https://doi.org/10.31925/farmacia.2026.2.6 **ORIGINAL ARTICLE**

DEVELOPMENTAL EXPOSURE TO REGULATORY PERMITTED DOSES OF GLYPHOSATE AND A GLYPHOSATE, DICAMBA, 2,4-D MIXTURE INDUCES HISTOPATHOLOGICAL ALTERATIONS IN TESTES AND PROSTATE OF MALE RATS

MOHAMAD ALAA HBOUS [1], LILIANA CERCELARU [2]*, RADU MITRUT [3], ANTONIA BLENDEA [4], ROBIN MESNAGE [5,6], MICHAEL N ANTONIOU [5], ANCA OANA DOCEA [7]

[1]*Doctoral School, University of Medicine and Pharmacy of Craiova, Romania*
[2]*Department of Anatomy and Embryology, University of Medicine and Pharmacy of Craiova, Romania*
[3]*Department of Cardiology, County Emergency Clinical Hospital of Craiova, Craiova, Romania*
[4]*Department of Pharmaceutical Botany, University of Medicine and Pharmacy of Craiova, 200349, Craiova, Romania*
[5]*King's College London, Gene Expression and Therapy Group, Department of Medical and Molecular Genetics, Faculty of Life Sciences and Medicine, Guy's Hospital, London, SE1 9RT, United Kingdom*
[6]*Buchinger Wilhelmi Clinic, Wilhelmi-Beck-Straße 27, 88662 Überlingen, Germany*
[7]*Department of Toxicology, University of Medicine and Pharmacy of Craiova, Romania*

**corresponding author: liliana.cercelaru@umfcv.ro*

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Abstract

Glyphosate is the most widely used herbicide worldwide and is frequently applied in combination with dicamba and 2,4-dichlorophenoxyacetic acid (2,4-D) in agricultural settings. Although regulatory reference values have been established for these compounds, concerns persist regarding their potential reproductive effects, particularly following prolonged exposure during sensitive developmental periods. This study evaluated whether chronic developmental exposure to glyphosate at European Union (EU) regulatory reference doses, namely the acceptable daily intake (ADI) and the no-observed-adverse-effect level (NOAEL), as well as to a mixture of glyphosate, dicamba, and 2,4-D at their respective EU ADI levels, induces hormonal and histopathological alterations in male reproductive organs. Pregnant Wistar rats were exposed from gestational day 6 through lactation, and male offspring continued treatment after weaning until postnatal day 111. At the end of the exposure period, serum testosterone levels, relative testis weight, and histopathological changes in testes and prostate were assessed. The herbicide mixture significantly decreased serum testosterone and increased relative testis weight. Histological analysis revealed progressive testicular and prostatic lesions, suggesting that developmental exposure to this herbicide mixture may disrupt male reproductive structure and endocrine homeostasis.

Rezumat

Glifosatul este erbicidul cel mai utilizat la nivel mondial și este frecvent aplicat în combinație cu dicamba și acidul 2,4-diclorofenoxiacetic (2,4-D) în practica agricolă. Deși au fost stabilite valori de referință admise prin reglementări pentru acești compuși, persistă îngrijorări privind potențialele lor efecte asupra aparatului reproducător, în special în urma expunerii prelungite în perioade sensibile ale dezvoltării. Acest studiu a evaluat dacă expunerea cronică, în perioada dezvoltării, la glifosat în doze de referință stabilite de Uniunea Europeană (UE), respectiv doza zilnică acceptabilă (ADI) și nivelul fără efect advers observabil (NOAEL), precum și la un amestec de glifosat, dicamba și 2,4-D la nivelurile corespunzătoare ADI, induce modificări hormonale și histopatologice la nivelul organelor reproducătoare masculine. Femelele gestante de șobolan Wistar au fost expuse începând cu ziua gestațională 6 până la sfârșitul lactației, iar masculii proveniți din descendență au continuat tratamentul după înțărcare până în ziua postnatală 111. La finalul perioadei de expunere au fost evaluate nivelurile serice de testosteron, greutatea relativă a testiculelor și modificările histopatologice testiculare și prostatice. Amestecul de erbicide a determinat o scădere semnificativă a testosteronului seric și o creștere a greutății relative a testiculelor. Analiza histologică a evidențiat leziuni progresive la nivel testicular și prostatic, sugerând că expunerea developmentală la acest amestec de erbicide poate perturba structura aparatului reproducător masculin și homeostazia endocrină.

