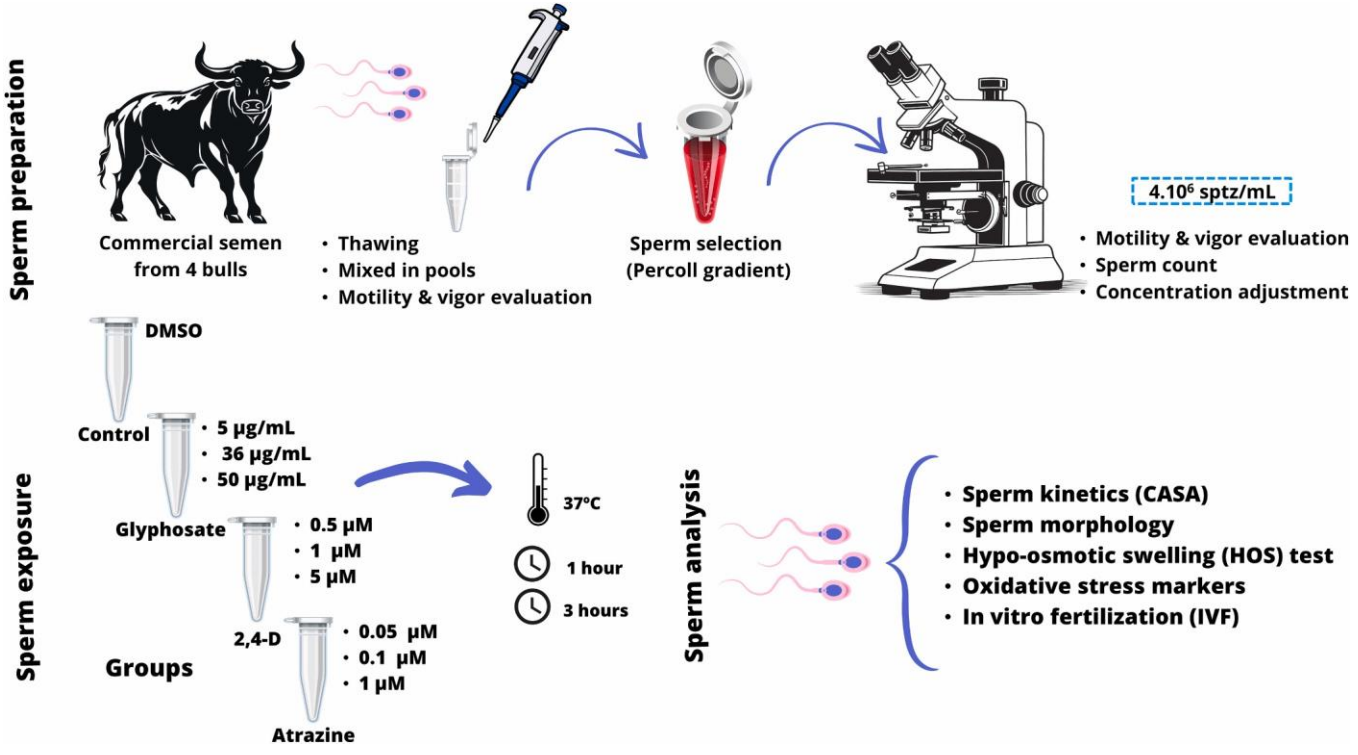


Fig. 1. Experimental design of the study.

D.F.Biccaetal.
Animal Reproduction Science 281 (2025) 107993

spectrophotometrically at 532 nm. In the reaction, 60 µL of the sample was used in a medium containing 100 µL of 0.8 % TBA, 100 µL of HCL-acetic acid buffer (pH, 1.2), and 40 µL of 0.8 % sodium dodecylsulfate (SDS) and incubated at 95°C for 90 min. The results are expressed as nmol MDA/mL.

2.9. Antioxidant potential

Total antioxidant capacity was evaluated using the ferric-reducing antioxidant potential (FRAP) technique according to Benzie and Strain (1996). In this assay, antioxidants present in the sample were evaluated as reducers of Fe^{3+} to Fe^{2+}, which were chelated by 2,4, 6-tri(2-pyridyl)-s-triazine (TPTZ) to form a Fe^{2+}-TPTZ complex with maximum absorption at 593 nm (Benzie and Strain, 1996). Ascorbic acid was used to obtain a standard curve and the results are expressed in micrograms (µg) of ascorbic acid equivalents (µg e.q AA).

2.10. Fertilization capacity (IVF)

The fertilizing capacity of the sperm was assessed using the following steps: obtaining and maturing bovine oocytes, IVF, and evaluating the fertilization rate. Bovine ovaries were obtained from a slaughterhouse and transported to the laboratory in physiological saline (0.9 %) with antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) at 30°C. Selection and maturation of bovine oocytes procedures were performed according to Stojkovic et al. (2001). The cumulus-oocyte complexes (COCs) were aspirated from follicles of 3–5 mm in diameter using a vacuum pump (20 mL of water per minute). The COCs were recovered, and only oocytes with a homogeneous ooplasm and intact cumulus layers were selected for maturation. Groups of COCs were transferred to 3-well plates containing 400 µL of TCM-199 medium with 20 % estrous cow serum (Biotech, Uruguaiana, RS, Brazil) for in vitro maturation. This medium was supplemented with 5 µg/mL of porcine FSH, NIH-FSH-P1 (Folltropin-V®, Bioniche Animal Health, Ontario, Canada), 5 µg/mL of porcine LH, LH-P (Lutropin-V®, Bioniche Animal Health), and 22 µg/mL of pyruvate and gentamicin. The COCs were cultured for 24 h at 39°C in an atmosphere of 5 % CO_2 and saturated humidity. During the IVF phase, a 3 h incubation period at 37 °C was applied to all treated and control sperm groups. To investigate the effects of herbicides on the fertilizing capacity of bovine sperm, one concentration from each herbicide group was selected, based on prior analyses from this study (morphology and HOS test) and previous literature. Semen samples from the G50 group (50 µg/mL glyphosate) were chosen according to findings from previous studies with the active ingredient (Nerozzi et al., 2020), in which sperm effects were observed only at higher doses. In contrast, for 2, 4-D and ATZ, the D05 (0.5 µM 2,4-D) and A005 (0.05 µM atrazine) groups were selected due to sperm alterations reported at lower concentrations (Tan et al., 2016; Komsky-Elbaz and Roth, 2017). Drops containing 150 µL of modified TALP-Fert medium (Parrish et al., 1995), 22 µg/mL of pyruvate, 6 mg/mL of bovine serum albumin, 10 µg/mL of heparin, 20 µM/mL of penicillamine, 10 µM/mL of hypotaurine, and 2 µM/mL of epinephrine were prepared in 30 × 10 mm plates (Corning Incorporated Life Sciences) under mineral oil for the fertilization of 20–25 COCs per treatment with 2×10^6 sperm/mL. The semen and oocytes were co-cultured at 39°C with saturated humidity and an atmosphere of 5 % CO_2 in air. Fertilization rate was evaluated after 18 h of co-incubation of oocytes with sperm. According to Missio et al. (2018), potential zygotes (non-exposed oocytes incubated with treated sperm) were selected and stained with 15 µg/mL of bisbenzimide (Hoechst 33342; Life Technologies, Japan) in buffered saline. Images of potential zygotes were examined under an epifluorescence microscope at 400x magnification with excitation at 365 nm and emission at 410 nm. Evaluations were performed considering a normal fertilization rate owing to the presence of a completely penetrated spermatozoon, two pronuclei or a fused nucleus in potential zygotes (Missio et al., 2018). The percentage of fertilization was calculated for the total number of potential zygotes evaluated. This protocol was repeated four times and percentage means were used for statistical evaluation.

2.11. Statistical analysis

All continuous variables were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests, respectively. The normality of residuals was further assessed using quantile-quantile (Q-Q) plots. Variables with residuals that did not follow a normal distribution were transformed using the sinh-arcsinh (SHASH) transformation, with gamma, delta, theta, and sigma parameters estimated to normalize each distribution.

Sperm kinematic parameters and oxidative stress markers were analyzed as repeated measures using mixed models for repeated data. The models included pesticide, dose nested within pesticide, time, and the interaction between group and time as fixed effects. Semen pool was included as the subject effect, and time was specified as the repeated variable. Different covariance structures were tested for each model, and the one with the lowest Akaike Information Criterion (AIC) was selected. Post-hoc analysis was performed using Student's t pairwise comparisons with multiple comparisons adjusted using the Tukey-Kramer method. All analyses were performed using JMP Pro 18 (SAS Institute Inc., Cary, NC, USA). Non-transformed variables are presented as least squares means ± standard error of the mean (SEM), whereas transformed variables are presented as arithmetic means ± SEM. Statistical significance was set at 5 %.

Statistical analyses of morphological assessments, the HOS test, and IVF outcomes were performed using GraphPad Prism version 8.0.2. Prior to these analyses, the Shapiro–Wilk test was applied to evaluate data normality. For all parameters, a one-way ANOVA was conducted, given that only the 3 h incubation period was considered. When appropriate, Dunnett's multiple comparisons post hoc test was used. Results are presented as mean ± standard error of the mean (SEM), and statistical significance was defined as $P < 0.05$.

3. Results

3.1. Exposure to herbicides causes different patterns of change in the kinematic parameters of spermatozoa

Based on the evaluation conducted using the CASA system, we verified that herbicide exposure affected several sperm kinematic parameters, with variations depending on treatment and incubation time, as shown in Table 1. To assess the effects of herbicides, incubation time, and their interaction on sperm kinematics, a mixed-model statistical analysis was performed, considering the treatment groups (concentration data available in Supplementary Table 1).

In general, all sperm kinematic parameters were influenced by incubation time, showing significant reductions between the 1 h and 3 h periods (Table 1). Regarding the effects of treatment, total motility (TM) was significantly impaired by ATZ exposure (reduction of 26.4 %; $P = 0.0283$). A significant effect of treatment group was also observed for progressive motility (PM), with decreases induced by GLP (39.8 %; $P=0.0075$), 2,4-D (33.2 %; $P = 0.0244$), and ATZ (39.1 %; $P=0.0085$) relative to the control (Table 1).

The analysis of velocity-related kinematic parameters revealed a treatment group effect for VSL and VAP, and a concentration (dose) effect for VAP (Table 1). Additionally, Student's *t*-test indicated that, compared with the control group, reductions in VCL (25.3 %; $P = 0.0388$), VSL (44.5 %; $P = 0.0046$), and VAP (36.5 %; $P = 0.0075$) occurred as a result of GLP treatment. In addition, a difference in VCL was also observed between the GLP and 2,4-D treatments ($P = 0.0439$).

As shown in Table 1, no differences were observed among treatment groups for ALH and BCF parameters. Nevertheless, hyperactivity (Hyp) results revealed a significant treatment group effect, with marked decreases caused by GLP (53.5 %; $P = 0.0068$), 2,4-D (38.3 %; $P = 0.0183$), and ATZ (44.8 %; $P = 0.0122$) compared to the control group (Table 1).

3.2. Sperm morphology was affected after exposure to herbicides

Sperm morphology in the different treatment groups was analyzed after incubation period for 3 h owing to the alterations in kinematics. The results presented in Fig. 2 indicate a high frequency of sperm with at least one morphological defect in cells exposed to high concentrations of herbicides in groups G50 (25.0 ± 3.25; $P = 0.0197$), D5 (22.17 ± 2.18; $P = 0.0207$), A1 (25.0 ± 2.75; $P = 0.0086$) and in the lowest concentration group of ATZ (A005; 23.5 ± 0.50; $P = 0.0210$) when compared to the control (15.33 ± 0.83).

The classification of defects presented in Fig. 3 demonstrates that the increase in number of abnormalities coincided with a greater occurrence of major defects (detached or missing acrosomes and strongly coiled tails) in groups G50 (17.83 ± 2.33; $P = 0.0194$), A1 (20.33 ± 2.42; $P = 0.0025$), and A005 (17.50 ± 0.28, $P = 0.0152$), which presented a significant difference in relation to the control (9.83 ± 0.72). Nevertheless, group A01 also showed a significant increase only in the frequency of major defects (15.83 ± 1.45; $P = 0.0488$). No significant differences in minor defects were observed among groups (Fig. 3).

3.3. Sperm membrane integrity is altered only after exposure to ATZ

Similar to the morphological evaluation, an HOS test was performed at an exposure time of 3 h. As show in Fig. 4, both GLP and 2,4- D concentrations did not promote significant changes in relation to sperm membrane permeability and values were comparative to those of the control group (23.33 ± 1.66).

Table 1
Effects of herbicide groups on bovine sperm kinematics considering two incubation periods. Data are reported as means ± SEM of three repetitions.

Parameter	Hour		Group				*P*-value			
	1	3	Control	GLP	2,4-D	ATZ	Time	Group	Time X Group	Dose (Group)
TM (%)	58.4 ± 3	40.1 ± 3	59.3 ± 6^{a}	45.8 ± 3.5^{ab}	48.4 ± 3.5^{ab}	43.6 ± 3.5^{b}	< 0.0001	0.16	0.77	0.41
PM (%)	42 ± 2.7	21.2 ± 2.7	43.9 ± 5.4^{a}	26.4 ± 3.1^{b}	29.3 ± 3.1^{b}	26.7 ± 3.1^{b}	< 0.0001	0.04	0.96	0.47
VCL (µm/s)	60.2 ± 2.9	40.4 ± 2.9	57.2 ± 5.9^{a}	42.7 ± 3.4^{b}	52.7 ± 3.4^{a}	48.5 ± 3.4^{ab}	< 0.0001	0.10	0.50	0.14
VSL (µm/s)	38.3 ± 2.7	25.2 ± 2.7	42 ± 5.4^{a}	23.3 ± 3.1^{b}	31.8 ± 3.1^{ab}	30 ± 3.1^{ab}	< 0.01	0.03	0.81	0.16
VAP (µm/s)	41.6 ± 2.5	29 ± 2.5	44.1 ± 5^{a}	28 ± 2.9^{b}	35.1 ± 2.9^{ab}	34 ± 2.9^{ab}	< 0.001	0.04	0.50	0.03
ALH (µm)	2.3 ± 0.2	1.6 ± 0.2	2.1 ± 0.3^{a}	1.7 ± 0.2^{a}	2 ± 0.2^{a}	1.9 ± 0.2^{a}	< 0.01	0.62	0.25	0.70
BCF (Hz)	9.4 ± 0.4	6.4 ± 0.7	8.9 ± 1.1^{a}	6.9 ± 0.6^{a}	7.3 ± 0.6^{a}	8.5 ± 0.6^{a}	< 0.001	0.21	0.13	0.10
Hyp (%)	16.7 ± 1.7	5.0 ± 0.9	18.5 ± 3.7^{a}	8.6 ± 1.6^{b}	11.4 ± 2.7^{b}	10.2 ± 2.0^{b}	< 0.0001	0.04	0.53	0.06

Groups: GLP (glyphosate); 2,4-D; ATZ (atrazine). Parameters: TM (Total Motility); PM (Progressive Motility); VCL (Curvilinear Velocity); VSL (Straight-line Velocity); VAP (Mean Path Velocity); ALH (Amplitude of Lateral Head Displacement; BCF (Beat Cross Frequency); Hyp (Hyperactivity). *P*-values assess the strength of evidence against the null hypothesis for the fixed effects in the repeated measures mixed model, with semen pool as the subject and hour as the repeated factor (statistical significance was defined as $P < 0.05$). Groups labeled with different lowercase letters differ significantly, according to the post hoc Student's *t*-test.

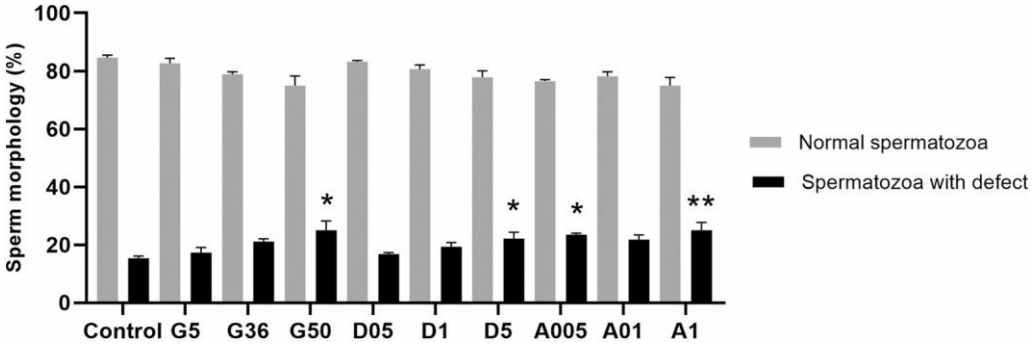


Fig. 2. Morphological analysis of sperm exposed to different concentrations of herbicides after 3 h of incubation. Data are reported as mean ± SEM of three repetitions. One way ANOVA followed by Dunnett's post hoc test identified statistical differences. * $P < 0.05$ vs control group. ** $P < 0.01$ vs control group.

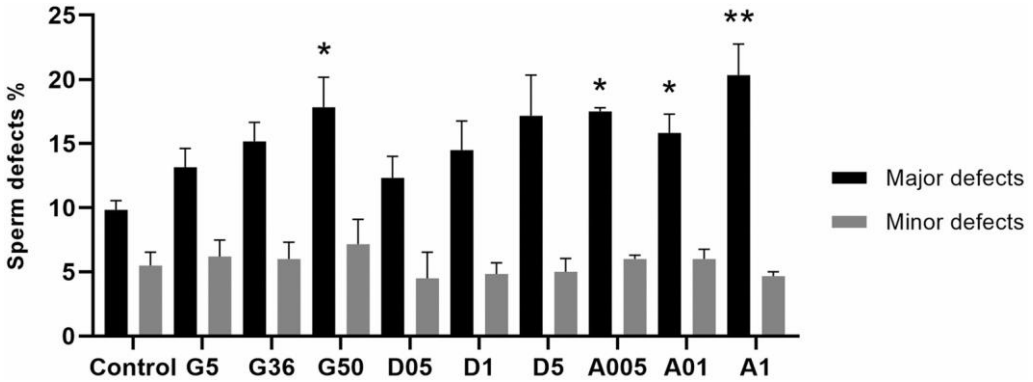


Fig. 3. Sperm defects results in different groups of treatment classified in major and minor defects. Data are reported as mean ± SEM of three repetitions. One way ANOVA followed by Dunnett's post hoc test identified statistical differences. * $P < 0.05$ vs control group. ** $P < 0.01$ vs control group.

The group ATZ, however, promoted a reducing effect on the permeability of sperm membranes at A005 (11.17 ± 0.92; $P = 0.0093$) and A01 (11.50 ± 1.60; $P = 0.00117$) concentrations, differing significantly from the control group (reductions of 52,1 % and 50.7 %) as shown in Fig. 4.

3.4. Effects on sperm function may result from impaired antioxidant capacity caused by 2,4-D exposure

Similar to the kinematic analysis, the evaluation of oxidative stress was conducted using mixed models, taking into account the effects of treatment groups and incubation time on bovine sperm (individual concentration data are available in Supplementary Table 2).

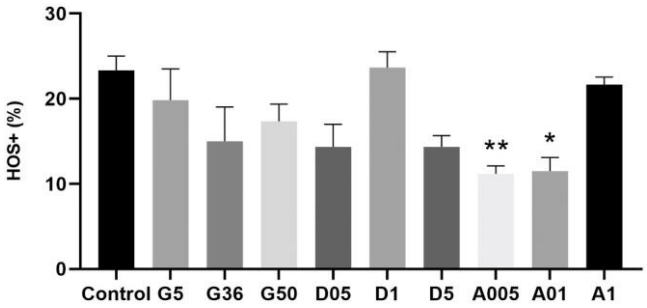


Fig. 4. Hypo-osmotic swelling (HOS) test results, showing the percentage of spermatozoa with coiled tail (HOS+) after 3 h of exposure to glyphosate, 2,4-D and atrazine. One way ANOVA followed by Dunnett's post hoc test identified statistical differences. Data are reported as percentage mean ± SEM of three repetitions. * $P < 0.05$ vs control group. ** $P < 0.01$ vs control group.

The analysis of oxidative stress markers showed no significant differences in ROS generation and lipid peroxidation parameters between the treatment groups or incubation periods when compared to the control group (Table 2).

A significant decrease, however, was observed in total antioxidant capacity (TAC) with respect to incubation time and treatment. Post hoc analysis identified a reduction promoted by the 2,4-D treatment ($P = 0.0149$), resulting in a 24.1 % decrease in TAC compared to the control group (Table 2).

3.5. Fertilization capacity is impaired in sperm cells exposed to the lowest concentration of 2,4-D

After IVF procedures and analysis of the fertilization rates of potential zygotes, the results revealed that there were no significant differences among groups G50 (40.1 % ±3.97, n = 82, $P = 0.0871$) and A005 (39.8 % ± 7.38, n = 90, $P = 0.0816$) when compared to the control group (57.0 % ± 2.64, n = 83), as shown in Fig. 5. However, a reduction in the percentage of fertilized oocytes (40.6 %) was observed in group D05 (36.6 % ± 7.22, n = 75, $P = 0.0403$) demonstrating the capacity of 2,4-D to impair bovine sperm function at lower concentrations (Fig. 5).

4. Discussion

Exposure to pesticides is a recurring theme in scientific research given the growing use of these compounds and the consequences on health and the environment. In this study, we evaluated the effects of direct exposure to the active ingredients of the three most commonly used herbicides in Brazil (GLP, 2,4-D, and ATZ) on bull sperm function and quality and verified the impairment of the evaluated parameters. Farm animals, including bovines, are continually exposed to chemicals used in agricultural activities that contaminate the environment or are present in their feeding (Sørensen et al., 2021; Bruinenberg et al., 2023), raising a concern because some chemicals can accumulate in organs and tissues (Giulioni et al., 2022; Tirpak et al., 2021). Herbicides were detected in exposed animals, such as the study of Krüger et al. (2014) who reported the active ingredient of glyphosate in the urine of cattle (average value of 35 µg/mL), and Urseler et al. (2022) who detected atrazine in milk samples of dairy cows (2.5–20.97 µg/L). Herbicides have the potential to affect non-target species and impair biological processes, such as reproduction (Giulioni et al., 2022). Based on these arguments we used concentrations derived from data exposure and low concentrations tested in previous experiments with other species, such as pigs (Nerozzi et al., 2020), humans (Tan et al., 2016), and cattle (Komsky-Elbaz and Roth, 2017). Although the literature on the *in vitro* and *in vivo* effects of pesticides is vast, there is a diversity of peculiarities involved, such as the nature of the compounds (i.e., active ingredient vs. commercial formulation), concentrations, time of exposure, model, and species used. These facts reinforced our need to investigate the consequences of sperm exposure to active ingredients in bovine species and to confirm the compounds' roles without the influence of inert components present in commercial formulations, which can be variable and sometimes unavailable.

First, the results obtained from the analysis using the CASA system showed that all groups of herbicides have the potential to impair sperm kinematics to some extent. Sperm kinematic parameters are relevant features of sperm function because they represent movement patterns associated with improved reproductive performance (Chakraborty and Saha, 2022). The impairments in total motility caused by ATZ, progressive motility affected by all herbicide treatments, velocity-related kinematics altered by GLP, and the overall reduction in hyperactivity revealed that herbicides could reduce sperm quality due to their ability to interfere with motion-related characteristics. Although incubation time alone caused a significant reduction from 1 to 3 h, the analysis results support the conclusion that herbicide treatments themselves also contributed independently to changes in sperm parameters.

Alterations in sperm kinematic parameters have frequently been reported in the literature regarding the effects of pesticide exposure. The disturbance of mitochondrial activity and ATP depletion, as well as redox imbalance, seem to cause a reduction in sperm motility using Roundup (Nerozzi et al., 2020). Ferramosca et al. (2021) verified the ability of glyphosate to decrease mitochondrial sperm energy efficiency and oxygen consumption rates, even at low concentrations (100 nM). In the case of GLP, however, the effects on kinematics were presented only with the use of high concentrations such as 360 µg/mL in pigs (Nerozzi et al., 2020) and 720 µg/mL in horses (Spinaci et al., 2022). In these studies, a comparison of commercial formulations confirmed that toxicity to cells of the formulation was higher than that of the active ingredient alone, which was also demonstrated by Torres-Badia et al. (2021), who attributed the toxicity of Roundup® to the surfactant. Using a commercial formulation, Anifandis et al. (2018) verified the effect of reduced motility in human semen even at low concentrations (0.36 µg/mL). As demonstrated in our study, the effects on bovine spermatozoa occurred from exposure to the active ingredient and, in comparison with the other studies, using lower concentrations which could be related to a species-specific sensitivity.

Table 2
Effects of exposure to herbicide groups on oxidative stress parameters in bovine sperm, considering two incubation periods. Results from reactive oxygen species (ROS), lipid peroxidation (LP) and total antioxidant capacity (TAC) evaluations are reported as means ± SEM of three repetitions.

Parameter	Hour		Group				*P*-value			
	1	3	Control	GLP	2,4-D	ATZ	Time	Group	Time X Group	Dose (Group)
ROS (UF)	63.3 ± 2	64 ± 3.6	56.6 ± 5.8^{a}	70.1 ± 3.4^{a}	65.6 ± 3.4^{a}	62.1 ± 3.4^{a}	0.86	0.17	0.58	0.85
LP (nmol MDA/mL)	4.6 ± 0.04	4.7 ± 0.04	4.7 ± 0.1^{a}	4.6 ± 0.05^{a}	4.7 ± 0.04^{a}	4.6 ± 0.05^{a}	0.22	0.51	0.84	0.70
TAC (µg e.q AA)	197.7 ± 10.1	161.5 ± 5.6	219.7 ± 28^{a}	182.3 ± 13$_{ab}$	166.7 ± 9$_{b}$	176.5 ± 8.9^{ab}	< 0.0001	0.04	0.53	0.06

Groups: GLP (glyphosate); 2,4-D; ATZ (atrazine). *P*-values assess the strength of evidence against the null hypothesis for the fixed effects in the repeated measures mixed model, with semen pool as the subject and hour as the repeated factor (statistical significance was defined as $P < 0.05$).
Groups labeled with different lowercase letters differ significantly, according to the post hoc Student's *t*-test.

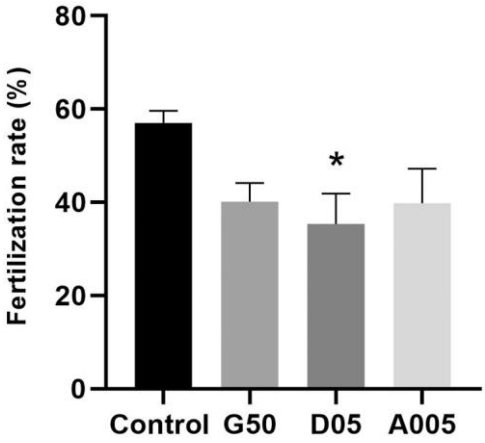


Fig. 5. Fertilization rate of i*n vitro* fertilization (IVF) with sperm from groups G50 (50 µg/mL of glyphosate)**,** D05 (0.5 µM of 2,4-D) and A005 (0.05 µM of atrazine) after 3 h of incubation. One way ANOVA followed by Dunnett's post hoc test identified statistical differences. Data are reported as mean ± SEM of four repetitions. * $P < 0.05$ vs control group.

The reduction in motility of human sperm exposed to 2,4-D was reported by Tan et al. (2016), but only detected at concentrations greater than 10 µM. The levels of 2,4-D in urine and semen were found to be lower as shown by Arbuckle et al. (1999), in the semen of rural workers with a detection limit of 2.94 µM. As a potent oxidative stress inducer, 2,4-D can cause excessive production of ROS and damage cell membranes, thereby affecting sperm motility and function (Zhang et al., 2017; Chakraborty and Saha, 2022). In the case of ATZ, the impairment in sperm kinematics can be related to a reduction in sperm energy metabolism owing to its capacity to bind to ATP synthase, inhibiting the phosphorylation process (Hase et al., 2008; Song et al., 2014). Experiments with pig spermatozoa exposure to atrazine showed a decrease in progressive motility at concentrations above 100 µM (Betancourt et al., 2006) with values much higher than those used in the present study.

Similar to kinematics, morphological evaluation of spermatozoa constitutes an important routine evaluation of sperm quality, because the occurrence of sperm abnormalities could be correlated to impairments in fertilization capacity (Barth and Oko, 1989; Johnson, 1997). Sperm morphology is also an important parameter when considering exposure to exogenous agents, as the structure of cells can be affected by toxic substances (Moretti et al., 2022). Castellini et al. (2009) demonstrated the i*n vitro* effects of different metals on rabbit spermatozoa morphology which, together with kinematics, indicated the potential of spermatozoa to be used as a toxicological monitor. A recent review by Giulioni et al. (2022) reported morphological alterations in studies of human semen exposed to pesticides. We found that higher concentrations of GLP and 2,4-D promoted morphological alterations, whereas ATZ significantly altered the morphology, even at the lowest concentration. These alterations were expressed in terms of a greater number of major defects, which, according to the Manual for Andrological Examination and Evaluation of Animal Semen (BCAR 2013), can constitute up to 20 % of the evaluated spermatozoa. Although morphology alone does not eliminate the need for other tests to evaluate sperm quality, fertilizing capacity, and, consequently, pregnancy in cattle (Sellem et al., 2015), possible morphological damage to bovine sperm caused by herbicide exposure, as detected in our study, could contribute to hindering its approval and use in cattle breeding.

Membrane permeability, as evaluated by the HOS test, was affected only by ATZ, as evidenced by the low number of sperm reactive to the hypotonic solution, at the lowest (A005) and intermediate (A01) concentrations. Sperm membrane permeability is a widely used parameter to evaluate damage to spermatozoa because adequate membrane integrity and functionality are necessary for fusion with oocytes (Zubair et al., 2015). The capacity of pesticides to disrupt sperm membranes and interfere with biological processes, such as capacitation and the acrosome reaction is of great concern because the impairment of sperm function is not detectable in routine evaluations (El-Taieb et al., 2015), and could be a cause of idiopathic infertility. Using fluorometric techniques, the plasma and acrosomal membrane of bovine spermatozoa were observed to be significantly affected by low concentrations of ATZ (0.1 and 1 µM), leading to a loss of acrosome activation capacity, an event necessary for fertilization (Komsky-Elbaz and Roth, 2017). The effect of this pesticide was also described by Maravilla-Galvan et al. (2009)´ in pig semen reported by the potential to destabilize the membrane and impair capacitation which was increased in the presence of progesterone. The reduction in capacitation and acrosomal reaction was also described by Tan et al. (2016), including at low concentrations of 2,4-D (1 µM), but only in conjunction with the same hormone, having no effects alone.

The promotion of oxidative stress is one of the main mechanisms involved in the action of pesticides (Sule et al., 2022) and is strictly related to sperm quality. Indeed, the occurrence of an oxidative environment owing to exogenous factors, such as pesticides, is associated with compromised sperm structure (morphology), nuclear content (DNA status), metabolism (mitochondrial activity) and function (kinematics and fertilizing ability) (Tirpak et al., 2021; Selvaraju et al., 2021). The generation of ROS resulting from dysfunctional mitochondrial activity can damage sperm membrane integrity, making the spermatozoon unviable depending on the extent of damage. This is reflected in the high levels of lipid peroxidation, given the chemical composition of plasma membranes, resulting in the loss of fertilization capacity (Agarwal et al., 2014; Mannucci et al., 2021).

In our study, similar to the evaluation of kinematic parameters, we considered two incubation periods to detect possible changes in oxidative status over time. The 2,4-D treatment effects indicate that oxidative stress may be a contributing mechanism, as demonstrated by the decreased antioxidant capacity in sperm from the exposed group. The promotion of oxidative stress owing to 2,4-D treatment was reported previously by Mesnage et al. (2021), which compared the *in vitro* effects of GLP, 2,4-D and dicamba pesticides, and attributed a more pronounced effect on oxidative stress to the latter two pesticides. In reproductive organs, Bongiovanni et al. (2012) reported that 2,4-D is able to induce oxidative stress in the rat prostate, with extensive effects on the testicles throughout the development period; increased lipid peroxides, hydroxyl radicals, and protein oxidation; and stimulation of antioxidant system,

especially glutathione S-transferase (GST). The antioxidant capacity, however, was affected in rats treated with high doses (100 and 200 mg/kg), leading to reduced catalase and SOD levels as well as increased MDA in the testicles (Zhang et al., 2017).

Conversely, the GLP and ATZ treatments did not have a significant effect on oxidative stress markers. Previous studies demonstrated the involvement of GLP in ROS generation in the testicles of exposed rats (Clair et al., 2012; Avdatek et al., 2018) and in the direct exposure of spermatozoa to commercial formulations (Anifandis et al., 2018, Nerozzi et al., 2020). Regarding ATZ, the findings of Song et al. (2014) reported decreased reduced gluthatione (GSH) levels and increased MDA levels in rat testicles, whereas Bautista et al. (2018) reported a reduction in superoxide dismutase 2 (SOD2) and *GPX4B* gene expression involved in the antioxidant response in zebrafish testes. Lipid peroxidation was not detected in any of the pesticide treatments compared to the control group. Although the possible involvement of oxidative stress in 2,4-D treatment and membrane integrity being affected by exposure to concentrations of ATZ demonstrated by the HOS test, these results cannot be explained by an increase in lipid damage.

Lastly, for the evaluation of fertility, we selected sperm from the highest concentration of GLP (G50) and the lowest concentrations of 2,4-D (D05) and ATZ (A005). The 3 h incubation period was also considered due to its influence on promoting effects. From this, we verified that 2,4-D had the capacity to impair fertilization at the lowest concentration, in contrast to the treatments with GLP and ATZ tested. Evaluation of fertilization capacity after protocols of pesticide exposure were conducted previously by Dovolou et al. (2024) with Roundup®, which also did not verify differences in the percentage of oocytes fertilized by exposed spermatozoa. Komsky-Elbaz et al. (2021) tested fertilization competence of bovine sperm exposed to atrazine and its metabolite diaminochlorotriazine (DACT), finding a reduction of zygote cleavage rates in groups of 1 µM of atrazine, 1 µM and 10 µM of DACT. Other experiments with rodents using fipronil *in vivo* (Bae and Kwon, 2020) and nicotinoids insecticides (Gu et al., 2013) *in vitro*, resulted in the impairment of male fertility, with reduced cleavage and blastocyst formation. Fertilization is a complex process, and sperm quality plays a crucial role in its success. The literature has demonstrated the potential of pesticides to impact fertilization through various mechanisms of action, such as endocrine disruption, impaired spermatogenesis and germ cell maturation, and more directly through pro-oxidant effects (Selvaraju et al., 2021; Giulioni et al., 2022).

Based on the above results, we show that exposure to these herbicides compromised important parameters of sperm quality in cattle. Sperm structure and function were negatively affected by the three herbicides; however, only 2,4-D showed reduced fertilization rates and could be related to oxidative stress mechanisms. Owing to the chemical diversity of pesticides, a more mechanistic approach through adequate techniques is important to investigate the herbicides' mode of action in promoting sperm impairments and to comprehend the impacts and risks of exposure to these compounds in non-target species. Simultaneously, it is necessary to consider the possibility of evaluating the effects of pesticide mixtures, which is consistent with the current context of agrochemical use and consequent exposures.

5. Conclusions

In this study, we demonstrated that exposure to GLP, 2,4-D, and ATZ, the most widely used herbicides in Brazil, reduced the quality of bovine semen. Important parameters of semen quality were negatively affected after exposure to concentrations of the active ingredients of the herbicides and significantly altered after 3 h of incubation. These results are of great concern because they could be related to the effects on the reproduction of this species based on the potential damage caused by pesticide exposure to bovine sperm performance and function.

Funding

The present study was financially supported by the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – Brazil (CAPES) – Finance Code 001, the National Council for Scientific and Technological Development – CNPQ for financial support to F.W.S.C and M. G.S. and Fundação de Amparo à Pesquisa do Rio Grande do Sul – FAPERGS is acknowledged for financial support to C.C.S.

CRediT authorship contribution statement

Francielli Weber Santos Cibin: Writing – review & editing, Resources, Project administration, Investigation, Data curation, Conceptualization. **Daniela dos Santos Brum:** Writing – review & editing, Investigation, Conceptualization. **Leivas Fabio Gallas:** Writing – review & editing, Investigation, Conceptualization. **Rogerio Ferreira:** ´ Writing – review & editing, Methodology, Formal analysis. **Laura Rohde Brondani:** Writing – review & editing, Methodology, Formal analysis. **Mariana Gimenez Santos:** Writing – review & editing, Methodology, Formal analysis. **Silva Clara de Carvalho:** Writing – review & editing, Methodology, Formal analysis. **Luiza Gazeta Passos:** Writing – review & editing, Methodology, Formal analysis. **Rafaela Dalmolin Menezes:** Writing – review & editing, Methodology, Formal analysis. **Diogo Ferreira Bicca:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

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

Acknowledgements

This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior ˜– Brazil (CAPES) – Finance Code 001. We acknowledge the National Council for Scientific and Technological Development (CNPQ) and Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) for financial support.

Competing interests

The authors declare that they have no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2025.107993.

References

Agarwal, A., Virk, G., Ong, C., du Plessis, S.S., 2014. Effect of oxidative stress on Male reproduction. World J. Mens. Health 32, 1–17. https://doi.org/10.5534/wjmh.2014.32.1.1.

Anifandis, G., Katsanaki, K., Lagodonti, G., Messini, C., Simopoulou, M., Dafopoulos, K., Daponte, A., 2018. The effect of glyphosate on human sperm motility and sperm DNA fragmentation. Int. J. Environ. Res. Public Health 15 (6), 1117. https://doi.org/10.3390/ijerph15061117.

ANVISA. Brazilian Health Surveillance Agency. 2016. Accessed September 22. Available at: http://portal.anvisa.gov.br/resultado-de-busca?p_p_id=101&p_p_lifecycle=0&p_p_state=maximized&p_p_mode=view&p_p_col_id=column-1&p_p_col_count=1&_101_struts_action=%2Fasset_publisher%2Fview_content&_101_ assetEntryId=2838557&_101_type=document.

ANVISA, Brazilian Health Surveillance Agency, 2018. Preliminary Technical Note on the Conclusions of the Reassessment of Glyphosate. https://www.gov.br/anvisa/ pt-br/centraisdeconteudo/publicacoes/agrotoxicos/notas-tecnicas/nota-tecnica-23-de-2018-glifosato.pdf. (Accessed in Jun 23).

Arbuckle, T.E., Schrader, S.M., Cole, D., Hall, J.C., Bancej, C.M., Turner, L.A., Claman, P., 1999. 2,4-dichlorophenoxyacetic acid residues in semen of ontario farmers. Reprod. Toxicol. 13, 421–429. https://doi.org/10.1016/S0890-6238(99)00057-X.

Avdatek, F., Birdane, Y.O., Türkmen, R., Demirel, H.H., 2018. Ameliorative effect of resveratrol on testicular oxidative stress, spermatological parameters and DNA damage in glyphosate-based herbicide-exposed rats. Andrologia 50, e13036. https://doi.org/10.1111/and.13036.

Bae, J.-W., Kwon, W.-S., 2020. Investigating the effects of fipronil on Male fertility: insight into the mechanism of capacitation. Reprod. Toxicol. 94, 1–7. https://doi.org/10.1016/j.reprotox.2020.04.002.

Barth, A.D., Oko, R.J., 1989. Abnormal Morphology of Bovine Spermatozoa. Iowa State University Press, Ames, p. 285.

Bautista, F.E.A., Varela Junior, A.S., Corcini, C.D., Acosta, I.B., Caldas, S.S., Primel, E.G., Zanette, J., 2018. The herbicide atrazine affects sperm quality and the expression of antioxidant and spermatogenesis genes in zebrafish testes. Comp. Biochem. Physiol. C. Toxicol. Pharm. 206–207, 17–22. https://doi.org/10.1016/j.cbpc.2018.02.003.

BCAR (Brazilian College of Animal Reproduction), 2013. Manual de Procedimentos Para Exame Andrologico E Avaliação de Sêmen Animal, 3 ed. CBRA, Belo˜ Horizonte, p. 104.

Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70–76. https:// doi.org/10.1006/abio.1996.0292.

Betancourt, M., Re´sendiz, A., Fierro, E.C.Y.R., 2006. Effect of two insecticides and two herbicides on the porcine sperm motility patterns using computer-assisted semen analysis (CASA) in vitro. Reprod. Toxicol. 22, 508–512. https://doi.org/10.1016/j.reprotox.2006.03.001.

Bittencourt, R.F., Filho, A.L.R., Ferreira, A.D., Chalhoub, S.M., Alves, S.G.G., Vasconcelos, M.F., Leandro, E.E.S., Guimaraes, J.D., 2005. Utilização do teste hiposmotico para avaliar a eficácia de diferentes protocolos de criopreservação do sêmen caprino. Cien. Anim. Bras. 6, 213–218. https://doi.org/10.5216/cab.v6i3.362.

Bongiovanni, B., Konjuh, C., Pochettino, A., Ferri, A., 2012. Oxidative stress as a possible mechanism of toxicity of the herbicide 2,4-dichlorophenoxyacetic acid (2,4- D), in: Hasaneen, M.N. (Ed.), Herbicides – Properties, Synthesis and Control of Weeds, vol 2., IntechOpen, London, pp. 315-334. https://doi.org/10.5772/32524.

Bruinenberg, M., van Agtmaal, M., Hoekstra, N., van Eekeren, N., 2023. Residues of pesticides in dairy cow rations and Fly treatments reduce the number of coleoptera in dung. Agric. Ecosyst. Environ. 344, 108307. https://doi.org/10.1016/j.agee.2022.108307.

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. Pharm. 55, 148–155. https://doi.org/10.1016/j.etap.2017.07.015.

Castellini, C., Mourvaki, E., Sartini, B., Cardinali, R., Moretti, E., Collodel, G., Fortaner, S., Sabbioni, E., Renieri, T., 2009. In vitro toxic effects of metal compounds on kinetic traits and ultrastructure of rabbit spermatozoa. Reprod. Toxicol. 27, 46–54. https://doi.org/10.1016/j.reprotox.2008.12.003.

Chakraborty, S., Saha, S., 2022. Understanding sperm motility mechanisms and the implication of sperm surface molecules in promoting motility. Middle East Fertil. Soc. J. 27, 4. https://doi.org/10.1186/s43043-022-00094-7.

Chhillar, S., Batra, V., Kumaresan, A., Kumar, R., Pal, A., Datta, T.K., 2023. Acute exposure to organophosphorus pesticide metabolites compromises buffalo sperm function and impairs fertility. Sci. Rep. 13, 9102. https://doi.org/10.1038/s41598-023-35541-6.

Clair, E., Mesnage, R., Travert, C., S´eralini, G.E., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. Vitr. 26, 269–279. https://doi.org/10.1016/j.tiv.2011.12.009.

Cook, L.E., Finger, B.J., Green, M.P., Pask, A.J., 2019. Exposure to atrazine during puberty reduces sperm viability, increases weight gain and alters the expression of key metabolic genes in the liver of Male mice. Reprod. Fertil. Dev. 31, 920–931. https://doi.org/10.1071/RD18505.

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. Sci. Total Environ. 700, 134868. https://doi.org/10.1016/j.scitotenv.2019.134868.

Dovolou, E., Nanas, I., Giannoulis, T., Fytsilli, A., Ntemka, A., Anifandis, G., Tsakmakidis, I., Amiridis, G.S., 2024. The effects of a glyphosate-based herbicide on the bovine gametes during an in vitro embryo production model. Environ. Pollut. 350, 123967. https://doi.org/10.1016/j.envpol.2024.123967.

El-Taieb, M.A., Ali, M.A., Nada, E.A., 2015. Oxidative stress and acrosomal morphology: a cause of infertility in patients with normal semen parameters. Middle East Fertil. Soc. J. 20, 79–85. https://doi.org/10.1016/j.mefs.2014.05.003.

Ferramosca, A., Lorenzetti, S., Di Giacomo, M., Murrieri, F., Coppola, L., Zara, V., 2021. Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency. Reprod. Toxicol. 99, 48–55. https://doi.org/10.1016/j.reprotox.2020.11.011.

Friedrich, K., Silveira, G.R.D., Amazonas, J.C., Gurgel, A.D.M., Almeida, V.E.S., Sarpa, M., 2021. International regulatory situation of pesticides authorized for use in Brazil: potential for damage to health and environmental impacts. Cad. Saude Publica 37, e00061820. https://doi.org/10.1590/0102-311X00061820.

Giulioni, C., Maurizi, V., Castellani, D., Scarcella, S., Skrami, E., Balercia, G., Galosi, A.B., 2022. The environmental and occupational influence of pesticides on Male fertility: a systematic review of human studies. Andrology 10, 1250–1271. https://doi.org/10.1111/andr.13228.

Gonçalves, C.G.M., Leivas, F.G., Missio, D., Santos, F.W., Schwengber, E.B., Folchini, N.P., Pavin, C.U.M., Brum, D.D.S., 2018. Use of a colloid to optimize centrifugation in the selection of bovine sperm for IVF. Semin. Ciˆenc. Agr. aR. 39, 1607´ –1616. https://doi.org/10.5433/1679-0359.2018v39n4p1607.

Gu, Y.-H., Li, Y., Huang, X.-F., Zheng, J.-F., Yang, J., Diao, H., Yuan, Y., Xu, Y., Liu, M., Shi, H.-J., Xu, W.-P., 2013. Reproductive effects of two neonicotinoid insecticides on mouse sperm function and early embryonic development in vitro. PLOS One 8 (7), e70112. https://doi.org/10.1371/journal.pone.0070112.

Harper, A.P., Finger, B.J., Green, M.P., 2020. Chronic atrazine exposure beginning prenatally impacts liver function and sperm concentration with multi-generational consequences in mice. Front. Endocrinol. 11, 580124. https://doi.org/10.3389/fendo.2020.580124.