Keywords: glyphosate, endocrine disruptors, reproductive toxicity, dicamba, 2,4-D

Introduction

Glyphosate is the most extensively used herbicide worldwide and plays a central role in modern agricultural practices (1). Due to its widespread application, environmental contamination and human exposure have become subjects of increasing public, scientific and regulatory concern (2). Regulatory agencies have established safety thresholds for glyphosate exposure, including the European Union (EU) acceptable daily intake (ADI) of 0.5 mg/kg body weight/day based on a no-observed-adverse-effect level (NOAEL) of 50 mg/kg body weight/day derived from conventional

toxicological studies focused on systemic toxicity in adult animals (3). However, growing evidence suggests that prolonged exposure, particularly during sensitive developmental periods, may reveal subtle biological effects not captured in traditional toxicity studies (4-9).

In agricultural environments, glyphosate is frequently applied together with other herbicides such as dicamba and 2,4-dichlorophenoxyacetic acid (2,4-D) in order to combat the vast emergence of glyphosate resistant weeds (10, 11) and thus improve weed control efficiency and manage genetically modified herbicide-resistant crops engineered to tolerate glyphosate plus dicamba and glyphosate plus 2,4-D application (12). Consequently, environmental exposure often occurs as mixtures rather than individual compounds (13). Several experimental studies have suggested that combined exposure to herbicides may produce additive or synergistic biological effects, especially when exposure occurs during developmental stages (14, 15).

Increasing toxicological evidence indicates that glyphosate and glyphosate-based herbicides (GBHs) may interfere with endocrine regulation and reproductive function (9, 16). Experimental studies in rodents have reported that developmental exposure to glyphosate can alter testosterone levels, impair spermatogenesis, and induce structural alterations in testicular tissue (17). Moreover, glyphosate exposure has been associated with disruption of the hypothalamic–pituitary–gonadal axis, oxidative stress, and mitochondrial dysfunction in reproductive tissues (18). Similarly, auxinic herbicides such as 2,4-D and dicamba have been linked to endocrine-disrupting effects and reproductive toxicity, including alterations in testicular morphology and steroidogenesis (19, 20).

Male reproductive organs, particularly the testes and prostate gland, are highly sensitive to endocrine disruption and oxidative stress (21). Perturbations of hormonal homeostasis during critical developmental windows may impair spermatogenesis and modify the structural integrity of reproductive tissues, potentially resulting in long-term reproductive dysfunction (22). Nevertheless, relatively few studies have investigated the consequences of prolonged exposure to regulatory reference doses of herbicides during prenatal and postnatal development.

Developmental exposure is of particular toxicological relevance because prenatal and early postnatal stages represent critical phases for reproductive organ maturation and endocrine programming (23, 24). Alterations occurring during these windows may lead to persistent structural and functional changes that become evident later in life (22).

Therefore, the aim of the present study was to evaluate whether chronic developmental exposure to glyphosate at EU regulatory reference doses (ADI and NOAEL), as well as exposure to a mixture of glyphosate, dicamba, and 2,4-D at their respective EU ADI, induces structural alterations in male reproductive organs. Using a rat developmental exposure model, animals were exposed from gestational day 6 through lactation and continued exposure after weaning until postnatal day 111. At the end of the exposure period, histopathological changes in the testes and prostate were evaluated, and serum testosterone levels determined to assess potential effects on male reproductive function.

Materials and Methods

Animals and experimental design

Adult Wistar rats were obtained from the Animal Facility of the University of Medicine and Pharmacy of Craiova, Romania. Animals were maintained under controlled laboratory conditions (temperature 21 ± 2°C, relative humidity 55 ± 10%, and 12 h light/12 h dark cycle) with free access to standard food for rodent (Cantacuzino Institute, Bucharest, Romania) and filtered tap water. Prior to the start of the experiment, animals were allowed to acclimatize for 14 days.