Hase, Y., Tatsuno, M., Nishi, T., Kataoka, K., Kabe, Y., Yamaguchi, Y., Ozawa, N., Natori, M., Handa, H., Watanabe, H., 2008. Atrazine binds to F1F0-ATP synthase and inhibits mitochondrial function in sperm. Biochem. Biophys. Res. Commun. 366, 66–72. https://doi.org/10.1016/j.bbrc.2007.11.107.

IBAMA (Instituto Brasileiro de Recursos Naturais e Renovaveis), 2023. Relat´orio de Comercializaç´ao de Agrot˜oxicos (Boletim 2023). Os 10 IAs mais vendidos em 2023 ´ (Agrotoxicos químicos).https://www.gov.br/ibama/pt-br/assuntos/quimicos-e-biologicos/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos/relatorios- ´de-comercializacao-de-agrotoxicos#boletinsanuais (Accessed in July 2023).

IBGE (Instituto Brasileiro de Geografia e Estatística), 2023. Pesquisa de Pecuaria Municipal: efetivo de Rebanhos por tipo (cabeças). ´ 〈http://www.ibge.gov.br/estatisticas/economicas/agricultura-e-pecuaria/9107-producao-da-pecuaria-municipal.html〉. (Accessed in July 2023).

Johnson, W., 1997. The significance to bull fertility of morphologically abnormal sperm. Vet. Clin. North Am. Food Anim. Pr. 13 (2), 255–270. https://doi.org/10.1016/S0749-0720(15)30339-X.

Komsky-Elbaz, A., Kalo, D., Roth, Z., 2021. Carryover effect of atrazine and its metabolite—from treated bovine spermatozoa to the embryo's transcriptome. Biol. Reprod. 104, 1162–1180. https://doi.org/10.1093/biolre/ioab027.

Komsky-Elbaz, A., Roth, Z., 2017. Effect of the herbicide atrazine and its metabolite DACT on bovine sperm quality. Reprod. Toxicol. 67, 15–25. https://doi.org/10.1016/j.reprotox.2016.11.001.

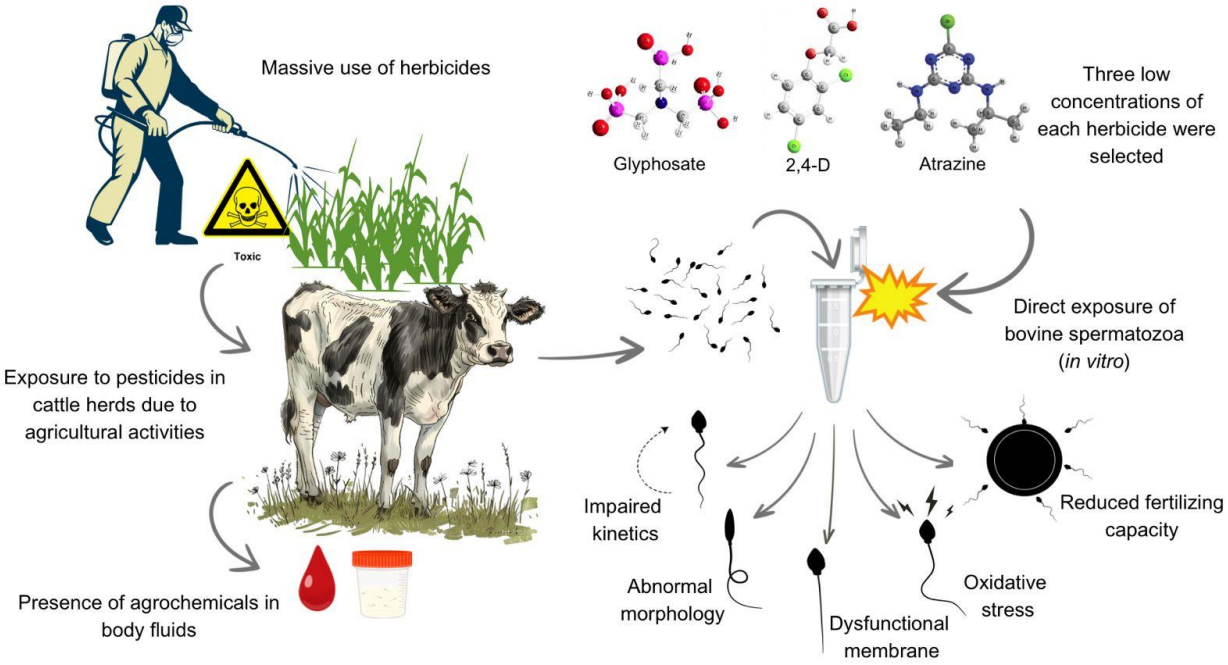
Krüger, M.K., Schledorn, P., Schrodl, W., Hoppe, H.-W., Lutz, W., Shehata, A.A., 2014. Detection of glyphosate residues in animals and humans. J. Environ. Anal. ¨ Toxicol. 4, 210. https://doi.org/10.4172/2161-0525.1000210.
Loetchutinat, C., Kothan, S., Dechsupa, S., Meesungnoen, J., Jay-Gerin, J.-P., Mankhetkorn, S., 2005. Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay. Radiat. Phys. Chem. 72, 323–331. https://doi.org/10.1016/j.radphyschem.2004.06.011.
Mannucci, A., Argento, F.R., Fini, E., Coccia, M.E., Taddei, N., Becatti, M., Fiorillo, C., 2021. The impact of oxidative stress in Male infertility. Front. Mol. Biosci. 8, 799294. https://doi.org/10.3389/fmolb.2021.799294.
Maravilla-Galvan, R., Fierro, R., Gonz´ alez-M´ arquez, H., G´ omez-Arroyo, S., Jim´ enez, I., Betancourt, M., 2009. Effects of atrazine and fenoxaprop-ethyl on capacitation ´ and the acrosomal reaction in boar sperm. Int. J. Toxicol. 28, 24–32. https://doi.org/10.1177/1091581809333138.
Marcu, D., Keyser, S., Petrik, L., Fuhrimann, S., Maree, L., 2023. Contaminants of emerging concern (CECs) and Male reproductive health: challenging the future with a double-edged sword. Toxics 11, 330. https://doi.org/10.3390/toxics11040330.
Marouani, N., Tebourbi, O., Cherif, D., Hallegue, D., Yacoubi, M.T., Sakly, M., Benkhalifa, M., Ben Rhouma, K., 2017. Effects of oral administration of 2,4-dichlorophenoxyacetic acid (2,4-D) on reproductive parameters in Male wistar rats. Environ. Sci. Pollut. Res. Int. 24, 519–526. https://doi.org/10.1007/s11356-016- 7656-3.
Mesnage, R., Brandsma, I., Moelijker, N., Zhang, G., Antoniou, M.N., 2021. Genotoxicity evaluation of 2,4-D, dicamba and glyphosate alone or in combination with cell reporter assays for DNA damage, oxidative stress and unfolded protein response. Food Chem. Toxicol. 157, 112601. https://doi.org/10.1016/j.fct.2021.112601.
Missio, D., Folchini, N., Leivas, F., Pavin, C., Pinto, H., Cibin, F., Brum, D., 2018. Reduction in percoll volume increases recovery rate of sex-sorted semen of bulls without affecting sperm quality and early embryonic development. Anim. Reprod. Sci. 192, 146–153. https://doi.org/10.1016/j.anireprosci.2018.03.002.
Moreira, S., Pereira, S.C., Seco-Rovira, V., Oliveira, P.F., Alves, M.G., Pereira, M.L., 2021. Pesticides and Male fertility: a dangerous crosstalk. Metabolites 11, 799. https://doi.org/10.3390/metabo11120799.
Moretti, E., Signorini, C., Noto, D., Corsaro, R., Collodel, G., 2022. The relevance of sperm morphology in Male infertility. Front. Reprod. Health 4, 945351. https:// doi.org/10.3389/frph.2022.945351.
Mortimer, S.T., 2000. CASA – practical aspects. J. Androl. 21, 491–496. https://doi.org/10.1002/j.1939-4640.2000.tb02116.x.
Nerozzi, C., Recuero, S., Galeati, G., Bucci, D., Spinaci, M., Yeste, M., 2020. Effects of roundup and its main component, glyphosate, upon mammalian sperm function and survival. Sci. Rep. 10, 11026. https://doi.org/10.1038/s41598-020-67538-w.
Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., Hens, L., 2016. Chemical pesticides and human health: the urgent need for a new concept in agriculture. Front. Public Health 4, 148. https://doi.org/10.3389/fpubh.2016.00148.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem 95, 351–358. https://doi.org/10.1016/0003-2697(79)90738-3.
Oliveira, M.P., Silva, A.B.S., Rigueto, C.V.T., Loss, R.A., Guedes, S.F., Geraldi, C.A.Q., 2023. Pesticides in different environmental compartments in Brazil: a review. CeN 45, e2. https://doi.org/10.5902/2179460X70715.
Parrish, J.J., Krogenaes, A., Susko-Parrish, J.L., 1995. Effect of bovine sperm separation by either swim-up or percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44, 859–869. https://doi.org/10.1016/0093-691X(95)00271-9.
Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H., First, N.L., 1986. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 25, 591–600. https://doi.org/10.1016/0093-691x(86)90143-3.
Perry, M.J., 2008. Effects of environmental and occupational pesticide exposure on human sperm: a systematic review. Hum. Reprod. Update 14, 233–242. https://doi.org/10.1093/humupd/dmm039.
Roth, Z., Komsky-Elbaz, A., Kalo, D., 2020. Effect of environmental contamination on female and Male gametes – a lesson from bovines. Anim. Reprod. 17, e20200041. https://doi.org/10.1590/1984-3143-AR2020-0041.
Sass, J.B., Colangelo, A., 2006. European union bans atrazine, while the United States negotiates continued use. Int. J. Occup. Environ. Health 12, 260–267. https:// doi.org/10.1179/oeh.2006.12.3.260.
Sellem, E., Broekhuijse, M.L., Chevrier, L., Camugli, S., Schmitt, E., Schibler, L., Koenen, E.P., 2015. Use of combinations of in vitro quality assessments to predict fertility of bovine semen. Theriogenology 84, 1447–1454.e5. https://doi.org/10.1016/j.theriogenology.2015.07.035.
Selvaraju, V., Baskaran, S., Agarwal, A., Henkel, R., 2021. Environmental contaminants and Male infertility: effects and mechanisms. Andrologia 53, e13646. https:// doi.org/10.1111/and.13646.
SenGupta, P., Banerjee, R., 2014. Environmental toxins: alarming impacts of pesticides on Male fertility. Hum. Exp. Toxicol. 33, 1017–1039. https://doi.org/10.1177/ 0960327113515504.
Song, Y., Jia, Z.C., Chen, J.Y., Hu, J.X., Zhang, L.S., 2014. Toxic effects of atrazine on reproductive system of Male rats. Biomed. Environ. Sci. 27, 281–288. https:// doi.org/10.3967/bes2014.050.
Sørensen, M.T., Poulsen, H.D., Katholm, C.L., Højberg, O., 2021. Review: feed residues of glyphosate – potential consequences for livestock health and productivity. Animal 15, 100026. https://doi.org/10.1016/j.animal.2020.100026.
Spinaci, M., Nerozzi, C., Mislei, B., Blanco-Prieto, O., Mari, G., Galeati, G., Bucci, D., 2022. Impact of glyphosate and its formulation Roundup® on stallion spermatozoa. Theriogenology 179, 197–203. https://doi.org/10.1016/j.theriogenology.2021.12.003.
Stojkovic, M., Machado, S.A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P.B., Wolf, E., 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization andculture. Biol. Reprod. 64, 904–909. https://doi.org/10.1095/biolreprod64.3.904.
Sule, R.O., Condon, L., Gomes, A.V., 2022. A common feature of pesticides: oxidative stress-the role of oxidative stress in pesticide-induced toxicity. Oxid. Med. Cell. Longev. 2022, 5563759. https://doi.org/10.1155/2022/5563759.
Tan, Z., Zhou, J., Chen, H., Zou, Q., Weng, S., Luo, T., Tang, Y., 2016. Toxic effects of 2,4-dichlorophenoxyacetic acid on human sperm function in vitro. J. Toxicol. Sci. 41, 543–549. https://doi.org/10.2131/jts.41.543.

Tirpak, F., Greifov´ a, H., Luk´ a´˘c, N., Stawarz, R., Massanyi, P., 2021. Exogenous factors affecting the functional integrity of Male reproduction. Life 11, 213. https://doi. org/10.3390/life11030213.
Torres-Badia, M., Solar-Malaga, S., Martin-Hidalgo, D., Hurtado de Llera, A., Gomez-Candelo, A., Garcia-Marin, L.J., Gonzalez-Fern´andez, L., Bragado, M.J., 2021. ´ Impaired mammalian sperm function and lower phosphorylation signaling caused by the herbicide Roundup®Ultra plus are due to its surfactant component. Theriogenology 172, 55–66. https://doi.org/10.1016/j.theriogenology.2021.05.026.
Urseler, N., Bachetti, R., Biol´e, F., Morgante, V., Morgante, C., 2022. Atrazine pollution in groundwater and raw bovine milk: water quality, bioaccumulation and human risk assessment. Sci. Total Environ. 852, 158498. https://doi.org/10.1016/j.scitotenv.2022.158498.
Zhang, D., Wu, Y., Yuan, Y., Liu, W., Kuang, H., Yang, J., Yang, B., Wu, L., Zou, W., Xu, C., 2017. Exposure to 2,4-dichlorophenoxyacetic acid induces oxidative stress and apoptosis in mouse testis. Pestic. Biochem. Physiol. 141, 18–22. https://doi.org/10.1016/j.pestbp.2016.10.006.
Zubair, M., Ahmad, M., Jamil, H., 2015. Review on the screening of semen by hypo-osmotic swelling test. Andrologia 47, 744–750. https://doi.org/10.1111/and.12335.

Glossary

2,4-D: 2,4-dichlorophenoxyacetic acid
ATZ: atrazine
BCF: beat cross frequency
CASA: computer-assisted semen analysis
DCF: dichlorofluorescein
DMSO: dimethyl
sulfoxide *GLP:*
glyphosate
HOS: hypo-
osmotic swelling
IVF: in vitro
fertilization
ROS: reactive
oxygen species
SCA: Sperm Class
Analyzer *VAP:*
mean path
velocity.

Graphical abstract



Reproductive Toxicology 137 (2025) 109037

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Effects of herbicide mixtures on the fertilizing capacity of bovine sperm

Diogo Ferreira Bicca [a,b], Rafaela Dalmolin Menezes [a], Laura Rohde Brondani [a], Monike Quirino [c], Larissa Thaísa Weide [a], Fabio Gallas Leivas´ [a], Daniela dos Santos Brum [a], Francielli Weber Santos Cibin [a,b,*]

[a] *Laboratorio de Biotecnologia da Reproduç´ ao (Biotech), Campus Uruguaiana, Universidade Federal do Pampa (UNIPAMPA), Uruguaiana, RS CEP 97500-970, Brazil˜* [b] *Laboratorio de Estresse Oxidativo, Campus Uruguaiana, Universidade Federal do Pampa (UNIPAMPA), Uruguaiana, RS CEP 97500-970, Brazil´* [c] *Instituto Federal Catarinense, IF-Catarinense, Brazil*

ARTICLEINFO

Keywords:

Herbicides
Glyphosate
2
4-D
Atrazine
Bovine
Sperm
Fertility

ABSTRACT

Herbicides are the most commonly used pesticide type worldwide. In Brazil, glyphosate-,dichlorophenoxyacetic acid (2,4-D)-, and atrazine-based pesticide formulations are intensively applied to crops, and mixtures of these compounds occur frequently in the environment. Owing to their proximity to these areas and management practices, bovines are exposed to these pesticide mixtures, and their impact on their health is unknown. In this study, we evaluated the *in vitro* effects of herbicide mixtures on bovine sperm. Semen from four bulls was prepared as a pool and divided into groups: control, dimethyl sulfoxide (DMSO), glyphosate (Gly; 50 µg/mL), 2,4-D (0.11 µg/mL), atrazine (Atz; 0.0107 µg/mL), Gly +2,4-D (GD), Gly +Atz (GA), Atz+2,4-D (AD) and Gly +2,4-D+Atz (GDA). Sperm cells were evaluated after 3 h of incubation with the different treatments at 37°C. Results showed that the Gly, 2,4-D, and AD groups decreased progressive motility, mean path velocity, and beat cross frequency compared to the control group. Similarly, Gly and 2,4-D reduced curvilinear and straight-line velocities, and 2,4-D affected the amplitude of lateral head displacement. Although no differences in reactive species levels were detected, an overall reduction in antioxidant capacity was observed. Membrane integrity, acrosome damage, and mitochondrial membrane potential results did not differ significantly among the groups. However, the mixture diminished sperm fertilization capacity in groups GD, AD, and GDA, when compared to the control. No effects appeared in the DMSO group. Herbicides showed distinct impacts on bovine sperm, emphasizing the importance of evaluating pesticide mixtures thoroughly

1. Introduction

Herbicides are an important class of agrochemicals used to minimize or eliminate plant species that are considered harmful to crops [1]. Brazil is a major consumer of herbicides owing to its vast agricultural production area, and most formulations contain the active compounds glyphosate (Gly), dichlorophenoxyacetic acid (2,4-D), and atrazine (Atz), which are in the top selling herbicides according to IBAMA [2]. However, the widespread use of these pesticides has raised concerns about increased exposure, as—despite their intended herbicidal purpose—literature has demonstrated their ability to cause deleterious effects on non-target organisms, disrupting various physiological systems, including the reproductive system [3,4].

Reproductive effects due to exposure to Gly [5], 2,4-D [6], and Atz [7] have been reported to cause impairments in both sexes in various animal models by acting as endocrine disruptors, promoting damage to reproductive organs, and reducing gamete quality. Studies involving exposure to Gly [8,9], 2,4-D [10] and Atz [11] with rodent models have shown that their effects on the male reproductive system are primarily associated with disruptions in testosterone metabolism, damage to the testis and epididymis, and alterations in the structure and function of Sertoli and Leydig cells, ultimately affecting the germinal cell line. As consequence, a reduction in sperm quality occurs, as demonstrated in studies with Gly [13], 2,4-D [6,14] and Atz [12,14] which is evidenced by findings in semen such as decreased concentration, motility impairments and morphological abnormalities. These results show that pesticides exposure could contribute to a reduction in the global fertility status, through direct and indirect mechanisms [15].

* Corresponding author at: Laboratorio de Biotecnologia da Reproduç´ ao (Biotech), Campus Uruguaiana, Universidade Federal do Pampa (UNIPAMPA), Uruguaiana, RS CEP 97500-970, Brazil.
E-mail address: francielliweber@yahoo.com.br (F.W.S. Cibin). https://doi.org/10.1016/j.reprotox.2025.109037

Received 17 June 2025; Received in revised form 20 August 2025; Accepted 21 August 2025
Available online 22 August 2025

Despite the knowledge about the impact of herbicides on reproduction, information on these chemical actions in a mixed context is lacking owing to the variability of the compounds and numerous interaction possibilities [16]. Recently, the presence of glyphosate, 2,4-D, and atrazine was reported in the ecosystems of different regions of Brazil, which is a cause for concern owing to environmental characteristics and compound properties, but also because these herbicides are commonly associated with agricultural procedure, leading to exposure with unknown health consequences [17,18,19]. Studies considering mixtures of chemicals are relevant because they are consistent with reality, as these compounds are frequently used and found together in the environment [20,21].

Cattle raising is an important economic activity in most countries [22] and the size of herds is increasing globally. In the context of rural production, most herds are frequently exposed to pesticides and contact often occurs through natural resources and feeding with pesticide-contaminated feed as well as through routine management [23,24]. Moreover, cattle are peculiarly exposed to pesticide mixtures via different routes; however, the possible impacts of exposure on their health and reproductive physiology remain unknown.

Due to their morphofunctional characteristics, spermatozoa represent a highly useful *in vitro* model for assessing the effects of exposure to various combinations of pesticide mixtures. The detection of herbicides in biological fluids such as semen supports the use of sperm parameter evaluation to investigate the potential of these compounds to induce disturbances that may compromise the structure and function of these cells, even after spermatogenesis has been completed [12, 25]. Therefore, in this study we evaluated the effects of the herbicides glyphosate, 2,4-D, and atrazine alone and in different combinations (mixtures), using bovine sperm parameters as risk assessment markers, to determine the impact of pesticide combinations on the reproductive features of this species.

2. Material and methods

2.1. Chemicals

The active ingredients of the herbicides—glyphosate (No. 45521, CAS: 1071–83–6), 2,4-D (No. 31518, CAS: 94–75–7), and atrazine (No. 45330, CAS: 1912–24–9)—as well as dimethyl sulfoxide (DMSO), the diluent used to prepare the solutions, were purchased from Sigma- Aldrich (St. Louis, MO, USA), unless otherwise specified. Fluorescein- isothiocyanate conjugated Pisum sativum agglutinin (FITC-PSA; Sigma-Aldrich, USA), carbonyl cyanide 3-chlorphenylhydrazone (CCCP; Sigma-Aldrich, USA) solution, propidium iodide (PI; Sigma-Aldrich, USA), MitoStatus TMRE (BD Pharmingen™, BD Biosciences, USA), Syto-24 (Invitrogen, Thermo Fisher Scientific, USA), and phosphate buffered saline (PBS; pH 7,2, Laborclin, Brazil) were purchased for flow cytometry. The fluorochrome solutions were prepared in PBS (FITC-PSA and PI) or DMSO (MitoStatus TMRE and Syto-24).

2.2. Sperm selection

Bovine semen was provided by a commercial establishment (Renascer Biotecnologia ®/Brazil). Samples were obtained from four bulls (Bos taurus) with proven fertility, in frozen straws from the same batch and collection. In the laboratory, the four frozen straws containing the semen of each bull were thawed at 37◦C for 30 s, homogenized and mixed to form a sperm pool, and then observed under a phase contrast microscope (CX-31; Olympus, Tokyo, Japan) for motility and vigor, which were confirmed using a computer-assisted semen analysis (CASA) system with Sperm Class Analyzer (SCA) software (version 5.1; Microptotic, Barcelona, Spain). Using the protocol described by Gonçalves et al. [26], sperm from the pool were selected using a discontinuous Percoll gradient solution. Briefly, 350 µL of sperm pool sample was layered on top of the gradient solution and centrifuged at 4000 ×g for 5 min. Thereafter, 100 µL of the pellet was resuspended in pre-warmed modified TALP-Fert medium (300 µL; pH 7.2–7.4, 270–290 mOsm, with 87 mM NaCl, 3.10 mM KCl, 2 mM CaCl2, 0.3 mM NaH2PO4, 0.4 MgCl2, 10 mM NaHCO-3, 40 mM HEPES, 1 mM pyruvic acid, 21.6 mM lactic acid, and 6 mM BSA) according to the method described by Parrish et al. [27], and centrifuged for 1 min at 4000 ×g. Finally, 100 µL of the pellet containing selected spermatozoa was reevaluated for motility and vigor to ensure the cells were in optimal conditions for the subsequent analysis. To adjust the concentration, 5 µL of the pooled sample was fixed in 95 µL formaldehyde and counted using a Neubauer chamber, and the volume of sample necessary to achieve a concentration of 4 ×106 sperm/mL in 400 µL of modified TALP-Fert medium, was calculated. This sperm pool preparation procedure was performed for each experimental repetition, using frozen straws collected from the same four bulls.