The exposure protocol followed the experimental design as previously described for a developmental exposure model system (8, 25). Briefly, pregnant female rats were randomly assigned to four experimental groups beginning on gestational day 6 (GD6), with 5 animals *per* group. The control group received untreated drinking water. The ADI group received glyphosate at 0.5 mg/kg body weight/day, corresponding to the EU acceptable daily intake (ADI). The NOAEL group received 50 mg/kg body weight/day glyphosate, corresponding to the EU no-observed-adverse-effect level (NOAEL). The herbicide Mixture group received a combination of glyphosate (0.5 mg/kg bw/day), dicamba (0.3 mg/kg bw/day), and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.02 mg/kg bw/day), corresponding to the EU ADI values. The exposure to herbicides was *via* drinking water.

Following birth and lactation, offspring were weaned and maintained in treatment groups derived from each litter. Ten male offspring from each group (approximately 2 male animals from each litter (n = 5) from respective group) continued to receive the same treatment regimen as their dams for an additional 13-week period, resulting in continuous exposure from gestational day 6 until postnatal day 111 (PND111). At the end of the exposure period, male animals were sacrificed and tissues and blood were collected for histopathological examination and hormonal analysis respectively.

The protocol for the animal study has the approval of the Ethical Committee of the University of Medicine and Pharmacy of Craiova and was conducted based on the current guidelines for the use of laboratory animals in research.

Tissue collection

At the end of the experimental period, animals were deeply anesthetized with ketamine and xylazine (Alfasan Int., the Netherlands) and euthanized by exsanguination. Blood samples were collected for hormonal analysis, and the testes and prostate glands were carefully excised, trimmed of surrounding connective tissue, and processed for histological examination. Left and right testes were collected, dried with paper towels and weighed with relative testis weight calculated by expressing the weight in grams *per* 100 grams of body weight.

Histopathological evaluation of testes and prostate

For the histopathological analysis, tissue samples were collected from 10 male rats *per* experimental group. The samples were fixed in 10% neutral buffered formalin for 24 hours and subsequently processed using standard paraffin-embedding techniques.

Serial sections with a thickness of 4 µm were obtained from the paraffin blocks and stained with hematoxylin and eosin (H&E) following conventional histological procedures. The stained sections were examined under a Nikon Eclipse Si light microscope (Nikon Europe, The Netherlands), and representative images were captured for documentation.

Microscopic evaluation focused on structural alterations of the reproductive tissues. In the testes, particular attention was given to the morphology of seminiferous tubules, spermatogenic cell layers, interstitial tissue, and Leydig cells. In the prostate, evaluation included the assessment of glandular architecture, epithelial integrity, stromal components, and the presence of inflammatory or degenerative changes. Histopathological lesions were identified and described in accordance with the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) guidelines for rodent brain pathology (26) and scored using a semi-quantitative system commonly applied in experimental toxicology studies (27) and based on extent and predominance of the observed changes: 0 – no lesion/absent; 1 – minimal/ focal changes (< 25%); 2 – mild changes (25 - 50%); 3 – moderate changes (50 - 75%); 4 – severe/diffuse changes (> 75%).

Assessment of serum testosterone concentration

Blood samples were collected at the end of the exposure period and centrifuged to obtain serum, which was stored at - 20°C until analysis. Quantification of testosterone concentrations was performed using a commercial testosterone ELISA kit (DRG Instruments GmbH, Marburg, Germany; catalog number EIA-1559) according to the manufacturer's instructions.

Briefly, serum samples and calibration standards were added to microplate wells coated with antibodies specific for testosterone. After incubation and washing steps, enzyme conjugate and substrate solutions were added, allowing the development of a colorimetric reaction proportional to the hormone concentration present in the sample. The absorbance was measured using a microplate reader at 450 nm, and testosterone concentrations were calculated using the standard calibration curve provided with the kit. Hormone levels were expressed as ng/mL of serum.

Statistical analysis

Statistical analyses were conducted using R software (version 4.0.0, R Foundation for Statistical Computing, Vienna, Austria). Testosterone levels and testes relative weights were summarized as mean ± standard deviation. The changes in these parameters were analyzed using one-way analysis of variance (ANOVA). When significant differences were detected, Dunnett's post hoc test was applied to compare herbicide exposure groups with the un-exposed control group. Graphical representation of the results was generated using the ggplot2 package in R.

Results and Discussion

Serum testosterone concentrations

The evaluation of serum testosterone levels showed a trend of decreased concentration in a dose dependent manner, reaching statistical significance in the herbicide Mixture group (Figure 1).