2.3. Experiment design

After the selection and concentration adjustment phases, spermatozoa were divided into different treatment groups (Fig. 1). Herbicide solutions were prepared in DMSO (10 µL, 2,5 %) and added to the TALP- Fert medium (400 µL) used for the groups. The concentrations of the active ingredients of the herbicides used in this study were based on previous research involving the detection of these compounds and in vitro analyses. For glyphosate, the average concentration detected in the urine of dairy cows, as reported by Kruger et al. [28], along with the evaluations conducted by Nerozzi et al. [29] using the active ingredient, served as the basis for selecting the glyphosate concentration used in this study (50 µg/mL). The concentration of 2,4-D was defined according to the in vitro assessment studies by Tan et al. [30], with a chosen concentration of 0.11 µg/mL, equivalent to 0.5 µM, taking into account the minimum detection levels of 2.94 µM in human semen [31]. Finally, for atrazine, the concentration of 0.0107 µg/mL was established based on the findings of Komsky-Elbaz and Roth [32] regarding the effects of in vitro exposure on bovine spermatozoa, and the detection data reported by Urseler et al. [33]in bovine milk samples. The groups were divided as follows: control (only medium), DMSO (10 µL of DMSO), glyphosate (Gly, 50 µg/mL), 2,4-D (0.11 µg/mL), atrazine (Atz, 0.0107 µg/mL). The mixture groups were prepared using the same concentrations of the isolated compounds. The combination groups were as follows: GD (Gly + 2,4-D), GA (Gly + Atz), AD (Atz + 2,4-D) and GDA (Gly + 2,4-D + Atz), as shown in Fig. 1. The selected sperm were added to a pre-warmed medium and to each treatment group and incubated in an oven at 37 ◦C for 3 h, and then immediately evaluated or frozen for subsequent analyses (Fig. 1). The experimental procedures (pool preparation, sperm selection, and exposure) were repeated for each experiment repetition.

2.4. Kinematic parameters

Sperm kinematics were evaluated after exposure treatments using a Computer-Assisted Semen analysis system (CASA) with Sperm Class Analyzer (SCA) software (version 5.1; Microptotic, Barcelona, Spain). For each treatment, a microscope slide with a 5 µL aliquot of semen was prepared and analyzed at 100 × magnification under controlled temperature conditions (37 °C). Five different fields, with an average of 200 cells per treatment/replicate, were evaluated at 25 frames/field with a rate of 25 fields/s, and the sperm parameters were analyzed considering the following criteria: head area, 25–70 µm2; velocity limit for slow spermatozoa, 10 µm/s; velocity limit for medium spermatozoa, 25 µm/ s; velocity limit for fast spermatozoa, 50 µm/s; minimal straightness for progressive spermatozoa, 70 %; and the maximal percentage of linearity, 50 %. The kinematic parameters of total motility (TMot, %), progressive motility (PMot,%), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), mean path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), and beat cross frequency (BCF, Hz) were analyzed using the method described by Mortimer [34].

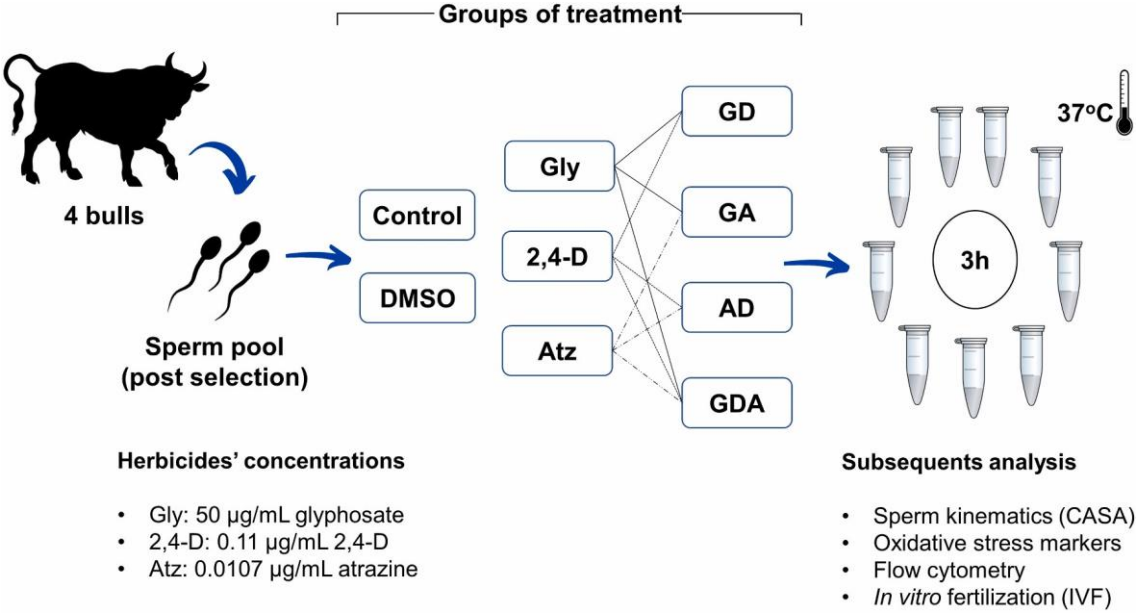


Fig. 1. Study's experimental design and procedures. A pool of semen from four bulls with proven fertility was used to evaluate the effects of concentrations of the active ingredients glyphosate, 2,4-D, and atrazine, both individually and in combination. Following treatment across the different experimental groups, the samples were subjected to subsequent analyses to investigate the impacts on bovine sperm function. Groups: Control (medium); DMSO (dimethyl sulfoxide); Gly (glyphosate); 2,4-D; Atz (atrazine); GD (Gly + 2,4-D); GA (Gly + Atz); AD (Atz + 2,4-D); and GDA (Gly + 2,4-D + Atz).

2.5. Oxidative stress analysis

The effects of herbicide exposure on the different treatment groups were analyzed using previously described protocols for oxidative stress markers. To determine reactive oxygen species (ROS) levels we used 2′,7′-dichlorofluorescein diacetate (DCF-DA) according to the method described by Loetchutinat et. al [35]. Briefly, 50 µL samples were incubated in the dark with 5 µL of DCF-DA (1 mM) and Tris HCl buffer (10 mM, pH 7.4). The resulting oxidation of DCF-DA to fluorescent DCF by ROS was measured after 120 min at 520 nm (with excitation at 488 nm) using a Shimadzu spectrofluorometer (model RF5301PC; Kyoto, Japan) and the results were expressed in fluorescence units (FU). Total antioxidant capacity (TAC) was analyzed using the ferric-reducing antioxidant potential (FRAP) assay described by Benzie and Strain [36], based on the ability of antioxidants present in samples to reduce Fe^{+3} to Fe^{+2}, which forms a complex with 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), measurable at an absorption of 593 nm. A standard curve with ascorbic acid was used and the results were expressed in micrograms of ascorbic acid equivalents (µg e.q AA).

2.6. Flow cytometry

For flow cytometry analysis, sperm samples of each treatment were homogenized and two 250 µL aliquots were obtained. Each aliquot was diluted in 250 µL of warmed and filtered PBS (37 °C; final concentration, 1×10^6 sperm cells/mL). The first sample was stained with FITC-PSA (1 µL of a working solution composed of 75 µg FITC-PSA/mL) and PI (10 µL of a working solution consisting of 50 µg PI/mL) to assess acrosomal status (FITC-PSA) and plasma membrane integrity (PI). This sample was homogenized by vortexing for 2 s, and then incubated in the dark for 10 min at 37 °C. The second sample was stained with MitoStatus TMRE (1 µL of a working solution at 125 µM) to assess the mitochondrial membrane potential. It was homogenized by vortexing for 2 s, and incubated in the dark for 20 min at 37 °C. After the incubation period, 250 µL of warmed filtered PBS (37 °C) was added

to each stained sample to reach a final concentration of 0.75×10^6 sperm cells/mL. The samples were again homogenized by vortexing for 2 s and then analyzed using a BD FACSLyric™ equipped with two lasers (blue, 488 nm and red, 640 nm). For each sample, a total of 10,000 events into the 'sperm population' gate was obtained at a medium flow rate (60 µL/s). The sperm population that was previously established based on a control sample stained with a cell marker (Syto-24; final concentration: 15 nM), was gated by referring to the expected forward- and side-scatter signals (FSC and SSC) after excluding agglutinations by forward-scatter gating (FSC-area vs. FSC-height).

All fluorochromes were excited by the blue laser, and the fluorescence signals of FITC-PSA, PI, and MitoStatus TMRE were detected using band-pass filters of 525/40 nm (FITC), 700/54 nm (PerCP-Cy 5.5), and 586/42 nm (PE), respectively. Signals were plotted on logarithmic scales, and the percentage of cells with non-intact plasma membranes (positive for PI), damaged acrosome (positive for FITC-PSA), and high mitochondrial membrane potential (high fluorescence for MitoStatus TMRE), were recorded. All data were recorded and analyzed using the BD FACSLyric™ software.

Before each routine ($n = 4$), the equipment was calibrated to verify the optical alignments and fluidic system using specific calibration beads provided by the manufacturer. All gates established for the fluorochromes were defined during a control routine using samples from the control group to prepare unstained, positive, and negative controls for all fluorochromes used. For PI and FITC-PSA, the negative control was a sample stained with each fluorochrome individually (low percentage of cells with positive fluorescence for both fluorochromes), whereas the positive control was a flash-frozen sample (sample subjected to three rounds of a flash-freezing process in liquid nitrogen followed by rapid thawing at 37 °C) stained with each fluorochrome individually. The flash-frozen sample consisted of a sample subjected to three rounds of a flash-freezing process in liquid nitrogen followed by rapid thawing at 37 °C to cause damage in both acrosome and plasma membrane (high percentage of cells with positive fluorescence for PI and FITC-PSA) [37]. For MitoStatus TMRE, the control procedure was performed using a sample stained with MitoStatus TMRE (leading to a high percentage of cells with MitoStatus TMRE fluorescence) and a sample stained with MitoStatus TMRE after the addition of CCCP (final concentration, 1 mM) and incubated for 5 min at 37 °C [38] to disrupt the mitochondrial membrane potential, decreasing the MitoStatus TMRE fluorescence. Control samples were also used to correct any spectral overlap (for the panels using FITC-PSA and PI).

2.7. Fertilization capacity

The fertilization capacity of treated sperm from the mixture groups was analyzed using the *in vitro* fertilization (IVF) protocol. Bovine ovaries were collected from a commercial slaughterhouse and transported to laboratory in controlled conditions (0.9 % physiological saline with 100 IU/mL penicillin and 100 IU/mL streptomycin at 30 °C). Using a vacuum pump (20 mL of water/min), the cumulus-oocyte complexes (COCs, follicles 3–5 mm in diameter) were aspirated, recovered, and selected for maturation, considering the homogeneous ooplasm and intact cumulus layers [39]. In this phase, groups of COCs were transferred to 3-well plates, each one with 400 µL of TCM-199 medium with 20 % estrous cow serum (Biotech, Uruguaiana, RS, Brazil) containing 5 µg/mL of porcine follicle stimulating hormone, NIH-FSH-P1 (Folltropin-V®, Bioniche Animal Health, Ontario, Canada), 5 µg/mL of pituitary luteinizing hormone, LH-P (Lutropin-V®, Bioniche Animal Health), and 22 µg/mL of pyruvate and gentamicin. COCs were cultured for 24 h at 39 °C in a 5 % CO_2 atmosphere and saturated humidity. Matured COCs were transferred to a solution containing 150 µL of modified TALP-Fert medium [40], 22 µg/mL of pyruvate, 6 mg/mL of bovine serum albumin (BSA), 10 µg/mL of heparin, 20 µM/mL of penicillamine, 10 µM/mL of hypotaurine, and 2 µM/mL of epinephrine, which was prepared in 30 × 10 mm plates (Corning Incorporated Life Sciences) with mineral oil. The matured COCs were co-cultured with 2×10^6 sperm/mL in groups of 20–25 COCs per treatment and incubated at 39 °C in saturated humidity and a 5 % CO_2 atmosphere. After 18 h of co-incubation, the potential zygotes (non-treated oocytes exposed to pesticide-treated sperm) were selected and stained using 15 µg/mL of bisbenzimide (Hoechst 33342; Life Technologies, Japan) in PBS and individually analyzed under an epifluorescence microscope (IX-1; Olympus, Tokyo, Japan) at 400 × magnification with excitation and emission at 365 nm and 410 nm, respectively, to evaluate the fertilization rate, according to the method described by Missio et al. [41]. The fertilization was considered normal if oocytes were completely penetrated by spermatozoon, and if two pronuclei or a fused nucleus were observed. The results were expressed as the percentage of fertilization relative to the total potential zygotes observed.

2.8. Statistical analysis

The results of the analyses are expressed as the mean ± standard error of the mean (SEM) from at least three experimental replicates. Statistical analyses were performed using GraphPad Prism (version 8.0.2). First, the results were evaluated using the Shapiro–Wilk normality test. Subsequently, one-way analysis of variance (ANOVA) and Tukey's post hoc tests were used to evaluate the kinematic parameters, oxidative stress markers, and flow cytometry results. Dunnett's post hoc test was used to analyze the IVF results. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of treatments in sperm kinematics

The kinematic parameters were affected by herbicide exposure in specific treatment groups (Fig. 2). To assess the effects of the treatments, the control group (spermatozoa and medium) was used as the baseline reference. Additionally, the DMSO group (spermatozoa, medium and DMSO) was included to ensure that the diluent itself exerted no influence on sperm kinematics, as shown by the absence of statistical differences ($P > 0.05$) in all evaluations in comparison with control group (Fig. 2). Regarding sperm parameters, although total motility (TMot) did not show any statistical differences among the groups ($P = 0.6646$), progressive motility (PMot) was significantly reduced in the Gly (17.7 % ± 5.23, $P = 0.0048$), 2,4-D (24 % ± 4.42, $P = 0.0422$) and AD (22.7 % ± 2.90, $P = 0.0276$) groups, compared with the control group (45.3 % ± 1.34). Interestingly, these results showed that the isolated glyphosate and 2,4-D groups differed significantly (respectively, $P = 0.0027$ and $P = 0.0242$) from the GD group (47 % ± 1.56)

revealing a possible inhibitory effect of these chemicals on each other. This group also significantly differed from AD ($P = 0.0157$), which demonstrated a heterogeneous influence on progressive motility depending on the composition of the mixture (Fig. 2B).

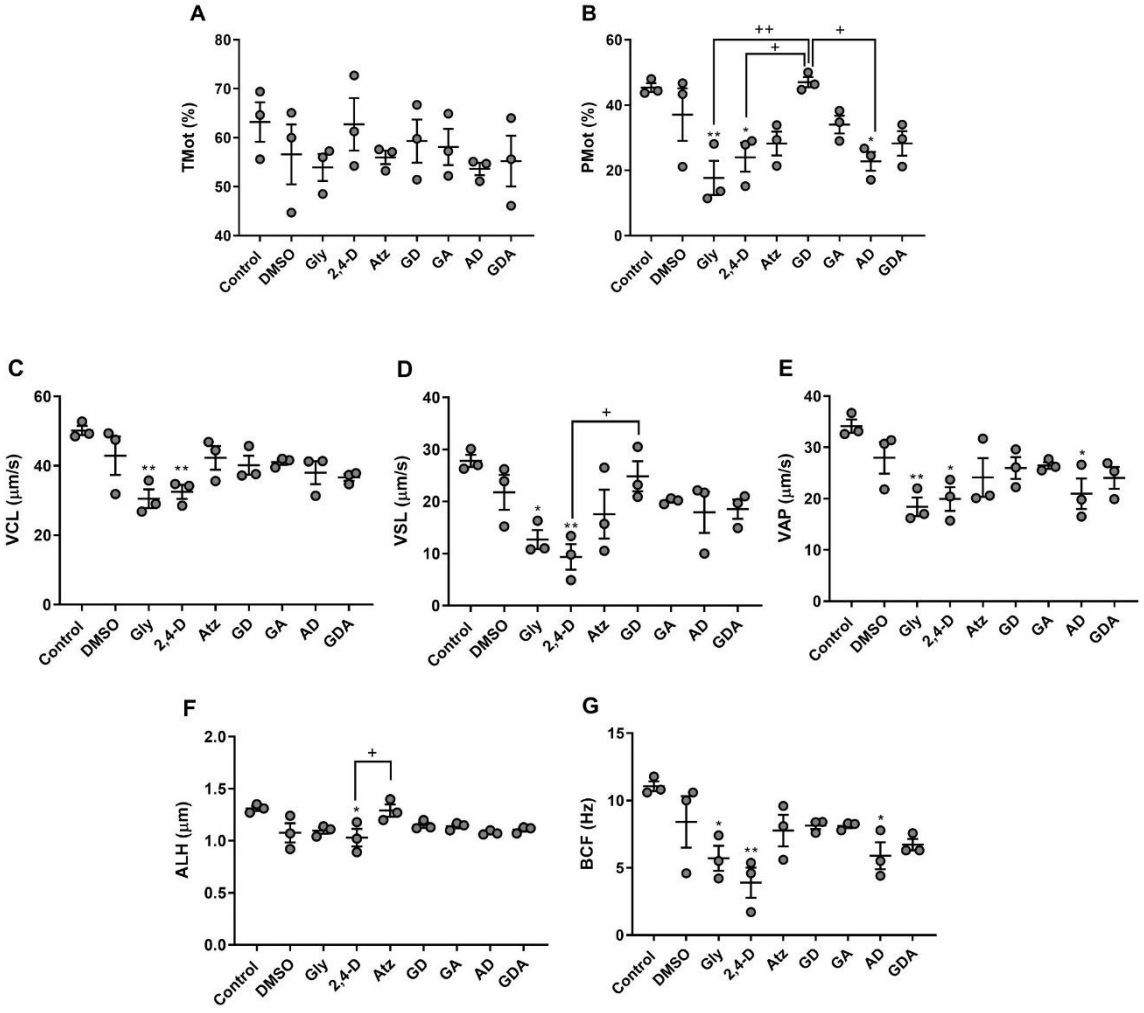


Fig. 2. Kinematic analysis of bovine spermatozoa exposed to isolated compounds and mixtures. Groups: Control (medium); DMSO (dimethyl sulfoxide); Gly (glyphosate); 2,4-D; Atz (atrazine); GD (Gly + 2,4-D); GA (Gly + Atz); AD (Atz + 2,4-D); and GDA (Gly + 2,4-D + Atz). (A) Total motility (TMot). (B) Progressive motility (PMot). (C) Curvilinear Velocity (VCL). (D) Straight-Line Velocity (VSL). (E) Mean Path Velocity (VAP). (F) Amplitude of Lateral Head Displacement (ALH). (G) Beat Cross Frequency (BCF). Data are reported as means ± SEM, after 3 repetitions. One way ANOVA followed by Tukey post hoc test identified statistical differences (* $P < 0.05$ vs control group; ** $P < 0.01$ vs control group; + $P < 0.05$ vs groups; ++ $P < 0.01$ vs groups).

A similar pattern was observed for the velocity parameters. VCL was reduced (Fig. 2C) in the Gly (30.5 µm/s ± 2.70, $P = 0.0036$) and 2,4-D (32.5 µm/s ± 2.0, $P = 0.0098$) groups compared to the control group (50.1 µm/s ± 1.32). VSL was reduced in the Gly (12.7 µm/s ± 1.8, $P = 0.0288$) and 2,4-D (9.3 µm/s ± 2.45, $P = 0.0051$) groups compared to the control group (27.8 µm/s ± 1.16), and a marked difference between the 2,4-D and GD groups (24.8 µm/s ± 2.89, $P = 0.0234$) was also observed (Fig. 2D). VAP was reduced in the Gly (18.4 µm/s ± 1.81, $P = 0.005$) and 2,4-D (19.9 µm/s ± 2.32, $P = 0.0133$) groups compared to the control (34.1 µm/s ± 1.28) and AD (20.9 µm/s ± 2.97, $P = 0.0246$; Fig. 2E) groups.

For ALH (Fig. 2F), only the 2,4-D group shown a significant reduction (1.01 µm ± 0.08, $P = 0.0183$) compared to the control (1.31 µm ± 0.02) and Atz (1.29 µm ± 1.54, $P = 0.0326$) group. Finally, BCH was reduced in the Gly (5.7 Hz ± 0.93, $P = 0.0231$), 2,4-D (3.9 Hz ± 1.11, $P = 0.0015$), and AD (5.9 Hz ± 0.89, $P = 0.0305$) groups compared to the control group (11 Hz ± 0.36) (Fig. 2G).

3.2. Oxidative stress findings

After the sperm was incubated for 3 h with each herbicide and their mixtures, the quantification of ROS levels in the samples did not show any significant differences ($P = 0.9820$) among the groups exposed to isolated compounds or the groups exposed to compound mixtures, compared to the control group (Fig. 3A).

However, we verified that the total antioxidant capacity was significantly reduced in all groups after the incubation period ($P < 0.0001$) compared to the control group (608 Eq. AA ± 21.54), regardless of exposure to isolated compounds or mixtures (Fig. 3B). The effect of herbicide treatments on antioxidant capacity is supported by the observation that the DMSO group (665.7 Eq. AA ± 21.94; P = 0.6239) did not exhibit any significant difference compared to the control group (Fig. 3B). In addition, a significant

difference (P = 0.0497) was observed in the reduction of antioxidant capacity in the Atz group (356 Eq. AA ± 23.86) compared to the Gly group (462 Eq. AA ± 29.14) (Fig. 3B).

3.3. Flow cytometry evaluations

According to the flow cytometry analysis, the effects of treatments on the percentage of sperm cells with non-intact plasma membrane, acrosomal damage and high mitochondrial activity in all groups showed no significant differences compared to the control group. The results of this analysis are presented in Table 1.

3.4. Fertilization rate outcomes

Fertilization capacity was evaluated only for sperm exposed to the compound mixtures. It was verified that fertilization rates in the DMSO- treated group remained statistically indistinguishable (47.7 % ± 4.85, n = 83) from those observed in the control group (59.5 % ± 3.22, n = 82), eliminating a possible diluent adverse effect on sperm fertilization capacity (Fig. 4). However, the IVF procedure results showed that the percentage of oocytes fertilized by sperm from the GD (36.1 % ± 7.95, n = 86, P = 0.0353), AD (34.2 % ± 8.19, n = 85, P = 0.0217), and GDA (28.8 % ± 4.48, n = 83, P = 0.0051) groups were significantly lower than that from the control group (Fig. 4). The only mixture group that did not show a reduction in fertilization rates compared to the control group was GA (43.9 % ± 2.39, n = 82, P = 0.2265), in which 2,4-D was not present (Fig. 4).

4. Discussion

In this study, we evaluated the effect of direct exposure of bovine sperm cells to herbicide mixtures to determine whether these compounds contribute to losses in sperm quality and function. We showed that most of the mixture groups analyzed compromised bovine sperm fertilization capacity. The environmental contamination status of glyphosate, 2,4-D, and atrazine in Brazil represents a concerning risk of exposure to these compounds currently, which becomes even more significant when the context of mixtures is taken into account. [19]. Intensive use, application management, environmental conditions, and the characteristics of the chemicals have led to the ubiquitous presence of these compounds in the environment, thereby increasing exposure [18, 42, 43]. Uncertain consequences to the health of non-target species such as bovines necessitate the evaluation of the impacts of pesticide mixtures to model a more realistic exposure scenario [24,44], and to clarify whether compounds with different modes of actions and chemical compositions could result in additive or interaction effects (synergism or antagonism) [16].

To investigate the effects of isolated and combined herbicides on sperm parameters, solutions were prepared using DMSO as the diluent. To eliminate potential confounding effects attributable to the diluent itself, a control group containing the same DMSO concentration used in the herbicide formulations was included throughout the study. The findings confirmed that the alterations observed in the groups treated with isolated compounds and mixtures were not solely attributable to the presence of DMSO.

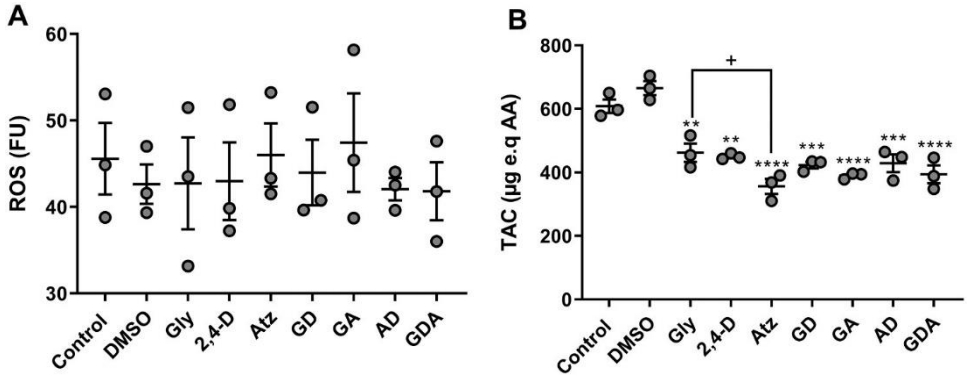


Fig. 3. Effects on oxidative stress markers in bovine sperm treated with isolated herbicide active ingredients and their mixtures. Groups: Control (medium); DMSO (dimethyl sulfoxide); Gly (glyphosate); 2,4-D; Atz (atrazine); GD (Gly + 2,4-D); GA (Gly + Atz); AD (Atz + 2,4-D); and GDA (Gly + 2,4-D + Atz). (A) ROS levels. (B) Total antioxidant capacity (TAC). Data are reported as means ± SEM, after 3 repetitions. One way ANOVA followed by Tukey post hoc test identified statistical differences (** P < 0.01 vs control group; *** P < 0.001 vs control group; **** P < 0.0001 vs control group. + P < 0.05 vs groups).

The evaluations of post-spermatogenesis verified that the sperm exposed to the mixture groups were not necessarily impaired to a greater extent than those treated with isolated compounds. This was clearly demonstrated by the kinematic results; the Gly and 2,4-D groups showed more frequent reductions in sperm motility parameters compared with the mixture groups, except for the AD group. The occurrence of an antagonistic effect in pesticide mixtures of compounds is not rare and has already been described in the literature concerning plant metabolism, especially in the case of herbicides containing a mixture of glyphosate and 2,4-D [45,46]. Simultaneous application of these compounds may interfere with their herbicidal effectiveness, as the capacity of 2,4-D to impair glyphosate translocation in plants. However, an antagonistic interaction between ammonium glufosinate (a glyphosate contact herbicide) and 2,4-D has also been reported, indicated by a reduced activation of the aspartate oxidase (APX) enzyme. This enzyme has an affinity for hydrogen peroxide, which—alongside superoxide radicals—tends to accumulate in response to the mode of action of auxin-type herbicides [47,48]. The generation of ROS and promotion of oxidative stress is also reported as consequence to 2,4-D exposure in non-target species, and is related to the impairments caused by the herbicide [10,49]. Similarly, the association between Gly and Atz showed a possible antagonism owing to the results in sperm kinematics in mixture group. A study involving both

compounds showed that treatment of male rats with glyphosate impaired sperm parameters and reduced testosterone levels more significantly than treatment with atrazine or the combined mixture [50]. This type of interaction in these groups could be reinforced because the AD group (2,4-D + Atz) was the only group in which the sperm kinematic parameters were impaired.

Despite the differences found between the isolated and mixed groups, we verified the ability of herbicides to impair kinematic parameters, which are sperm-specific features that are strictly related to their performance [51]. Previous studies that used *in vitro* models from different animal species have demonstrated that exposure to Gly [29,52, 53], 2,4-D [30] and Atz [32] interfere with sperm motility. Deficits in energy production in human sperm due to exposure to Gly have been reported, as it can inhibit succinate dehydrogenase in the electron transfer chain, thereby reducing ATP production [54]. Conversely, Atz affects the bioenergetics of human sperm cells without impairing mitochondrial respiration because of its ability to bind to F1-F0 ATP synthase, leading to ATP depletion [55]. Similarly, 2,4-D has demonstrated the capacity to reduce ATP levels in mitochondria of rat liver cells without affecting the mitochondrial respiratory chain. However, this occurs due to interactions with the lipid bilayer of the organelle membranes, leading to changes in their permeability and disruption of the mitochondrial membrane potential [56]. In this study, we did not observe significant differences in the mitochondrial membrane potential of the isolated and mixed compound groups at the concentrations tested.

Table 1
Effects of treatments to the percentual of bovine sperm cells with non-intact plasma membrane, acrosome damage and high mitochondrial membrane potential, assessed by flow cytometry. Data are expressed as means ± SEM from four repetitions.

Group	Non-intact plasma membrane			Acrosomal damage			High mitochondrial membrane potential		
	Individual value, % of cells	*P*	Value relative to the positive control sample*, %	Individual value, % of cells	*P*	Value relative to the positive control sample*, %	Individual value, % of cells	*P*	Value relative to the control samples*, %
Control	32.2 ± 1.13	-	32.4	13.4 ± 0.47	-	17.0	71.7 ± 2.06	-	28.9
DMSO	34.2 ± 1.64	0.9933	34.4	14 ± 1.22	> 0.9999	17.8	64.7 ± 6.04	0.6808	36.1
Gly	33.7 ± 1.73	0.9991	33.9	13.8 ± 0.94	> 0.9999	17.5	66.9 ± 2.70	0.9415	33.9
2,4-D	31.3 ± 1.86	> 0.9999	31.5	12.6 ± 0.88	0.9999	16.0	65.3 ± 1.26	0.7715	35.5
Atz	36.4 ± 0.66	0.6823	36.7	14.1 ± 0.71	> 0.9999	17.9	67.6 ± 1.27	0.9745	33.1
GD	33.6 ± 2.02	0.9995	33.8	13.9 ± 1.33	> 0.9999	17.7	67.2 ± 2.12	0.9565	33.5
GA	31.4 ± 2.23	> 0.9999	31.6	13.3 ± 1.57	> 0.9999	16.9	67.3 ± 2.54	0.9604	33.4
AD	33.8 ± 1.49	0.9987	34.0	13.5 ± 1.54	> 0.9999	17.2	64.0 ± 1.85	0.5632	36.8
GDA	33.0 ± 1.41	> 0.9999	33.2	12.9 ± 1.33	> 0.9999	16.4	64.8 ± 1.31	0.6965	36.0

DMSO (2.5 % dimethyl sulfoxide); Gly (50 µg/mL glyphosate); 2,4-D (0.11 µg/mL 2,4-D); Atz (0.0107 µg/mL atrazine); GD (Gly + 2,4-D); GA (Gly + Atz); AD (Atz + 2,4-D); and GDA (Gly + 2,4-D + Atz).
P:*p*-value related to the comparison between the individual value of each treatment and the individual value of the control group (reference group).
*Relative values were calculated based on the formula: (Individual value of treatment / Value of FF sample) × 100 or ((Value of Mitostatus TMRE sample - Individual value of treatment) / (Value of Mitostatus TMRE + CCCP sample - Individual value of treatment))*100.
FF sample: sample subjected to three rounds of a flash-freezing process in liquid nitrogen followed by rapid thawing at 37°C and stained with PI and FITC-PSA individually (99.3 % of cells with positive fluorescence for PI; 78.7 % of cells with positive fluorescence for FITC-PSA).
Mitostatus TMRE sample: sample stained with MitoStatus TMRE (99.8 % cells with positive fluorescence for Mitostatus TMRE).
CCCP sample: sample stained with MitoStatus TMRE after the addition of CCCP incubated for 5 min (2.62 % cells with positive fluorescence for Mitostatus TMRE).

Similarly, evaluation of membrane integrity showed no statistically significant differences among the groups. Sperm membranes also play a special role in sperm function because, beyond acting as a physiological barrier, they regulate relevant sperm processes such as motility pattern, capacitation, acrosome reaction, and fusion with the oocyte [57]. Previous studies have demonstrated that exposure to Atz increases the number of non-viable sperm in bovines [32], goats [58], and zebrafish [59], even at low doses. Conversely, the effects of glyphosate on the membrane integrity of porcine and equine spermatozoa have only been reported at higher concentrations and in commercial formulations [25, 53], which are considered more toxic due to components such as surfactants [60]. Exposure to 2,4-D has been shown not to affect the viability of human spermatozoa, despite a reduction in motility [30].

Evaluation of the acrosome structure is relevant as a marker of sperm function because fertilization capacity depends on the ability of the sperm to fuse with the oocyte, which is dependent on the acrosome's enzymes [61]. Consequently, acrosomal damage, as well as perturbation of capacitation and acrosome reaction processes, have been correlated with impaired fertilization of sperm in different species [62, 63], and this could be a result of pesticide exposure [29, 32, 64]. No damage to the acrosomal membrane of non-capacitated sperm cells was observed in this study; however, the hypothesis that the herbicide mixtures tested in this study can impair the acrosomal status must not be discarded. The PSA lectin binds to the acrosomal matrix through pores formed in the acrosomal membrane; therefore, only spermatozoa with compromised acrosomal membranes at the time of staining are detected, not allowing distinguishing the cells that have completely lost the acrosome prior to staining from those with intact acrosome at the time of staining. In this way, further investigations considering the use of fluorochromes binding the outer acrosomal membrane [65, 66], such as the peanut agglutinin conjugated with fluorescein (FITC-PNA), are recommended.

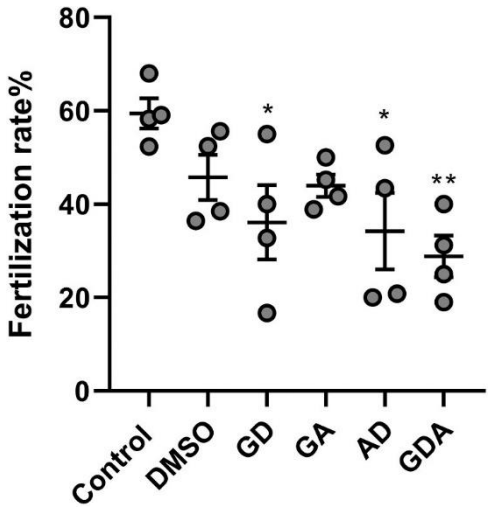


Fig. 4. Fertilization rates percentages of mixtures groups in comparison with control, measured with IVF protocol. Groups: Control (medium); DMSO (dimethyl sulfoxide); GD (Gly + 2,4-D); GA (Gly + Atz); AD (Atz + 2,4-D); and GDA (Gly + 2,4-D + Atz). Data are reported as means ± SEM, after 4 repetitions. One way ANOVA followed by Dunnett's post hoc test identified statistical differences (* $P < 0.05$ vs control group; ** $P < 0.01$ vs control group).

Regarding oxidative stress parameters, we verified that despite no differences in ROS levels in the groups during the evaluation period (3 h), sperm exposure to the isolated and mixed compounds resulted in an overall reduction in antioxidant capacity. Herbicides are commonly referred to as potential oxidative stress inductors [67], and the diminished status of antioxidant defenses in exposed sperm could be related to the maintenance of the redox balance. Sperm cells are recognized for their lower antioxidant capacity and suitability for oxidative stress because of their plasma membrane, which is rich in polyunsaturated fatty acids (PUFAs) [68,69]. Therefore, an imbalance between the oxidative forms and the antioxidant response is often associated with damage to sperm cells which significantly reduces their quality and function. Previous studies involving exposure to glyphosate, 2,4-D, and atrazine demonstrated a reduction in the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as decreased glutathione (GSH) levels in the testes of treated rodents. The occurrence of this oxidative environment was reflected in sperm parameters, such as concentration, motility, and morphology [9,10,11]. In this study, we confirmed the impact of exposure to herbicide concentrations on reducing the total antioxidant capacity of treated spermatozoa, and observed that this condition persisted in the context of mixtures.

Nevertheless, the loss of sperm quality is correlated with a compromised fertilization capacity. Using the IVF procedure, we verified a significant reduction in sperm fertility in the mixture groups containing 2,4-D, which was confirmed by the absence of a decrease in fertilization rates in the GA (Gly + Atz) group. These results showed that 2,4-D could play a singular role in promoting negative effects on fertility through mechanisms that were not evident in this study. Even with the kinematic results, no direct correlation could be made with the groups affected by the IVF, except for AD, and despite the reduction in antioxidant capacity, it could not be related to oxidative damage because there were no observable effects on the integrity of sperm membranes.

However, a possible perturbation in the regulation of sperm function could result from the capacity to alter Ca^+ signaling through the activation of CatSper Ca^+- channel, which has been attributed to a large number of pesticides [70]. The disturbance of Ca^+ levels is linked to the impairment of relevant sperm functions, such as motility, capacitation, and acrosome reaction, and consequently, to fertilization capacity [57]. Despite Gly or 2,4-D were not reported to disrupt Ca^+ signaling in human sperm [70], recently, the treatment with 2,4-D was found to promote the inhibition of Catsper1 expression in rat testes, which could partly explain the impairments reported in sperm function [71].

Nevertheless, despite the limitations of the present study in verifying the specific mechanisms of action of the herbicides on sperm function, our findings reveal valuable information about the behavior of compound mixtures in terms of sperm kinematics, which is an important sperm evaluation tool, owing to the verification of the solo effect of compounds and the occurrence of antagonism in the mixed compound groups. However, this was not verified by the other study parameters, as shown by the overall reduction in antioxidant capacity and decreased fertilization rates of most of the mixture groups. The presence of 2,4-D in these mixture groups revealed that it has the potential to impair fertilization capacity through the disruption of sperm functions, without necessarily promoting cell damage. Therefore, further investigations on the potential of these herbicides to disrupt sperm regulation are required to identify the impacts of exposure in depth.

5. Conclusion

The massive use and diversity of compounds applied in agricultural activities increases the exposure of non-target species to pesticides and mixtures of the pesticides, which have been associated with impairments in physiological processes such as reproduction. In this study, we analyzed the effects of active ingredients of the most commercialized herbicides (glyphosate, 2,4-D, and atrazine) in Brazil on bovine sperm using different mixtures of the compounds, prepared in DMSO solutions. The absence of adverse effects in the DMSO group allowed us to conclusively attribute the observed alterations in sperm parameters to the active ingredients of the herbicides. Then, we verified that impairments in kinematics, a routine sperm-quality evaluation, were more frequent in isolated compounds than in mixtures, showing a possible antagonistic effect. However, fertility capacity was compromised in all groups exposed to the mixtures, except for group GA (Gly + Atz), indicating the greater potential of 2,4-D to negatively influence sperm success in fertilization than Gly or Atz.

Further studies are required to confirm the herbicides mechanisms of interference in bovine sperm function and reproduction. This research demonstrates the usefulness of *in vitro* approach as an alternative tool to obtain more information on the impact of pesticide mixtures of target compounds and their biological relevance, serving as a reference for future studies.

Funding

This study was funded by the Coordenaçao de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES) in Brazil (Finance Code 001), the National Council for Scientific and Technological Development (CNPQ; financial support provided to F.W.S.C. (306037/2023–8).

CRediT authorship contribution statement

Laura Rohde Brondani: Writing – original draft, Methodology, Investigation, Formal analysis. **Rafaela Dalmolin Menezes:** Writing – original draft, Methodology, Investigation, Formal analysis. **Larissa Thaísa Weide:** Writing – original draft, Methodology, Investigation, Formal analysis. **Monike Quirino:** Writing – original draft, Methodology, Investigation, Formal analysis. **Diogo Ferreira Bicca:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Cibin Francielli W. S.:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Daniela dos Santos Brum:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Fabio Gallas Leivas:** ´ Writing – review & editing, Investigation, Formal analysis.

Declaration of Competing Interest

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

Acknowledgments

This study was partially funded by the Coordenaçao de Aperfeiçoa˜ mento de Pessoal de Nível Superior (CAPES), Brazil (Finance Code 001). We acknowledge the National Council for Scientific and Technological Development (CNPQ) and Fundaçao de Amparo ˜ a Pesquisa do Rio ` Grande do Sul (FAPERGS) for their financial support.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2025.109037.

Data availability

Data will be made available on request.

References

[1] M.M. Akashe, U.V. Pawade, A.V. Nikam, Classification of pesticides: a review, Int. J. Res. Ayurveda Pharm. 9 (2018) 144–150, https://doi.org/10.7897/2277-4343.094131.

[2] IBAMA (Instituto Brasileiro de Recursos Naturais e Renovaveis). Relat´ orio de ´ Comercializaçao de Agrot˜ oxicos (Boletim 2023). Os 10 IAs mais vendidos em 2023 ´ (Agrotoxicos químicos) ´ 〈https://www.gov.br/ibama/pt-br/assuntos/quimicos-e- biologicos/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos/relatorios- de-comercializacao-de-agrotoxicos#boletinsanuais〉 ; 2025 [accessed in 10 jun 2025].

[3] A. Fucic, R.C. Duca, K.S. Galea, T. Maric, K. Garcia, M.S. Bloom, H.R. Andersen, J. E. Vena, Reproductive health risks associated with occupational and environmental exposure to pesticides, Int. J. Environ. Res. Public Health 18 (2021) 6576, https:// doi.org/10.3390/ijerph18126576.

[4] C. Panis, A.C.B. Kawassaki, A.P.J. Crestani, C.R. Pascotto, D.S. Bortoloti, G. Vicentini, L.C. Lucio, M.O. Ferreira, R.T.C. Prates, V.K. Vieira, S.C. Gaboardi, L. Z.P. Candiotto, Evidence on human exposure to pesticides and the occurrence of health hazards in the Brazilian population: a systematic review, Front. Public Health 7 (9) (2022) 787438, https://doi.org/10.3389/fpubh.2021.787438.

[5] A.M. Stone, O.G. Camp, M.M. Biernat, D. Bai, A.O. Awonuga, H.M. Abu-Soud, Re- Evaluating the use of Glyphosate-based herbicides: implications on fertility, Reprod. Sci. 32 (2025) 950–964, https://doi.org/10.1007/s43032-025-01834-6.

[6] H.A. Vural, A. Baran, A. Altiner, General and reproductive toxicity of 2,4- Dichlorophenoxyacetic acid (2,4-D), Adv. Clin. Toxicol. 1 (2) (2016), https://doi.org/10.23880/act-16000108.

[7] H. Sadeghnia, S. Shahba, A. Ebrahimzadeh-Bideskan, S. Mohammadi, A. M. Malvandi, A. Mohammadipour, Atrazine neural and reproductive toxicity, Toxin Rev. 41 (4) (2022) 1290–1303, https://doi.org/10.1080/ 15569543.2021.1966637.

[8] E. Clair, R. Mesnage, C. Travert, G.E. S ´ eralini, A glyphosate-based herbicide ´ induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels, Toxicol. Vitr. 26 (2012) 269–279, https:// doi.org/10.1016/j.tiv.2011.12.009.

[9] F. Avdatek, Y.O. Birdane, R. Türkmen, H.H. Demirel, Ameliorative effect of resveratrol on testicular oxidative stress, spermatological parameters and DNA damage in glyphosate-based herbicide-exposed rats, Andrologia 50 (2018) e13036, https://doi.org/10.1111/and.13036.

[10] D. Zhang, Y. Wu, Y. Yuan, W. Liu, H. Kuang, J. Yang, B. Yang, L. Wu, W. Zou, C. Xu, Exposure to 2,4-dichlorophenoxyacetic acid induces oxidative stress and apoptosis in mouse testis, Pestic. Biochem. Physiol. 141 (2017) 18–22, https://doi.org/ 10.1016/j.pestbp.2016.10.006.

[11] Y. Song, Z.C. Jia, J.Y. Chen, J.X. Hu, L.S. Zhang, Toxic effects of atrazine on reproductive system of Male rats, Biomed. Environ. Sci. 27 (2014) 281–288, https://doi.org/10.3967/bes2014.050.

[12] Z.R. Jarrell, M.U. Ahammad, A.P. Benson, Glyphosate-based herbicide formulations and reproductive toxicity in animals, Vet. Anim. Sci. 10 (2020) 100126, https://doi.org/10.1016/j.vas.2020.100126.

[13] C. Giulioni, V. Maurizi, D. Castellani, S. Scarcella, E. Skrami, G. Balercia, A. B. Galosi, The environmental and occupational influence of pesticides on Male fertility: a systematic review of human studies, Andrology 10 (2022) 1250–1271, https://doi.org/10.1111/andr.13228.

[14] D. Marcu, S. Keyser, L. Petrik, S. Fuhrimann, L. Maree, Contaminants of emerging concern (CECs) and Male reproductive health: challenging the future with a double-edged sword, Toxics 11 (2023) 330, https://doi.org/10.3390/ toxics11040330.

[15] V. Selvaraju, S. Baskaran, A. Agarwal, R. Henkel, Environmental contaminants and Male infertility: effects and mechanisms, Andrologia 53 (2021) e13646, https:// doi.org/10.1111/and.13646.

[16] A.F. Hernandez, T. Parr´on, A.M. Tsatsakis, M. Requena, R. Alarc´on, O. L´opez- ´ Guarnido, Toxic effects of pesticide mixtures at a molecular level: their relevance to human health, Toxicology 307 (2013) 136–145, https://doi.org/10.1016/j. tox.2012.06.009.

[17] V.L.S.S. Castro, Agricultural pesticides mixtures use and its toxicological implications in health, Ecotoxicol. Environ. Contam. 4 (1) (2009) 87–94, https://doi.org/10.5132/jbse.2009.01.012.

[18] C.L.F. Fernandes, L.M. Volcao, P.F. Ramires, R.R. Mouraab, F.M. Junior, ˜ Distribution of pesticides in agricultural and urban soils of Brazil: a critical review, Environ. Sci. Process. Impacts 22 (2020) 256, https://doi.org/10.1039/ c9em00433e.

[19] E.M. Brovini, B.C.T. Deus, J.A. Vilas-Boas, G.R. Quadra, L. Carvalho, R. Mendonça, R.O. Pereira, S.J. Cardoso, Three-bestseller pesticides in Brazil: freshwater concentrations and potential environmental risks, Sci. Total Environ. 771 (2021) 144754, https://doi.org/10.1016/j.scitotenv.2020.144754.