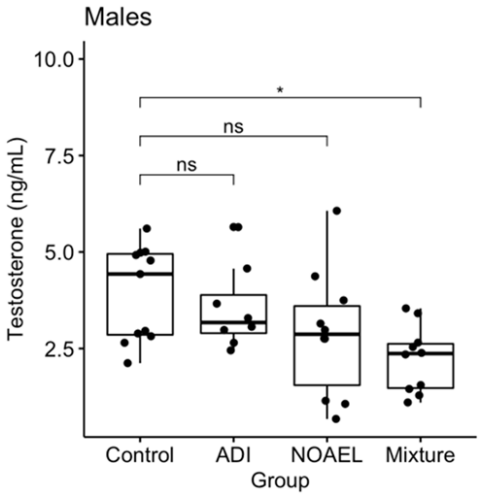

Figure 1.

Serum testosterone concentration in male rats at PND111. Serum was collected from animals exposed to the glyphosate EU acceptable daily intake (ADI), EU no-observed adverse effect level (NOAEL), and an EU ADI glyphosate, 2,4-D, dicamba Mixture. Control: unexposed control group; *p < 0.05 compared to Control

Testis weight

Evaluation of the relative weight of the testes demonstrated a significant increase in both the right and left testis in males exposed to the herbicide Mixture (Figure 2A, 2B). In contrast, no significant variation was observed in the glyphosate alone exposure groups compared with controls (Figure 2A, 2B).

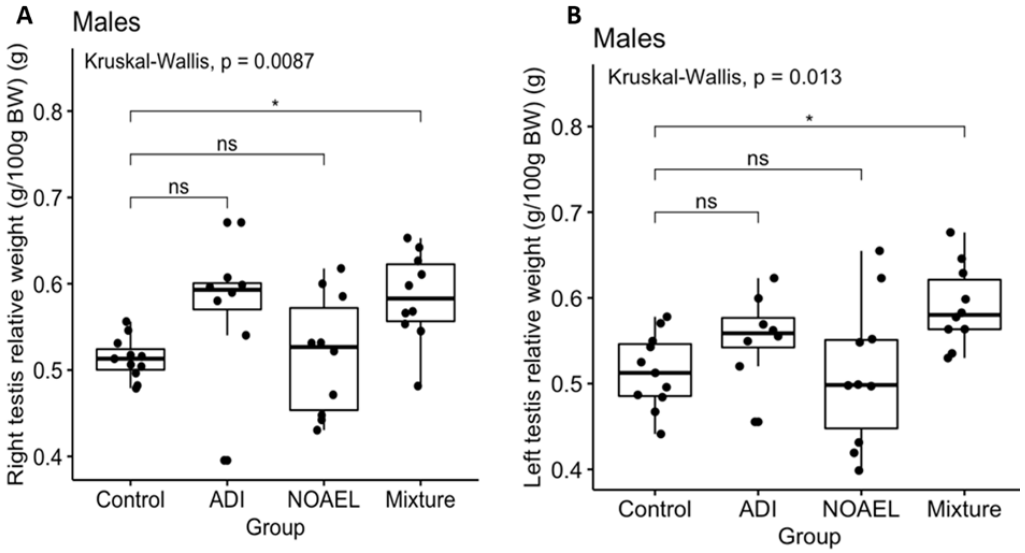

Figure 2.

Relative testis weight in male rats at PND111. Testes were collected from animals exposed to the glyphosate EU acceptable daily intake (ADI), EU no-observed adverse effect level (NOAEL), and an EU ADI glyphosate, 2,4-D, dicamba Mixture. Control: unexposed control group. **A.** Right testis. **B.** Left testis; *p < 0.05 compared to control

Histopathological assessment of testicular tissue

In the un-exposed control group, the testis exhibited normal histological architecture with seminiferous tubules regularly arranged and displaying a complete spermatogenic series with well-organized germinal epithelium and Leydig cells present in a normal distribution and morphology (Figure 3A, Table I).

In the glyphosate ADI exposure group, mild to moderate histopathological alterations were observed. Interstitial edema was evident, characterized by expansion of the intertubular spaces, accompanied by occasional hyperemic blood vessels. The tunica albuginea appeared mildly thickened, accompanied by occasional hyperemic blood vessels. Seminiferous tubules largely preserved their architecture, although focal epithelial changes were present (Figure 3B, Table I).

In the glyphosate NOAEL exposure group, the lesions were similar in type but more pronounced in severity. Marked interstitial edema led to clear separation of seminiferous tubules, and vascular congestion was more evident. Thickening of the tunica albuginea was more conspicuous. Seminiferous tubules exhibited degenerative changes, aspects of vacuoles in the epithelium and atrophy of the spermatogenic epithelium and partial disorganization of the normal stratified arrangement. Leydig cells were present within the edematous interstitium presenting as small clusters, without evident aspects of atypia (Figure 3C, Table I).

The glyphosate, 2,4-D, dicamba Mixture exposure group displayed the most severe histopathological changes. Interstitial edema was extensive, with visible expansion of the intertubular spaces and prominent vascular congestion. The tunica albuginea showed pronounced thickening with a dense fibrous appearance. Seminiferous tubules showed a reduction of the spermatogenic epithelium, with depletion of germ cells, and architectural disorganization regarding stratification. Focally, areas of necrosis were identified, characterized by loss of nuclear detail, increased cytoplasmic eosinophilia, and disruption of tissue architecture. Leydig cells were present, occasionally forming small aggregates (Figure 3D, Table I).

Table I

Histopathological scoring of testicular lesions. Testes were collected from animals exposed to the glyphosate EU acceptable daily intake (ADI), EU no-observed adverse effect level (NOAEL), and an EU ADI glyphosate, 2,4-D, dicamba Mixture. Control: unexposed control group.

Parameters	Experimental groups /scores			
	Control (n = 10)	ADI (n = 10)	NOAEL (n = 10)	Mixture (n = 10)
Tunica albuginea thickening	0	1	2	3
Interstitial edema	0	1	2	3 - 4
Vascular congestion	0	1	2	3
Seminiferous epithelial atrophy	0	1	2	2 - 3
Necrosis	0	0	0	1

0 – no lesion/absent, 1 – minimal/focal changes (< 25%), 2 – mild changes (25 - 50%), 3 – moderate changes (50 - 75%), 4 – severe/diffuse changes (> 75%)

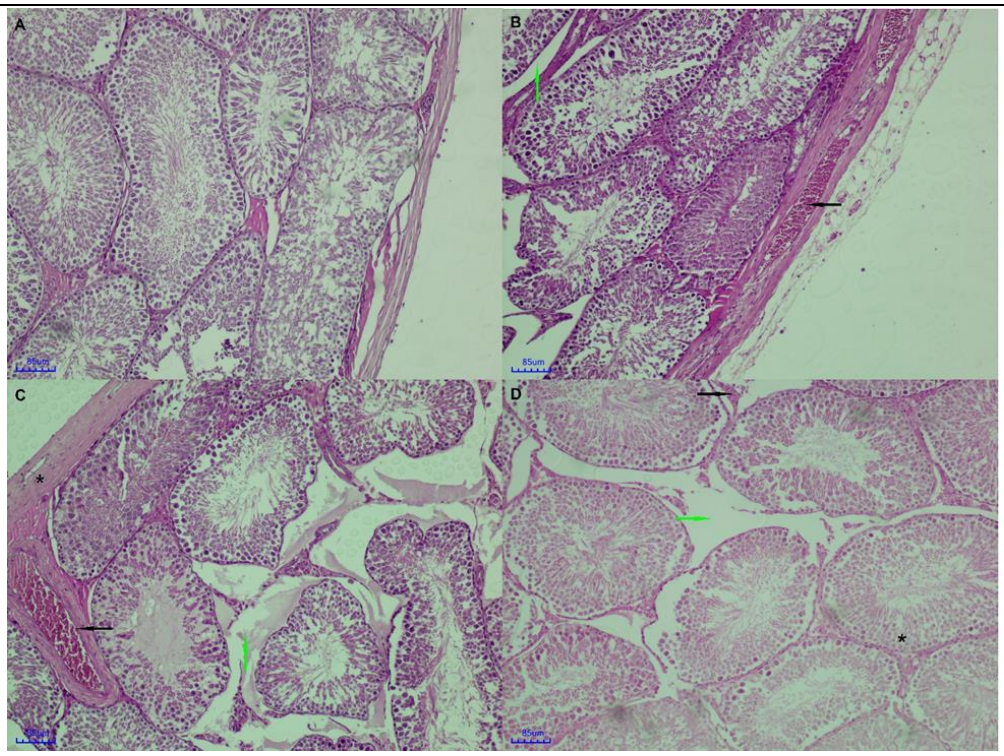

Figure 3.