[20] M. Alehashem, R. Peters, H.O. Fajana, S. Eslamizad, N. Hogan, M. Hecker, S. D. Siciliano, Herbicides and pesticides synergistically interact at low concentrations in complex mixtures, Chemosphere 353 (2024) 141431, https:// doi.org/10.1016/j.chemosphere.2024.141431.

[21] C. Gao, N. Sun, J. Xie, J. Li, L. Tao, L. Guo, L. Shi, X. He, X. Shen, H. Wang, P. Yang, A. Covaci, Y. Huang, Co-exposure to 55 endocrine-disrupting chemicals linking diminished sperm quality: mixture effect, and the role of seminal plasma docosapentaenoic acid, Environ. Int. 185 (2024) 108571, https://doi.org/ 10.1016/j.envint.2024.108571.

[22] IBGE (Instituto Brasileiro de Geografia e Estatística). Pesquisa de Pecuaria ´ Municipal: efetivo de Rebanhos por tipo (cabeças). 〈 http://www.ibge.gov.br/estati sticas/economicas/agricultura-e-pecuaria/9107-producao-da-pecuaria-municipal. html〉 ; 2025 [accessed in 10 jun 2025].

[23] M.T. Sørensen, H.D. Poulsen, C.L. Katholm, O. Højberg, Review: feed residues of glyphosate – potential consequences for livestock health and productivity, Animal 15 (2021) 100026, https://doi.org/10.1016/j.animal.2020.100026.

[24] M. Bruinenberg, M. van Agtmaal, N. Hoekstra, N. van Eekeren, Residues of pesticides in dairy cow rations and Fly treatments reduce the number of coleoptera in dung, Agric. Ecosyst. Environ. 344 (2023), https://doi.org/10.1016/j. agee.2022.108307.

[25] S. Kumar, V.V. Mishra, Review: toxicants in reproductive fluid and *in vitro* fertilization (IVF) outcome, Toxicol. Ind. Health 26 (8) (2010) 505–511, https://doi.org/10.1177/0748233710373081.

[26] C.G.M. Gonçalves, F.G. Leivas, D. Missio, F.W. Santos, E.B. Schwengber, N. P. Folchini, C.U.M. Pavin, D.D.S. Brum, Use of a colloid to optimize centrifugation in the selection of bovine sperm for IVF, Semin. Cienc. Agrar 39 (2018) 1607–1616, https://doi.org/10.5433/1679-0359.2018v39n4p1607.

[27] J.J. Parrish, J.L. Susko-Parrish, M.L. Leibfried-Rutledge, E.S. Critser, W. H. Eyestone, N.L. First, Bovine *in vitro* fertilization with frozen-thawed semen, Theriogenology 25 (1986) 591–600, https://doi.org/10.1016/0093-691x(86) 90143-3.

[28] M.K. Krüger, P. Schledorn, W. Schrodl, H.-W. Hoppe, W. Lutz, A.A. Shehata, ¨ Detection of glyphosate residues in animals and humans, J. Environ. Anal. Toxicol. 4 (2014), https://doi.org/10.4172/2161-0525.1000210.

[29] C. Nerozzi, S. Recuero, G. Galeati, D. Bucci, M. Spinaci, M. Yeste, Effects of roundup and its main component, glyphosate, upon mammalian sperm function and survival, Sci. Rep. 10 (2020) 11026, https://doi.org/10.1038/s41598-020- 67538-w.

[30] Z. Tan, J. Zhou, H. Chen, Q. Zou, S. Weng, T. Luo, Y. Tang, Toxic effects of 2,4- dichlorophenoxyacetic acid on human sperm function *in vitro*, J. Toxicol. Sci. 41 (2016) 543–549, https://doi.org/10.2131/jts.41.543.

[31] T.E. Arbuckle, S.M. Schrader, D. Cole, J.C. Hall, C.M. Bancej, L.A. Turner, P. Claman, 2,4-dichlorophenoxyacetic acid residues in semen of ontario farmers, Reprod. Toxicol. 13 (1999) 421–429, https://doi.org/10.1016/S0890-6238(99) 00057-X.

[32] A. Komsky-Elbaz, Z. Roth, Effect of the herbicide atrazine and its metabolite DACT on bovine sperm quality, Reprod. Toxicol. 67 (2017) 15–25, https://doi.org/ 10.1016/j.reprotox.2016.11.001.

[33] N. Urseler, R. Bachetti, F. Biole, V. Morgante, C. Morgante, Atrazine pollution in ´ groundwater and raw bovine milk: water quality, bioaccumulation and human risk assessment, Sci. Total Environ. 852 (2022) 158498, https://doi.org/10.1016/j. scitotenv.2022.158498.

[34] S.T. Mortimer, CASA – practical aspects, J. Androl. 21 (2000) 491–496, https:// doi.org/10.1002/j.1939-4640.2000.tb02116.x.

[35] C. Loetchutinat, S. Kothan, S. Dechsupa, J. Meesungnoen, J.-P. Jay-Gerin, S. Mankhetkorn, Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay, Radiat. Phys. Chem. 72 (2005) 323–331, https://doi.org/10.1016/j.radphyschem.2004.06.011.

[36] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76, https:// doi.org/10.1006/abio.1996.0292.

[37] M. Quirino, U. Jakop, A.P.G. Mellagi, F.P. Bortolozzo, M. Jung, M. Schulze, A 5- color flow cytometry panel to assess plasma membrane integrity, acrosomal status, membrane lipid organization and mitochondrial activity of boar and stallion spermatozoa following liquid semen storage, Anim. Reprod. Sci. 247 (2022) 107076, https://doi.org/10.1016/j.anireprosci.2022.107076.

[38] M. Quirino, V.N. Pereira, M.S.C. Tamanini, R.R. Ulguim, M. Schulze, A.P. Mellagi, F.P. Bortolozzo, Sperm concentration of boar semen doses and sperm quality: novel perspectives based on the extender type and sperm resilience, Anim. Reprod. Sci. 255 (2023) 107293, https://doi.org/10.1016/j.anireprosci.2023.107293.

[39] M. Stojkovic, S.A. Machado, P. Stojkovic, V. Zakhartchenko, P. Hutzler, P.B. Gonçalves, E. Wolf, Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture, Biol. Reprod. 64 (2001) 904–909, https://doi.org/10.1095/ biolreprod64.3.904.

[40] J.J. Parrish, A. Krogenaes, J.L. Susko-Parrish, Effect of bovine sperm separation by either swim-up or percoll method on success of *in vitro* fertilization and early embryonic development, Theriogenology 44 (1995) 859–869, https://doi.org/ 10.1016/0093-691X(95)00271-9.

[41] D. Missio, N. Folchini, F. Leivas, C. Pavin, H. Pinto, F. Cibin, D. Brum, Reduction in percoll volume increases recovery rate of sex-sorted semen of bulls without affecting sperm quality and early embryonic development, Anim. Reprod. Sci. 192 (2018) 146–153, https://doi.org/10.1016/j.anireprosci.2018.03.002.

[42] M.A. Pedlowski, M.C. Canela, M.A.C. Terra, R.M.R. Faria, Modes of pesticides utilization by Brazilian smallholders and their implications for human health and the environment, Crop Prot. 31 (1) (2012) 113–118, https://doi.org/10.1016/j. cropro.2011.10.002.

[43] D.L.P. Gazziero, Mixtures of pesticides in tank, in Brazilian farms, Planta Daninha 33 (2015) 83–92, https://doi.org/10.1590/S0100-83582015000100010.

[44] F. Penagos-Tabares, M. Sulyok, J. Faas, R. Krska, R. Khiaosa-ard, Q. Zebeli, Residues of pesticides and veterinary drugs in diets of dairy cattle from conventional and organic farms in Austria, Environ. Pollut. 316 (2023) 1200626, https://doi.org/10.1016/j.envpol.2022.120626.

[45] J.L. Flint, M. Barrett, Antagonism of glyphosate toxicity to johnsongrass (*sorghum halepense*) by 2,4-D and dicamba, Weed Sci. 37 (5) (1989) 700–705, https://doi. org/10.1017/S0043174500072660.

[46] J. Li, H. Han, L. Bai, Q. Yu, 2,4-D antagonizes glyphosate in glyphosate-resistant barnyard grass *echinochloa colona*, J. Pest. Sci. 45 (2) (2020) 109–113, https://doi.org/10.1584/jpestics.D20-013.

[47] A.C. Concato, W.A. Tamagno, C. Alves, N.T. Sutorillo, A.P. Vanin, L. Vargas, R. R. Kaizer, L. Galon, Enzymatic antioxidant defense system and ala-d enzyme activity in soybean Enlist™ line, Bragantia 81 (2022), https://doi.org/10.1590/ 1678-4499.20220010.

[48] K. Grossmann, Auxin herbicides: current status of mechanism and mode of action, Pest Manag. Sci. 66 (2) (2010) 113–120, https://doi.org/10.1002/ps.1860.

[49] B. Bukowska, Toxicity of 2,4-Dichlorophenoxyacetic acid – molecular mechanisms, Pol. J. Environ. Stud. 15 (2006) 365–374.

[50] S.O. Abarikwu, O.F. Akiri, M.A. Durojaiye, A. Adenike, 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 (1) (2015) 70–80, https://doi.org/10.3109/15376516.2014.989349.

[51] S. Chakraborty, S. Saha, Understanding sperm motility mechanisms and the implication of sperm surface molecules in promoting motility, Middle East Fertil. Soc. J. 27 (2022), https://doi.org/10.1186/s43043-022-00094-7.

[52] G. Anifandis, K. Katsanaki, G. Lagodonti, C. Messini, M. Simopoulou,K. Dafopoulos, A. Daponte, The effect of glyphosate on human sperm motility and sperm DNA fragmentation, Int. J. Environ. Res. Public Health 15 (2018) 1117, https://doi.org/10.3390/ijerph15061117.

[53] M. Spinaci, C. Nerozzi, B. Mislei, O. Blanco-Prieto, G. Mari, G. Galeati, D. Bucci, Impact of glyphosate and its formulation Roundup® on stallion spermatozoa, Theriogenology 179 (2022) 197–203, https://doi.org/10.1016/j.theriogenology.2021.12.003.

[54] A. Ferramosca, S. Lorenzetti, M. Di Giacomo, F. Murrieri, L. Coppola, V. Zara, Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency, Reprod. Toxicol. 99 (2021) 48–55, https:// doi.org/10.1016/j.reprotox.2020.11.011.

[55] Y. Hase, M. Tatsuno, T. Nishi, K. Kataoka, Y. Kabe, Y. Yamaguchi, N. Ozawa, M. Natori, H. Handa, H. Watanabe, Atrazine binds to F1F0-ATP synthase and inhibits mitochondrial function in sperm, Biochem. Biophys. Res. Commun. 366 (2008) 66–72, https://doi.org/10.1016/j.bbrc.2007.11.107.

[56] L.R.F. Polido, C.A. Miranda, D.A. França, F.E. Mingatto, L.C. Pereira, Effects of 2,4- dichlorophenoxyacetic acid (2,4-D), isolated and in a formulated product, on the functional parameters of isolated rat liver mitochondria, Toxicol. Vitr. 104 (2025) 105984, https://doi.org/10.1016/j.tiv.2024.105984.

[57] F.M. Pinto, A. Odriozola, L. Candenas, N. Subiran, The role of sperm membrane ´ potential and ion channels in regulating sperm function, Int. J. Mol. Sci. 24 (8) (2023) 6995, https://doi.org/10.3390/ijms24086995.

[58] A. Komsky-Elbaz, M. Saktsier, D. Biran, N. Argov-Argaman, H. Azaizeh, Y.S. Landau, Z. Roth, Atrazine-induced toxicity in goat spermatozoa is alleviated to some extent by polyphenol-enriched feed, Chemosphere 236 (2019) 124858, https://doi.org/10.1016/j.chemosphere.2019.124858.

[59] F.E.A. Bautista, A.S. Varela Junior, C.D. Corcini, I.B. Acosta, S.S. Caldas, E. G. Primel, J. Zanette, The herbicide atrazine affects sperm quality and the expression of antioxidant and spermatogenesis genes in zebrafish testes, Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 206–207 (2018) 17–22, https://doi.org/ 10.1016/j.cbpc.2018.02.003.

[60] M. Torres-Badia, S. Solar-Malaga, D. Martin-Hidalgo, A. Hurtado de Llera, A. Gomez-Candelo, L.J. Garcia-Marin, L. Gonzalez-Fern´andez, M.J. Bragado, ´ Impaired mammalian sperm function and lower phosphorylation signaling caused by the herbicide Roundup®Ultra plus are due to its surfactant component, Theriogenology 172 (2021) 55–66, https://doi.org/10.1016/j.theriogenology.2021.05.026.
[61] N.C. Bernecic, E. Donnellan, E. O'Callaghan, K. Kupisiewicz, C. O'Meara, K. Weldon, P. Lonergan, D.A. Kenny, S. Fair, Comprehensive functional analysis reveals that acrosome integrity and viability are key variables distinguishing artificial insemination bulls of varying fertility, J. Dairy Sci. 104 (2021) 11226–11241, https://doi.org/10.3168/jds.2021-20319.
[62] D.L. Egeberg Palme, A. Rehfeld, A.K. Bang, K.A. Nikolova, S. Kjærulff, M. R. Petersen, J.V. Jeppesen, M. Glensbjerg, A. Juul, N.E. Skakkebæk, S. Ziebe, N. Jørgensen, K. Almstrup, Viable acrosome-intact human spermatozoa in the ejaculate as a marker of semen quality and fertility status, Hum. Reprod. 33 (3) (2018) 361–371, https://doi.org/10.1093/humrep/dex380.
[63] W. Sun, S. Jiang, J. Su, J. Zhang, X. Bao, R. Ding, P. Shi, S. Li, C. Wu, G. Zhao, G. Cao, Q.Y. Sun, H. Yu, X. Li, The effects of cryopreservation on the acrosome structure, enzyme activity, motility, and fertility of bovine, ovine, and goat sperm, Anim. Reprod. 17 (4) (2020) 20200219, https://doi.org/10.1590/1984-3143- AR2020-0219.
[64] S. Chhillar, V. Batra, A. Kumaresan, R. Kumar, A. Pal, T.K. Datta, Acute exposure to organophosphorus pesticide metabolites compromises buffalo sperm function and impairs fertility, Sci. Rep. 13 (2023) 9102, https://doi.org/10.1038/s41598-023- 35541-6.
[65] D. Mortimer, E.F. Curtis, R.G. Miller, Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon, J. Reprod. Fertil. 81(1987) 127–135.
[66] M.E. Farlin, D.J. Jasko, J.K. Graham, E.L. Squires, Assessment of pisum sativum agglutinin in identifying acrosomal damage in stallion spermatozoa, Mol. Reprod. Dev. 32 (1992) 23–27, https://doi.org/10.1002/mrd.1080320105.
[67] R.O. Sule, L. Condon, A.V. Gomes, A common feature of pesticides: oxidative stress- the role of oxidative stress in pesticide-induced toxicity, Oxid. Med. Cell. Longev. (2022) 5563759, https://doi.org/10.1155/2022/5563759.
[68] A. Agarwal, G. Virk, C. Ong, S.S. du Plessis, Effect of oxidative stress on Male reproduction, World J. Mens. Health 32 (2014) 1–17, https://doi.org/10.5534/wjmh.2014.32.1.1.
[69] R.J. Aitken, E.G. Bromfield, Z. Gibb, Oxidative stress and reproductive function: the impact of oxidative stress on reproduction: a focus on gametogenesis and fertilization, Reproduction 164 (6) (2022) 79–94, https://doi.org/10.1530/REP- 22-0126.
[70] M.R. Birch, M. Johansen, N.E. Skakkebæk, A.M. Andersson, A. Rehfeld, *in vitro* investigation of endocrine disrupting effects of pesticides on Ca2+-signaling in human sperm cells through actions on the sperm-specific and steroid-activated CatSper Ca2+-channel, Environ. Int. 167 (2022) 107399, https://doi.org/10.1016/j.envint.2022.107399.
[71] E. Luis, V. Conde-Maldonado, E. García-Nieto, L. Juarez-Santacruz, M. Alvarado, ´ A. Anaya-Hernandez, Altered expression of Thyroid- and calcium ion Channels- ´ Related genes in rat testes by short-term exposure to commercial herbicides paraquat or 2,4-D, J. Xenobiotics 14 (4) (2024) 1450–1464, https://doi.org/ 10.3390/jox14040081.

Glossary

2,4-D: 2,4-dichlorophenoxyacetic acid
ALH: amplitude of lateral head displacement
Atz: atrazine
BCF: beat cross frequency
CASA: computer-assisted semen analysis
CCCP: carbonyl cyanide 3-chlorphenylhydrazone solution;
COC: cumulus-oocyte complex *DCF:* dichlorofluorescein
DMSO: dimethyl sulfoxide
FITC-PSA: fluorescein-isothiocyanate conjugated Pisum sativum agglutinin
Gly: glyphosate
IVF: in vitro fertilization; *PI:* propidium iodide
PBS: phosphate buffered saline *ROS:* reactive oxygen species *SCA:* Sperm Class Analyzer *PMot:* progressive motility
TAC: total antioxidant capacity *TMot:* total motility
VAP: mean path velocity
VCL: curvilinear velocity
VSL: straight-line velocity

6. DISCUSSÃO

Para a realização desta tese, selecionamos os ingredientes ativos glifosato, 2,4-D e atrazina utilizados na elaboração de compostos com finalidade herbicida, com o intuito de avaliar efeitos de concentrações similares àquelas encontradas em indivíduos expostos a estes compostos, sobre parâmetros espermáticos associados com a qualidade dos espermatozoides. A espécie bovina foi definida com base no grau de exposição que estes animais são submetidos durante a criação, bem como a proximidade a atividades com uso de pesticidas, o que tornando mais frequente o contato destes animais com estes químicos. A natureza da exposição, não consiste no contato com compostos isolados, mas sim em uma mistura de componentes, dos quais não se tem conhecimento das consequências. Por essa razão, avaliamos o efeito dos herbicidas na forma de misturas a fim de averiguar a ocorrência de efeitos aditivos ou interações.

No primeiro trabalho, visamos identificar concentrações dos ingredientes ativos com base nos dados de detecção e seus efeitos sobre os espermatozoides bovinos. Verificamos que o tempo de incubação influenciou os parâmetros cinéticos, no entanto os tratamentos com grupos de herbicidas também apresentaram diferentes impactos sobre os espermatozoides. A motilidade total foi reduzida pela atrazina, enquanto a progressiva foi diminuída em todos os tratamentos. Já os parâmetros cinéticos relativos à velocidade foram reduzidos significativamente pelo tratamento com glifosato, e a hiperatividade foi menor nos tratamentos com os três herbicidas em relação ao controle. A morfologia espermática também evidenciou maior ocorrência de alterações de natureza maior nos grupos tratados com as maiores concentrações de cada herbicida, e na menor concentração de atrazina, sendo expressas pelo maior número de células com acrossoma destacado ou ausente, ou com cauda fortemente enrolada. Os efeitos da atrazina também foram evidentes no teste hiposmótico (HOS), em que os tratamentos com as concentrações menor e intermediária coincidiram com o maior número de espermatozoides com membrana plasmática disfuncional. No caso das avaliações de estresse oxidativo, apenas a capacidade antioxidante foi reduzida pelo tratamento com 2,4-D, sendo igualmente influenciada pelo tempo de incubação. E por fim, selecionamos uma concentração de cada grupo para avaliar a capacidade de fertilização de oócitos não tratados, constatando novamente que as taxas de fertilização foram reduzidas no tratamento com a menor concentração de 2,4-D.

A seleção das concentrações utilizadas foi baseada em estudos anteriores com detecção destes herbicidas na urina (KRUGER et al., 2014) e leite (URSELER et al., 2022) de bovinos expostos ao glifosato e a atrazina, respectivamente, assim como a concentração de 2,4-D no esperma de trabalhadores rurais (ARBUCKLE et al., 1999). Igualmente, as faixas de concentração testadas para o glifosato (5, 36 e 50 µg/mL), 2,4-D (0,5, 1 e 5 µM) e atrazina (0,05, 0,1 e 1 µM), correspondem a valores mais baixos quando comparados com a maior parte da literatura relacionada à exposição a estes compostos, com efeitos sobre o sistema reprodutor masculino. Como exceções, KOMSKY-ELBAZ; ROTH (2017) descreveram os efeitos de baixas concentrações sobre espermatozoides bovinos, cujos prejuizos à viabilidade e danos à membrana plasmática e acrossomal ocorreram devido a tratamentos com 0,1 e 1 uM de atrazina. No caso do glifosato, o efeito da concentração de 1 ppm, descrito por DOVOLOU et al. (2024), reduziu a motilidade e, mais posteriormente, o desenvolvimento embrionário de oócitos fecundados pelos espeermatozoides expostos. Contudo, neste caso a formulação comercial foi utilizada, a qual é atribuida maior toxicidade devido aos componentes adjuvantes, como os surfactantes (TORRES-BADIA et al., 2021).

A cinética espermática pode ser afetada por fatores como a influência dos herbicidas sobre o metabolismo energético das células. Conforme FERRAMOSCA et al., (2021), mesmo concentrações mais baixas de glifosato (100 nM) foram capazes de reduzir a atividade mitocondrial de espermatozoides humanos, a qual tem relação com a habilidade do composto de inibir a succinato desidrogenase. De forma similar, o atrazina e 2,4-D demonstraram a capacidade de reduzir a produção ATP, sem alterar a cadeia transportadora de elétrons, mas a partir da ligação com a F1-F0 sintetase na mitocôndria de espermatozoides, no caso da atrazina (HASE et al. 2008), e de alterações na permeabilidade da membrana mitocondrial causadas pelo 2,4-D (POLIDO et al., 2025), como verificado em hepatócitos de ratos expostos.

As alterações morfológicas evidenciam que as células mesmo maduras estão sujeitas a sofrer modificações visíveis em sua estrutura, o que demonstra o potencial dos espermatozoides de servir como marcadores de exposição a agentes exógenos (CASTELLINI et al., 2009; MORETTI et al., 2022). A ocorrência de defeitos no acrossoma (destacados ou ausentes) bem como a cauda fortemente enrolada, podem ser decorrentes do processamento inadequado dos espermatozoides (GUN; BAKER, 2024), no entanto, o aumento destes defeitos em relação ao controle sugere associação com o tratamento com as concentrações dos herbicidas.

A redução do número de espermatozoides reativos ao meio hipotônico pelos tratamentos com atrazina demonstra que a células tiveram a função da membrana plasmática comprometida. A constatação de que esses efeitos ocorreram nas concentrações mais baixas, demonstra o potencial do herbicida de alterar a funcionalidade/integridade da membrana plasmática do espermatozoide como verificado por KOMSKY-ELBAZ; ROTH (2017), cujos danos podem influenciar na viabilidade e capacidade fertilização destes gametas visto o papel regulatório exercido pela membrana espermática (PINTO et al., 2023).