Testes were collected from animals exposed to the glyphosate EU acceptable daily intake (ADI) and EU no-observed adverse effect level (NOAEL), and an EU ADI glyphosate, 2,4-D, dicamba Mixture and subjected to histological analysis *via* H&E staining. **A**: Control group: normal architecture. **B**: ADI group: thickened tunica albuginea with hyperemic blood vessels – black arrow, interstitial edema – green arrow. **C**: NOAEL group: hyperemic blood vessels-black arrow, interstitial edema – green arrow, thickened tunica albuginea – black asterix. **D**: Mixture group: Leydig cells aggregate – black arrow, interstitial edema – green arrow, black asterix – small areas of necrosis. Magnification: x100

Prostate histological evaluation

The control group maintained a normal prostatic aspect, characterized by glandular structures lined by a uniform layer of cuboidal to columnar epithelial cells, with surrounding fibromuscular present (Figure 4A, Table II).

In the glyphosate ADI group, the glandular architecture appeared altered, with densely packed acini leading to epithelial folding. In several areas, intraluminal projections appearing as micropapillary structures were observed. Focal vascular hyperemia was observed within the interacinar stroma (Figure 4B, Table II).

In the glyphosate NOAEL group, the prostatic tissue presented acini of variable sizes and shapes and eosinophilic luminal secretions. Irregular epithelial folding was also maintained, with formation of micropapillary projections into the glandular lumen. For some acini, a reduction of the fibromuscular layer was observed. Interacinar edema was noted and there was a vascular component of small-caliber vessels with hyperemia (Figure 4C, Table II).

In the herbicide Mixture exposed group, the acini displayed marked variability in size, with notable focal gland dilation and containing eosinophilic secretory material or epithelial hypertrophy. The interstitial edema was more pronounced compared to other groups. Vascular structures were enlarged, with dilated lumina and thickened vascular walls, and were frequently associated with inflammatory cellular elements (Table II, Figure 4D).

The present study evaluated the potential effects of developmental exposure to glyphosate at regulatory reference doses (ADI and NOAEL), as well as to a mixture of glyphosate, dicamba, and 2,4-D also at the EU ADI, on male reproductive organs. Continuous exposure from gestational day 6 through lactation and post-weaning until postnatal day 111 resulted in measurable alterations in testicular and prostatic structure, accompanied by a reduction in circulating testosterone levels in herbicide Mixture-exposed animals. The decrease in serum testosterone observed in the Mixture group suggests interference with endocrine regulation of the male reproductive system (28). Testosterone plays a critical role in the maintenance of spermatogenesis and the structural integrity of reproductive tissues (29). Disruption of androgen production has been reported following exposure to several pesticides, including GBH formulations (30, 31).

Table II

Histopathological scoring of prostatic tissue lesions. Prostate tissue was collected from animals exposed to the glyphosate EU acceptable daily intake (ADI), EU no-observed adverse effect level (NOAEL), and an EU ADI glyphosate, 2,4-D, dicamba Mixture. Control: unexposed control group

Parameters	Experimental groups scores			
	Control (n = 10)	ADI (n = 10)	NOAEL (n = 10)	Mixture (n = 10)
Glandular athitecture	0	1	2	2-3
Epithelial changes	0	1	1-2	2
Interacinar edema	0	0	1	2-3
Vascular changes	0	1	1	2-3

0 – no lesion/absent, 1 – minimal/focal changes (< 25%), 2 – mild changes (25 - 50%), 3 – moderate changes (50 - 75%), 4 – severe/diffuse changes (> 75%)