As avaliações de estresse oxidativo permitiram verificar que apenas o tratamento com 2,4-D reduziu significativamente a capacidade antioxidante dos espermatozoides bovinos. O 2,4-D é comumente associado à capacidade de induzir o estresse oxidativo (MESNAGE et al. 2021), fator que propicia os danos reportados em órgãos reprodutivos em modelos animais tratados, com consequências sobre produção de espermatozoides (ZHANG et al., 2017; MAROUANI et al., 2017). A ausência de diferenças em relação a geração de ROS e os níveis de peroxidação lipídica quando comparados ao grupo controle permitiu inferir que os efeitos da exposição não promoveram danos aos espermatozoides. Apesar disso, com o tratamento com a 2,4-D foi constatada a redução das taxas de fertilização, fato que não ocorreu com os outros herbicidas testados.

Com base nos resultados das concentrações do primeiro estudo, elencamos as concentrações de trabalho do segundo estudo em que foco centralizou-se no efeito das misturas. As concentrações de 50 µg/mL de glifosato, 0,11 µg/mL de 2,4-D (0,5 µM) e 0,0107 µg/mL de atrazina (0,05 µM), foram testadas de forma isolada e em grupos de misturas. A partir da avaliação da cinética constatamos que o efeito dos compostos isolados Gli e 2,4-D em reduzir os parâmetros espermáticos foi evidente, diferentemente de quando associados. Assim, foi possível observar a ocorrência de um antagonismo pois apenas o grupo de mistura AD (atrazina+2,4-D), apresentou efeitos sobre parâmetros da cinética espermática. Apesar disso, a avaliação da capacidade antioxidante demonstrou que este parâmetro foi reduzido em todos os grupos de tratamento, de compostos isolados e misturas. Quando testados os efeitos sobre a capacidade de fertilização dos espermatozoides expostos as misturas de herbicidas, resultou que os grupos contendo 2,4-D na composição apresentaram menores taxas de fertilização em relação ao grupo controle.

A ocorrência de efeitos antagônicos de herbicidas é um achado recorrente no metabolismo vegetal, visto que estes compostos podem apresentar diferentes mecanismos

de ação para o desempenho de sua finalidade. No caso do glifosato e o 2,4-D, ambos podem exercer efeitos antagônicos dependendo do volume ou momento de aplicação, sendo relatado que a translocação do glifosato pelo vegetal pode ser impedida pelo 2,4-D (LI et al., 2020). Da mesma forma, a aplicação do glufosinato de amônio (herbicida a base de glifosato, de contato) com o 2,4-D, revelou uma menor indução do sistema antioxidante vegetal (CONCATO et al., 2022), permitindo sugerir que a inibição do efeito do 2,4-D, cujo mecanismo de ação decorre da geração de ROS, comum às auxinas (GROSSMANN, 2010). Já a atrazina e o glifosato demonstraram efeitos antagônicos em modelo de roedores expostos aos compostos isolados e em misturas, tendo como consequências danos testiculares e redução da testosterona, mais pronunciados quando o glifosato foi utilizado isoladamente (ABARIKWU et al., 2015).

Apesar disso, os efeitos sobre a cinética foram reproduzidos novamente neste segundo estudo, com exceção da atrazina. Neste sentido, o efeito dos tratamentos foi investigado no que se refere aos parâmetros de integridade de membrana, dano acrossomal e potencial de membrana mitocondrial. Contudo, não foram observados efeitos nestes parâmetros em quaisquer dos grupos. A integridade de membrana, avaliada pelo iodeto de propídio é também um indicador de viabilidade espermática, podendo também ser associada com a motilidade. Investigações com atrazina verificaram danos a membrana plasmática mesmo em baixas doses em bovinos (KOMSKY-ELBAZ; ROTH 2017), cabras (KOMSKY-ELBAZ et al., 2019) e peixes (BAUTISTA et al., 2018), enquanto no caso do glifosato, a integridade só foi afetada em altas concentrações e com a formulação comercial (NEROZZI et al., 2020; SPINACI et al., 2022). No caso do 2.4-D, não houveram prejuízos a viabilidade dos espermatozoides humanos, apesar da redução da motilidade (TAN et al., 2016). Apesar de não verificadas diferenças entre os grupos referente a ocorrência de dano acrossomal, torna-se importante mencionar que o fluorocromo utilizado para determinação deste parâmetro (FITC-PSA) possui afinidade com poros específicos da membrana acrossômica, para identificação dos danos. Assim subentende-se que as células que perderam o acrossoma em decorrência de alguma condição ou tratamento, não serão identificadas na citometria de fluxo. Por essa razão, o efeito dos tratamentos com herbicidas de forma isolada ou em conjunto, não pode ser descartado. Em relação ao potencial de membrana mitocondrial, apesar do possível envolvimento dos herbicidas no metabolismo energético exposto anteriormente, não foram constatadas alterações quanto a este parâmetro em nenhum dos tratamentos deste estudo.

A redução da capacidade antioxidante em todos os grupos revela a sensibilidade da célula espermática a agentes oxidantes como os herbicidas, devido ao aporte reduzido de defesas antioxidantes (AITKEN; BROMFIELD; GIBB Z, 2022) Apesar da ausência de diferenças quanto a geração de ROS entre os grupos após o período de incubação (3 horas), o decréscimo do potencial antioxidante nos grupos tratados com herbicidas isolados e em misturas evidencia que estas defesas foram mobilizadas anteriormente ao período de avaliação, a fim de contrapor o insulto oxidativo.

Os resultados da fertilização *in vitro* demonstram que o 2,4-D pode exercer um papel importante no comprometimento da fertilização devido a presença do herbicida nos grupos com taxas de oócitos fertilizados menores que o controle. A ausência de indicadores de danos, bem como de efeitos sobre organelas espermáticas (membrana plasmática, acrossoma e mitocôndrias), demonstram que o 2,4-D possa interferir em mecanismos de regulação das funções do espermatozoide. Como exemplo, a capacidade de alguns pesticidas de influenciar o canal iônico de cálcio, CatSper, revelam o potencial destes compostos de intervir na função espermática (BIRCH et al., 2022), podendo assim influenciar na motilidade, capacitação, reação acrossômica e, consequentemente, na capacidade de fertilização (PINTO et al., 2023). Apesar do 2,4-D não exercer influência sobre o CatSper em espermatozoides humanos (BIRCH et al., 2022), em ratos tratados com este herbicida, a expressão do gene CatSper1 foi reduzida significativamente (LUIS et al., 2024), reforçando a necessidade da investigação em mais espécies.

Por fim, os resultados desta tese permitem evidenciar as consequências da exposição aos herbicidas a partir de um modelo *in vitro* com espermatozoides bovinos demonstrando que estes compostos promovem diferentes efeitos em parâmetros espermáticos, comprometendo sua qualidade. Ao mesmo tempo, os resultados mostram o comportamento dos herbicidas em misturas, os quais, apesar de não acarretarem maiores danos que os compostos isolados considerando uma técnica de rotina, como a cinética espermática, comprometeram significativamente a capacidade de fertilização dependendo do componente adicionado, no caso, o 2,4-D.

CONCLUSÕES

Baseado nos resultados apresentados por esta tese, pode-se concluir que:

1) A exposição com a concentrações dos ingredientes ativos glifosato, 2,4-D e atrazina promoveram alterações significativas em parâmetros espermáticos bovinos, comprometendo a qualidade espermática;

2) Os efeitos promovidos pelos compostos ocorreram na concentração maior de glifosato, enquanto para 2,4-D e atrazina, as concentrações mais baixas testadas produziram alterações nos parâmetros analisados;

3) A comparação entre efeitos provocados pelos compostos isolados e em misturas permitiu verificar a ocorrência de um possível antagonismo entre os ingredientes ativos, considerando os parâmetros cinéticos;

4) A redução da capacidade antioxidante em grupos isolados e misturas, demonstra o potencial dos ingredientes ativos de comprometer as defesas antioxidantes das células espermáticas, contribuindo para sua vulnerabilidade;

5) Dos ingredientes ativos testados, o 2,4-D parece ter papel relevante na redução da capacidade de fertilização dos espermatozoides bovinos, visto sua capacidade de diminuir as taxas de oócitos fertilizados quando utilizados de forma isolada ou em mistura.

Em conjunto, os resultados desta tese contribuem para a compreensão do efeito de herbicidas por meio de um modelo *in vitro*, demonstrando sua utilidade na investigação de diferentes contextos de exposição. Além da possibilidade de experimentação com concentrações de interesse, o modelo permitiu verificar que as consequências da ação dos herbicidas sobre a célula espermática, não necessariamente incorrem em déficit na sua função reprodutiva, como exemplo do efeito das misturas na cinética não refletido na capacidade fertilização. Estas constatações geram a necessidade da busca pelo conhecimento de mecanismos envolvidos na regulação espermática passiveis de influência por xenobióticos, que não necessariamente inflijam em dano concomitante.

REFERÊNCIAS BIBLIOGRÁFICAS

ABARIKWU, S.O.; AKIRI, O.F.; DUROJAIYE, M.A.; ADENIKE, A. 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**. v. 25, n. 1, p. 70-80, 2015. https//doi.org/10.3109/15376516.2014.989349

AITKEN, R.J.; BROMFIELD, E.G.; GIBB, Z. Oxidative stress and reproductive function: The impact of oxidative stress on reproduction: a focus on gametogenesis and fertilization. **Reproduction**. v. 164, n. 6, p. 79-94, 2022. https://doi.org/10.1530/REP-22-0126

AKASHE, M. M.; PAWADE, U. V.; NIKAM, A. V. Classification of pesticides: a review. **Int. J. Res. Ayurveda Pharm**. v. 9, n. 4, p. 144-150, 2018. http://doi.org/10.7897/2277-4343.094131

ALCALA, C. S.; LICHTVELD, M. Y.; WICKLIFFE, J. K.; ZIJLMANS, W.; SHANKAR, A.; ROKICKI, E.; COVERT, H.; ABDOEL WAHID, F. Z.; HINDORI-MOHANGOO, A. D.; VAN SAUERS-MULLER, A.; VAN DIJK, C.; ROOSBLAD, J.; CODRINGTON, J.; WILSON, M. J. Characterization of Urinary Pesticide Metabolite Concentrations of Pregnant Women in Suriname. **Toxics**. v. 10, n. 11, 2022. https://doi.org/10.3390/toxics10110679

ALEHASHEM, M.; PETERS, R.; FAJANA, H.O.; ESLAMIZAD, S.; HOGAN, N.; HECKER, M.; SICILIANO, S.D. Herbicides and pesticides synergistically interact at low concentrations in complex mixtures. **Chemosphere**. v. 353, n. 141431., 2024. http://doi.org/10.1016/j.chemosphere.2024.141431

ALMEIDA, V. E. S.; FRIEDRICH, K.; TYGEL, A. F.; MELGAREJO, L.; CARNEIRO, F. F. Use of genetically modified crops and pesticides in Brazil: growing hazards. **Cien Saude Colet.** v. 22, n. 10, p. 3333-3339, 2017. https://doi.org/10.1590/1413-812320172210.17112017

ANIFANDIS, G.; KATSANAKI, K.; LAGODONTI, G.; MESSINI, C.; SIMOPOULOU, M.; DAFOPOULOS, K.; DAPONTE, A. The Effect of Glyphosate on Human Sperm Motility and Sperm DNA Fragmentation. **Int J Environ Res Public Health**. v. 15, n. 6, 2018. https://doi.org/10.3390/ijerph15061117

ARBUCKLE, T. E.; SCHRADER, S. M.; COLE, D.; HALL, J. C.; BANCEJ, C. M.; TURNER, L. A.; CLAMAN, P. 2,4-Dichlorophenoxyacetic acid residues in semen of Ontario farmers. **Reprod Toxicol.** v. 13, n. 6, p. 421-429, 1999. http://doi.org/10.1016/s0890-6238(99)00057-x

BAUTISTA, F. E. A.; VARELA JUNIOR, A. S.; CORCINI, C. D.; ACOSTA, I. B.; CALDAS, S. S.; PRIMEL, E. G.; ZANETTE, J. The herbicide atrazine affects sperm quality and the expression of antioxidant and spermatogenesis genes in zebrafish testes. **Comp Biochem Physiol C Toxicol Pharmacol**. v. 206-207, p. 17-22, 2018. https://doi.org/10.1016/j.cbpc.2018.02.003

BENBROOK, C. M. Trends in glyphosate herbicide use in the United States and globally. **Environ Sci Eur.** v. 28, n. 1, p. 3, 2016. https://doi.org/10.1186/s12302-016-0070-0

BETANCOURT, M.; RES´ENDIZ, A.; CASAS, E.; FIERRO, R. Effect of two insecticides and two herbicides on the porcine sperm motility patterns using computer-assisted semen analysis (CASA) in vitro. **Reprod Toxicol.** v. 22, p. 508–512, 2006. https://doi.org/10.1016/j.reprotox.2006.03.001

BIRCH, M.R.; JOHANSEN, M.; SKAKKEBÆK, N.E.; ANDERSSON, A.M.; REHFELD, A. In vitro investigation of endocrine disrupting effects of pesticides on Ca2+-signaling in human sperm cells through actions on the sperm-specific and steroid-activated CatSper Ca2+-channel. **Environ Intern**. v. 167, n. 107399, 2022. https://doi.org/10.1016/j.envint.2022.107399

BRANCO, S.R.; ALVES, M.G,; OLIVEIRA, P.F.; ZAMONER, A. Critical Review of Glyphosate-Induced Oxidative and Hormonal Testicular Disruption. **Antioxidants**. v. 14(9), n.1036, 2025. https://doi.org/10.3390/antiox14091036

BROVINI, E. M.; DE DEUS, B. C. T.; VILAS-BOAS, J. A.; QUADRA, G. R.; CARVALHO, L.; MENDONCA, R. F.; PEREIRA, R. O.; CARDOSO, S. J. Three-bestseller pesticides in Brazil: Freshwater

concentrations and potential environmental risks. **Sci Total Environ**. v. 771, n. 144754, 2021. http://doi.org/10.1016/j.scitotenv.2020.144754

BROVINI, E. M.; QUADRA, G. R.; PARANAIBA, J. R.; CARVALHO, L.; PEREIRA, R. O.; DE AQUINO, S. F. Occurrence and environmental risk assessment of 22 pesticides in Brazilian freshwaters. **Aquat Toxicol**. v. 260, n. 106566, 2023. https://doi.org/10.1016/j.aquatox.2023.106566

BRUINENBERG, M.; AGTMAAL, M. V.; HOEKSTRA, N.; EEKEREN, N. V. Residues of pesticides in dairy cow rations and fly treatments reduce the number of Coleoptera in dung. **Agr Ecosyst Environ**. v. 344, 2023. https://doi.org/10.1016/j.agee.2022.108307

CABRY, R.; MERVIEL, P.; MADKOUR, A.; LEFRANC, E.; SCHEFFLER, F.; DESAILLOUD, R.; BACH, V.; BENKHALIFA, M. The impact of endocrine disruptor chemicals on oocyte/embryo and clinical outcomes in IVF. **Endocr Connect**. v. 9, n. 6, p. 134-142, 2020. https://doi.org/10.1530/EC-20-0135

CAI, W.; JI, Y.; SONG, X.; GUO, H.; HAN, L.; ZHANG, F.; LIU, X.; ZHANG, H.; ZHU, B.; XU, M. Effects of glyphosate exposure on sperm concentration in rodents: A systematic review and meta-analysis. **Environ Toxicol Pharmacol**, v. 55, n., p. 148-155, 2017. https://doi.org/10.1016/j.etap.2017.07.015

CALAHORRANO-MORENO, M.B.; ORDOÑEZ-BAILON, J.J.; BAQUERIZO-CRESPO, R.J.; DUEÑAS-RIVADENEIRA, A.A.; MONTENEGRO, M.C.B.S.M; RODRÍGUEZ-DÍAZ, J.M. Contaminants in the cow's milk we consume? Pasteurization and other technologies in the elimination of contaminants. **F1000 Research**. v.11, n. 91, 2022. https://doi.org/10.12688/f1000research.108779.1

CASSAULT-MEYER, E.; GRESS, S.; SÉRALINI, G.; GALERAUD-DENIS, I. An acute exposure to glyphosate-based herbicide alters aromatase levels in testis and sperm nuclear quality, **Environ Toxicol Pharmacol.** v. 38, n.1, p.131-140, 2014.
https://doi.org/10.1016/j.etap.2014.05.007

CASTELLINI, C.; MOURVAKI, E.; SARTINI, B.; CARDINALI, R.; MORETTI, E.; COLLODEL, G.; FORTANER, S.; SABBIONI, E.; RENIERI, T. In vitro toxic effects of metal compounds on kinetic traits and ultrastructure of rabbit spermatozoa. **Reprod. Toxicol**. v. 27, p. 46–54, 2009. https://doi.org/10.1016/j.reprotox.2008.12.003.

CHAKRABORTY, S.; SAHA, S. Understanding sperm motility mechanisms and the implication of sperm surface molecules in promoting motility. **Middle East Fertil. Soc. J.**. v. 27, n. 4, 2022. https://doi.org/10.1186/s43043-022-00094-7

CLAIR, E.; MESNAGE, R.; TRAVERT, C.; SERALINI, G. E. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. **Toxicol In Vitro**. v. 26, n. 2, p. 269-279, 2012. https://doi.org/10.1016/j.tiv.2011.12.009

CONAB (Companhia Nacional de Abastecimento). Boletim da Safra de Grãos – Abril 2025. Disponível em: https://www.conab.gov.br

CONCATO, A.C.; TAMAGNO, W.A.; ALVES, C.; SUTORILLO, N.T.; VANIN, A.P.; VARGAS, L.; KAIZER, R.R,; GALON, L. Enzymatic antioxidant defense system and ala-d enzyme activity in soybean Enlist™ line. **Bragantia**. v. 81, 2022. https://doi.org/10.1590/1678-4499.20220010

COOK, L. E.; FINGER, B. J.; GREEN, M. P.; PASK, A. J. Exposure to atrazine during puberty reduces sperm viability, increases weight gain and alters the expression of key metabolic genes in the liver of male mice. **Reprod Fertil Dev**. v. 31, n. 5, p. 920-931, 2019. https://doi.org/10.1071/RD18505

DE ALBUQUERQUE, F. P.; DE OLIVEIRA, J. L.; MOSCHINI-CARLOS, V.; FRACETO, L. F. An overview of the potential impacts of atrazine in aquatic environments: Perspectives for tailored solutions based on nanotechnology. **Sci Total Environ**. v. 700, n. 134868, 2020. https://doi.org/10.1016/j.scitotenv.2019.134868

DOVOLOU, E.; NANAS, I.; GIANNOULIS, T.; FYTSILLI, A.; NTEMKA, A.; ANIFANDIS, G.; TSAKMAKIDIS, I.; AMIRIDIS, G. S. The effects of a glyphosate-based herbicide on the bovine gametes during an in vitro embryo production model. **Environ. Pollut**. v. 350, n. 123967, 2024. https://doi.org/10.1016/j.envpol.2024.123967

DUKE, S. O. The history and current status of glyphosate. **Pest Manag Sci**. v. 74, n. 5, p. 1027-1034, 2018. https://doi.org/10.1002/ps.4652

EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária). Panorama da Suinocultura. Concórdia: Embrapa Suínos e Aves, 2025. Disponível em: https://www.embrapa.br/suinos-e-aves/panorama-da-suinocultura. Acesso em: 2 set. 2025.

FERNANDES, C. L. F.; VOLCAO, L. M.; RAMIRES, P. F.; MOURA, R. R.; DA SILVA JUNIOR, F. M. R. Distribution of pesticides in agricultural and urban soils of Brazil: a critical review. **Environ Sci Process Impacts.** v. 22, n. 2, p. 256-270, 2020. https://doi.org/10.1039/C9EM00433E

FERRAMOSCA, A.; LORENZETTI, S.; DI GIACOMO, M.; MURRIERI, F.; COPPOLA, L.; ZARA, V. Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency. **Reprod Toxicol**. v. 99, n., p. 48-55, 2021. https://doi.org/10.1016/j.reprotox.2020.11.011

FAO (Food and Agriculture Organization of the United Nations). International code of conduct on the distribution and use of pesticides. Rome: Food and Agriculture Organization of the United Nations (1986a).

FAO (Food and Agriculture Organization of the United Nations). FAOSTAT: produção agrícola mundial por país. Roma: FAO, 2025a. Disponível em: https://www.fao.org/faostat/en/

FAO (Food and Agriculture Organization of the United Nations). FAOSTAT Database – Crops and livestock products. FAO, 2025b: https://www.fao.org/faostat

FAO – Food and Agriculture Organization of the United Nations. Pesticides Use and Trade 1990–2022. FAOSTAT Analytical Brief nº 89. Roma: FAO, 2025c. Disponível em: https://openknowledge.fao.org/bitstreams/a8a8c2c8-ee36-42e8-a619-7e73c8daf8a6/download

FREISTHLER, M. S.; ROBBINS, C. R.; BENBROOK, C. M.; YOUNG, H. A.; HAAS, D. M.; WINCHESTER, P. D.; PERRY, M. J. Association between increasing agricultural use of 2,4-D and population biomarkers of exposure: findings from the National Health and Nutrition Examination Survey, 2001-2014. **Environ Health.** v. 21, n. 1, p. 23, 2022. https://doi.org/10.1186/s12940-021-00815-x

FRIEDRICH, K.; SILVEIRA, G. R. D.; AMAZONAS, J. C.; GURGEL, A. D. M.; ALMEIDA, V. E. S.; SARPA, M. International regulatory situation of pesticides authorized for use in Brazil: potential for damage to health and environmental impacts. **Cad Saude Publica**. v. 37, n. 4, e00061820, 2021. http://doi.org/10.1590/0102-311X00061820

FUCIC, A.; DUCA, R. C.; GALEA, K. S.; MARIC, T.; GARCIA, K.; BLOOM, M. S.; ANDERSEN, H. R.; VENA, J. E. Reproductive Health Risks Associated with Occupational and Environmental Exposure to Pesticides. **Int J Environ Res Public Health**. v. 18, n. 12, 2021. https://doi.org/10.3390/ijerph18126576

GAO, C.; SUN, N.; XIE, J.; LI, J.; TAO, L.; GUO, L.; SHI, L.; HE, X.; SHEN, X.; WANG, H.; YANG, P.; COVACI, A.; HUANG, Y. Co-exposure to 55 endocrine-disrupting chemicals linking diminished sperm quality: Mixture effect, and the role of seminal plasma docosapentaenoic acid. **Environ Int**. v 185, n.108571, 2024. http://doi.org/10.1016/j.envint.2024.108571

GAZZIERO, D. L. P. Mixtures of Pesticides in Tank, in Brazilian Farms. **Planta daninha.** v. 33, n. 1, p. 83-92, 2015. https://doi.org/10.1590/S0100-83582015000100010

GIULIONI, C.; MAURIZI, V.; CASTELLANI, D.; SCARCELLA, S.; SKRAMI, E.; BALERCIA, G.; GALOSI, A. B. The environmental and occupational influence of pesticides on male fertility: A systematic review of human studies. **Andrology**. v. 10, n. 7, p. 1250-1271, 2022. https://doi.org/10.1111/andr.13228.Epub2022

GONÇALVES, B. B.; NASCIMENTO, N. F.; SANTOS, M. P.; BERTOLIN, R. M.; YASUI, G. S.; GIAQUINTO, P. C. Low concentrations of glyphosate-based herbicide cause complete loss of sperm motility of yellowtail tetra fish Astyanax lacustris. **Journal of Fish Biology.** v. 92, n. 4, p. 1218-1224, 2018. https://doi.org/10.1111/jfb.13571.Epub2018

GROSSMANN, K. Auxin herbicides: current status of mechanism and mode of action. **Pest Manag Sci**. v. 66, n. 2, p. 113-20, 2010. http://doi.org/10.1002/ps.1860

GUNN, A.; BAKERB, M. Effect of incorrect storage of bull semen samples on sperm morphology assessment. **Clinical Theriogenology**. v.16, n. 9972, 2024. http://dx.doi.org/10.58292/CT.v16.9972

HASE, Y.; TATSUNO, M.; NISHI, T.; KATAOKA, K.; KABE, Y.; YAMAGUCHI, Y.; OZAWA, N.; NATORI, M.; HANDA, H.; WATANABE, H. Atrazine binds to F1F0-ATP synthase and inhibits mitochondrial function in sperm. **Biochem. Biophys. Res. Commun**. v. 366, p. 66–72, 2008. https://doi.org/10.1016/j.bbrc.2007.11.107

HARPER, A. P.; FINGER, B. J.; GREEN, M. P. Chronic Atrazine Exposure Beginning Prenatally Impacts Liver Function and Sperm Concentration With Multi-Generational Consequences in Mice. **Front Endocrinol (Lausanne).** v. 11, n. 580124, 2020. https://doi.org/10.3389/fendo.2020.580124

HERNANDEZ, A. F.; GIL, F.; LACASANA, M. Toxicological interactions of pesticide mixtures: an update. **Arch Toxicol.** v. 91, n. 10, p. 3211-3223, 2017. https://doi.org/10.1007/s00204-017-2043-5

ILBOUDO, S.; FOUCHE, E.; RIZZATI, V.; TOE, A. M.; GAMET-PAYRASTRE, L.; GUISSOU, P. I. In vitro impact of five pesticides alone or in combination on human intestinal cell line Caco-2. **Toxicol Rep.** v. 1, n., p. 474-489, 2014. https://doi.org/10.1016/j.toxrep.2014.07.008

IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis). Boletim anual de comercialização de agrotóxicos: 2023. Brasília: IBAMA, 2024. Disponível em: https://www.gov.br/ibama/pt-br/assuntos/quimicos-e-biologicos/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos#boletinsanuais

IBGE (Instituto Brasileiro de Geografia e Estatística. Levantamento Sistemático da Produção Agrícola Estatística da Produção Agrícola. Brasil: IBGE (2023a). Disponível em: https://biblioteca.ibge.gov.br/visualizacao/periodicos/2415/epag_2023_jun.pdf

IBGE (Instituto Brasileiro de Geografia e Estatística). Pesquisa de Pecuária Municipal: Efetivo de Rebanhos por tipo (cabeças) 2021. Brasil: IBGE (2023). Disponível em: www.ibge.gov.br/estatisticas/economicas/agricultura-e-pecuaria/9107-producao-da-pecuaria-municipal.html

JARRELL, Z. R.; AHAMMAD, M. U.; BENSON, A. P. Glyphosate-based herbicide formulations and reproductive toxicity in animals. **Vet Anim Sci.** v. 10, n. 100126, 2020. https://doi.org/10.1016/j.vas.2020.100126

KEYSER, S.; MARCU, D.; DAVIDSE, M.T.D.; BENNETT, M.; PETRIK, L.; MAREE, L. Human sperm as an in vitro toxicity model: a versatile tool for assessing the risk of environmental contaminants. **Arch Toxicol**. v. 99, p. 2937–2952, 2025. https://doi.org/10.1007/s00204-025-04035-x

KOMSKY-ELBAZ, A.; ROTH, Z. Effect of the herbicide atrazine and its metabolite DACT on bovine sperm quality. **Reprod Toxicol**. v. 67, p. 15-25, 2017. https://doi.org/10.1016/j.reprotox.2016.11.001

KOMSKY-ELBAZ, A.; SAKTSIER, M.; BIRAN, D.; ARGOV-ARGAMAN, N.; AZAIZEH, H.; LANDAU, Y.S.; ROTH, Z. Atrazine-induced toxicity in goat spermatozoa is alleviated to some extent by polyphenol-enriched feed. **Chemosphere**. v. 236, n. 124858, 2019. https://doi.org/10.1016/j.chemosphere.2019.124858

KOMSKY-ELBAZ, A.; KALO, D.; ROTH, Z. Carryover effect of atrazine and its metabolite-from treated bovine spermatozoa to the embryo's transcriptome. **Biol Reprod**. v. 104, n. 5, p. 1162-1180, 2021. https://doi.org/10.1093/biolre/ioab027

KRÜGER, M.; SCHRÖDL, W.; NEUHAUS, J.; SHEHATA, A. A. Field Investigations of Glyphosate in Urine of Danish Dairy Cows. **J Environ Anal Toxicol** . v. 3, n. 186, 2013. https://doi.org/10.4172/2161-0525.1000186

KRÜGER, M.; SCHLEDORN, P.; SCHRÖDL, W.; HOPPE, H.-W.; LUTZ, W.; SHEHATA, A. A. Detection of Glyphosate Residues in Animals and Humans. **J Environ Anal Toxicol** , v. 4, n. 210, p., 2014. https://doi.org/10.4172/2161-0525.1000210

KUMAR, S.; SHARMA, A. K.; RAWAT, S.S.; JAIN, D.K.; GHOSH, S. Use of pesticides in agriculture and livestock animals and its impact on environment of India. **Asian J. Environ. Sci**. v. 8, n. 1, p.51-57, 2013.

LI, J.; HAN, H.; BAI, L.; YU, Q. 2,4-D antagonizes glyphosate in glyphosate-resistant barnyard grass Echinochloa colona. **J Pestic Sci**. v.45, n. 2, p.109-113, 2020. http://doi.org/10.1584/jpestics.D20-013

LIU, J.; ZAI, X.; TIAN, X.; LI, J.; YAN, S.; WANG, T. Practical implementation and impact of the 4R principles in ethnopharmacology: Pursuing a more humane approach to research. **Front Pharmacol**. v. 16, n. 1543316, 2025. https://doi.org/10.3389/fphar.2025

LUIS, E.; CONDE-MALDONADO, V.; GARCÍA-NIETO, E.; JUÁREZ-SANTACRUZ, L.; ALVARADO, M.; ANAYA-HERNÁNDEZ, A. Altered Expression of Thyroid- and Calcium Ion Channels-Related Genes in Rat Testes by Short-Term Exposure to Commercial Herbicides Paraquat or 2,4-D. **J. Xenobiot**. v. 14, n. 4, p. 1450-1464, 2024. https://doi.org/10.3390/jox14040081

MARAVILLA-GALVAN, R.; FIERRO, R.; GONZALEZ-MARQUEZ, H.; GOMEZ-ARROYO, S.; JIMENEZ, I.; BETANCOURT, M. Effects of atrazine and fenoxaprop-ethyl on capacitation and the acrosomal reaction in boar sperm. **Int J Toxicol.** v. 28, n. 1, p. 24-32, 2009. https://doi.org/10.1177/1091581809333138

MARCENOVICZ, P. C. Informe Avicultura: Avicultura Paranaense. Curitiba: SINDIAVIPAR; DERAL/SEAB, 2025. Disponível em: https://sindiavipar.com.br/wp-content/uploads/2025/02/Informe-avicultura_25-28_01_2025.pdf

MARCU, D.; KEYSER, S.; PETRIK, L.; FUHRIMANN, S.; MAREE, L. Contaminants of Emerging Concern (CECs) and Male Reproductive Health: Challenging the Future with a Double-Edged Sword. **Toxics**. v. 11, n. 4, p., 2023. https://doi.org/10.3390/toxics11040330

MAROUANI, N.; TEBOURBI, O.; CHERIF, D.; HALLEGUE, D.; YACOUBI, M. T.; SAKLY, M.; BENKHALIFA, M.; BEN RHOUMA, K. Effects of oral administration of 2,4-dichlorophenoxyacetic acid (2,4-D) on reproductive parameters in male Wistar rats. **Environ Sci Pollut Res Int.** v. 24, n. 1, p. 519-526, 2017. https://doi.org/10.1007/s11356-016-7656-3

MERVIEL, P.; CABRY, R.; CHARDON, K.; HARAUX, E.; SCHEFFLER, F.; MANSOURI, N.B.; DEVAUX, A.; CHAHINE, H.; BACH, V.; COPIN, H.; BENKHALIFA, M. Impact of oocytes with CLCG on ICSI outcomes and their potential relation to pesticide exposure. **J Ovarian Res**. 2017 v 10(1):42, 2017. https://doi.org/10.1186/s13048-017-0335-2

MERVIEL, P.; CABRY, R.; CHARDON, K.; LOURDEL, E.; SCHEFFLER, F.; MANSOURI, N.; DEVAUX, A.; BACH, V.; COPIN, H.; BENKHALIFA, M. Qualité ovocytaire en fécondation in vitro et exposition aux pesticides. **Me´decine de la Reproduction.** v 20 (3), p. 215-223, 2018. https://doi.org/10.1684/mte.2018.0711

MESNAGE, R.; BRANDSMA, I.; MOELIJKER, N.; ZHANG, G.; ANTONIOU, M. N. Genotoxicity evaluation of 2,4-D, dicamba and glyphosate alone or in combination with cell reporter assays for DNA damage, oxidative stress and unfolded protein response. **Food Chem Toxicol.** v. 157, n., p. 112601, 2021. http://doi.org/10.1016/j.fct.2021.112601

MOREIRA, S.; PEREIRA, S.C.; SECO-ROVIRA, V.; OLIVEIRA, P.F.; ALVES, M.G.; PEREIRA, M.L. Pesticides and male fertility: a dangerous crosstalk. **Metabolites**. v 11:799. 2021. https://doi.org/10.3390/metabo11120799

MORETTI, E.; SIGNORINI, C.; CORSARO, R.; GIAMALIDI, M.; COLLODEL, G. Human Sperm as an In Vitro Model to Assess the Efficacy of Antioxidant Supplements during Sperm Handling: A Narrative Review. **Antioxidants**. v. 12, n. 1098, 2023. https://doi.org/10.3390/antiox12051098

NEROZZI, C.; RECUERO, S.; GALEATI, G.; BUCCI, D.; SPINACI, M.; YESTE, M. Effects of Roundup and its main component, glyphosate, upon mammalian sperm function and survival. **Sci Rep.** v. 10, n. 1, p. 11026, 2020. https://doi.org/10.1038/s41598-020-67538-w

OLAYINKA, E. T.; ORE, A.; ADEWOLE, K. E.; OYERINDE, O. Evaluation of the toxicological effects of atrazine-metolachlor in male rats: in vivo and in silico studies. **Environ Anal Health Toxicol.** v. 37, n. 3, 2022. https://doi.org/10.5620/eaht.2022021

OLIVEIRA, M. P. D.; SILVA, A. B. S. E.; RIGUETO, C. V. T.; LOSS, R. A.; GUEDES, S. F.; GERALDI, C. A. Q. Pesticides in different environmental compartments in Brazil: a review. **Ciência e Natura.** v. 45, n. 2, p., 2023. https://doi.org/10.5902/2179460X70715

ÖZGÜR, M. E. The in vitro toxicity of atrazine on kinematics and DNA fragmentation in common carp (Cyprinus carpio) sperm cells. **J Hellenic Vet Med Soc**. v. 70, n. 3, p. 1639-1646, 2019. https://doi.org/ 10.12681/jhvms.21787

PANIS, C.; KAWASSAKI, A. C. B.; CRESTANI, A. P. J.; PASCOTTO, C. R.; BORTOLOTI, D. S.; VICENTINI, G. E.; LUCIO, L. C.; FERREIRA, M. O.; PRATES, R. T. C.; VIEIRA, V. K.; GABOARDI, S. C.; CANDIOTTO, L. Z. P. Evidence on Human Exposure to Pesticides and the Occurrence of Health Hazards in the Brazilian Population: A Systematic Review. **Front Public Health.** v. 9, n., p. 787438, 2021. https://doi.org/10.3389/fpubh.2021.787438

PANUWET, P.; LADVA, C.; BARR, D.B.; PRAPAMONTOL, T.; MEEKER, J.D.; D'SOUZA, P.E.; MALDONADO, H.; RYAN, P.B.; ROBSON, M.G. Investigation of associations between exposures to pesticides and testosterone levels in Thai farmers. **Arch Environ Occup Health**. v 73, n.4, p. 205-218, 2018. http://doi.org/10.1080/19338244.2017.1378606

PEDLOWSKI, M. A.; CANELA, M. C.; TERRA, M. A. D. C.; FARIA, R. M. R. D. Modes of pesticides utilization by Brazilian smallholders and their implications for human health and the environment. **Crop Protection.** v. 31, p. 113 - 118, 2012.
https://doi.org/10.1016/j.cropro.2011.10.002

PERRY, M.J. Effects of environmental and occupational pesticide exposure on human sperm: a systematic review. **Hum Reprod Update**. v 14(3), p. 233-242, 2008.
https://doi.org/10.1093/humupd/dmm039

PINTO, F.M.; ODRIOZOLA, A.; CANDENAS, L.; SUBIRÁN, N. The Role of Sperm Membrane Potential and Ion Channels in Regulating Sperm Function. **Int. J. Mol. Sci**. v. 24, n. 6995, 2023. https://doi.org/10.3390/ijms24086995

POLIDO, L.R.F., MIRANDA, C.A.; FRANÇA, D.A.; MINGATTO, F.E.; PEREIRA, L.C. Effects of 2,4-dichlorophenoxyacetic acid (2,4-D), isolated and in a formulated product, on the functional parameters of isolated rat liver mitochondria, **Toxicol. Vitr.** v. 104, n. 105984, 2025. https://doi.org/10.1016/j.tiv.2024.105984

RODRÍGUEZ-ROBLEDO, V.; VENDRELL, A.; GARCÍA-CIFUENTES, A.; VILLASECA-GONZÁLEZ, N.; GUIBERTEAU-CABANILLAS, C.; MARTÍNEZ, L.L.; GARDE, J.J.; FERNÁNDEZ-SANTOS, M.R. Determination of atrazine and propazine metabolites deemed endocrine disruptors in human seminal plasma by LC–ESI-MS/MS. **Chem. Biol. Technol. Agric**. v.9, n.22, 2022. https://doi.org/10.1186/s40538-022-00285-4

ROTH, Z.; KOMSKY-ELBAZ, A.; KALO, D. Effect of environmental contamination on female and male gametes - A lesson from bovines. **Anim Reprod**. v. 17, n. 3, 2020. https://doi.org/10.1590/1984-3143-AR2020-0041

ROUSTAN, A.; AYE, M.; DE MEO, M.; DI GIORGIO, C. Genotoxicity of mixtures of glyphosate and atrazine and their environmental transformation products before and after photoactivation. **Chemosphere**. v. 108, p. 93-100, 2014. https://doi.org/10.1016/j.chemosphere.2014.02.079

SADEGHNIA, H.; SHAHBA, S.; EBRAHIMZADEH-BIDESKAN, A.; MOHAMMADI, S.; MALVANDI, A.M.; MOHAMMADIPOUR, A. Atrazine neural and reproductive toxicity. **Toxin Reviews**. v. 41, n.4, p. 1290-1303, 2022. https://doi.org/10.1080/15569543.2021.1966637

SASS, J. B.; COLANGELO, A. European Union bans atrazine, while the United States negotiates continued use. **Int J Occup Environ Health.** v. 12, n. 3, p. 260-267, 2006. https://doi.org/10.1179/oeh.2006.12.3.260

SELVARAJU, V.; BASKARAN, S.; AGARWAL, A.; HENKEL, R. Environmental contaminants and male infertility: effects and mechanisms. **Andrologia**. v. 53, n. e13646, 2021. https://doi.org/10.1111/and.13646

SENGUPTA, P.; BANERJEE, R. Environmental toxins: alarming impacts of pesticides on male fertility. **Hum Exp Toxicol**. v. 33, n. 10, p. 1017-1039, 2014. https://doi.org/10.1177/0960327113515504

SERRA, L.; ESTIENNE, A.; BOURDON, G.; RAME, C.; CHEVALEYRE, C.; DIDIER, P.; CHAHNAMIAN, M.; EL BALKHI, S.; FROMENT, P.; DUPONT, J. Chronic Dietary Exposure of Roosters to a Glyphosate-Based Herbicide Increases Seminal Plasma Glyphosate and AMPA Concentrations, Alters Sperm Parameters, and Induces Metabolic Disorders in the Progeny. **Toxics.** v. 9, n. 12, 2021. https://doi.org/10.3390/toxics9120318

SNOJ, T.; KOBAL, S.; PREMROV BAJUK, B.; ZUZEK, M. C.; CEBULJ-KADUNC, N.; MAJDIC, G. Retrospective study of bull semen quality – possible correlation with pesticide use? **Acta Vet. Hung**. v. 61, n. 4, p. 495–504, 2013. https://doi.org/10.1556/AVet.2013.034

SONG, Y. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. **J Integr Plant Biol.** v. 56, n. 2, p. 106-113, 2014. https://doi.org/10.1111/jipb.12131

SONG, Y.; JIA, Z. C.; CHEN, J. Y.; HU, J. X.; ZHANG, L. S. Toxic effects of atrazine on reproductive system of male rats. **Biomed Environ Sci.** v. 27, n. 4, p. 281-288, 2014. https://doi.org/10.3967/bes2014.050

SORENSEN, M. T.; POULSEN, H. D.; KATHOLM, C. L.; HOJBERG, O. Review: Feed residues of glyphosate - potential consequences for livestock health and productivity. **Animal.** v. 15, n. 1, 2021. https://doi.org/10.1016/j.animal.2020.100026

SOUZA, M. C. O.; CRUZ, J. C.; CESILA, C. A.; GONZALEZ, N.; ROCHA, B. A.; ADEYEMI, J. A.; NADAL, M.; DOMINGO, J. L.; BARBOSA, F. Recent trends in pesticides in crops: A critical review of the duality of risks-benefits and the Brazilian legislation issue. **Environ Res**, v. 228, n. 115811, 2023. https://doi.org/10.1016/j.envres.2023.115811

SPINACI, M.; NEROZZI, C.; MISLEI, B.; BLANCO-PRIETO, O.; MARI, G.; GALEATI, G.; BUCCI, D. Impact of glyphosate and its formulation Roundup(R) on stallion spermatozoa. **Theriogenology**. v. 179, p. 197-203, 2022. https://doi.org/10.1016/j.theriogenology.2021.12.003

STONE, A.M.; CAMP, O.G.; BIERNAT, M.M.; BAI D.; AWONUGA, A.O.; ABU-SOUD, H.M. Re-Evaluating the Use of Glyphosate-based Herbicides: Implications on Fertility. **Reprod. Sci.** v. 32, p. 950-964, 2025. https://doi.org/10.1007/s43032-025-01834-6

TAN, Z.; ZHOU, J.; CHEN, H.; ZOU, Q.; WENG, S.; LUO, T.; TANG, Y. Toxic effects of 2,4-dichlorophenoxyacetic acid on human sperm function in vitro. **J Toxicol Sci.** v. 41, n. 4, p. 543-549, 2016. https://doi.org/10.2131/jts.41.543

TORRES-BADIA, M.; SOLAR-MALAGA, S.; MARTIN-HIDALGO, D.; HURTADO DE LLERA, A.; GOMEZ-CANDELO, A.; GARCIA-MARIN, L. J.; GONZALEZ-FERNANDEZ, L.; BRAGADO, M. J. Impaired mammalian sperm function and lower phosphorylation signaling caused by the herbicide Roundup(R) Ultra Plus are due to its surfactant component. **Theriogenology**. v. 172, p. 55-66, 2021. https://doi.org/10.1016/j.theriogenology.2021.05.026

URSELER, N.; BACHETTI, R.; BIOLE, F.; MORGANTE, V.; MORGANTE, C. Atrazine pollution in groundwater and raw bovine milk: Water quality, bioaccumulation and human risk assessment. **Sci Total Environ**. v. 852, n. 158498, 2022. https://doi.org/10.1016/j.scitotenv.2022.158498

USDA (UNITED STATES DEPARTMENT OF AGRICULTURE). World Agricultural Supply and Demand Estimates (WASDE). Washington, D.C.: USDA, 2025. Disponível em: https://www.usda.gov.

VASSEUR, C.; SERRA, L.; EL BALKHI, S.; LEFORT, G.; RAMÉ, C.; FROMENT, P.; DUPONT, J. Glyphosate presence in human sperm: First report and positive correlation with oxidative stress in an infertile French population. **Ecotoxicol Environ Saf**. v. 15, n. 278, 2024. https://doi.org/10.1016/j.ecoenv.2024.116410

VICENTE-CARRILLO, A. The usefulness of sperm kinematics in drug-induced toxicity assessment. **Basic Clin Pharmacol Toxicol**. v. 123, n. 1, p. 3-7, 2018. https://doi.org/10.1111/bcpt.12994

VURAL, H.A.; BARAN, A.; ALTINER, A. General and Reproductive Toxicity of 2,4-Dichlorophenoxyacetic acid (2,4-D). **Adv Clin Toxicol**. v. 1, n.2, 2016. http://doi.org/10.23880/act-16000108

WEI, X.; PAN, Y.; ZHANG, Z.; CUI, J.; YIN, R.; LI, H.; QIN, J.; JING LI, A.; QIU, R. Biomonitoring of glyphosate and aminomethylphosphonic acid: Current insights and future perspectives. **J. Hazard. Mater**. v. 463, n.132814, 2023. https://doi.org/10.1016/j.jhazmat.2023.132814

WILLIAMS, G. M.; KROES, R.; MUNRO, I. C. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. **Regul Toxicol Pharmacol.** v. 31, n. 2 Pt 1, p. 117-165, 2000. https://doi.org/10.1006/rtph.1999.1371

WHO (World Health Organization). Public Health Impact of Pesticides Used in Agriculture. England: World Health Organization (1990).

WHO (World Health Organization). Recommended classification of pesticides by hazard and guidelines to classification, 2019 edition. Geneva: World Health Organization (2020)

ZHAO, L.; ZHANG, J.; YANG, L.; ZHANG, H.; ZHANG, Y.; GAO, D.; JIANG, H.; LI, Y.; DONG, H.; MA, T.; WANG, X.; WU, M.; WANG, A.; JIN, Y.; YUAN, Y.; CHEN, H. Glyphosate exposure attenuates testosterone synthesis via NR1D1 inhibition of StAR expression in mouse Leydig cells, **Sci Total Environ**. v. 785, n. 147323, 2021. https://doi.org/10.1016/j.scitotenv.2021.147323

ZHANG, D.; WU, Y.; YUAN, Y.; LIU, W.; KUANG, H.; YANG, J.; YANG, B.; WU, L.; ZOU, W.; XU, C. Exposure to 2,4-dichlorophenoxyacetic acid induces oxidative stress and apoptosis in mouse testis. **Pestic Biochem Physiol**. v. 141, p. 18-22, 2017. https://doi.org/10.1016/j.pestbp.2016.10.006