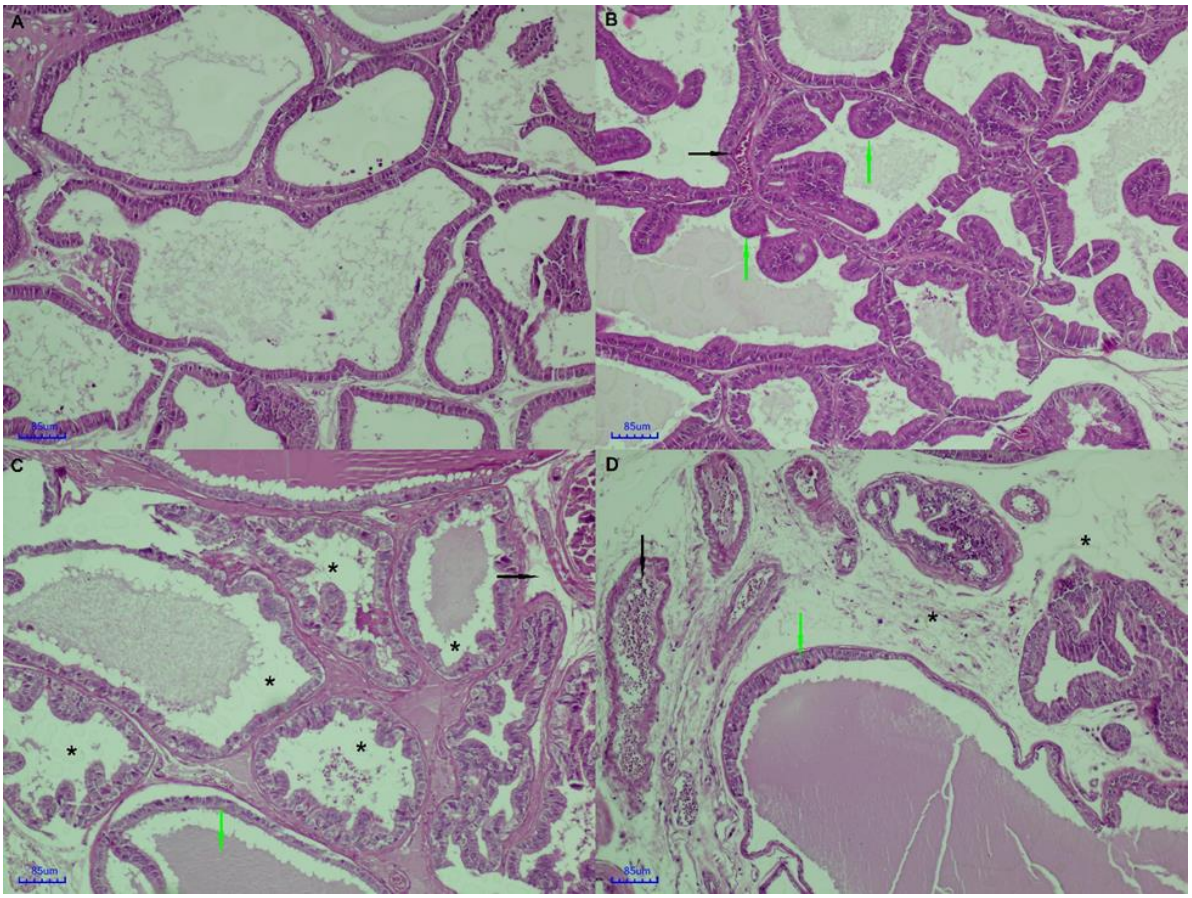

Figure 4.

Prostate tissue was collected from animals exposed to the glyphosate EU acceptable daily intake (ADI) and EU no-observed adverse effect level (NOAEL), and an EU ADI glyphosate, 2,4-D, dicamba Mixture and subjected to histological analysis *via* H&E staining. **A**: Control group: normal architecture. **B**: ADI group: interacinar vascular hyperemia – black arrow, intraluminal projections appearing as micropapillary structures – green arrow. **C**: NOAEL group: interacinar edema – black arrow, eosinophilic luminal secretions – green arrow, asterix – acini of variable sizes and shapes. **D**: Mixture group: vascular structure with inflammatory elements – black arrow, intra-acinar epithelial hypertrophy – green arrow, asterix – interacinar edema. Magnification: X100

Experimental studies have shown that glyphosate exposure may affect steroidogenesis through oxidative stress mechanisms and mitochondrial dysfunction in Leydig cells, leading to altered testosterone synthesis (32). More recent investigations have also demonstrated that glyphosate can interfere with the hypothalamic–pituitary–gonadal axis and promote oxidative damage in reproductive tissues (33, 34).

In the present study, histopathological examination revealed progressive structural alterations in testicular tissue across the exposure groups. Mild interstitial edema and vascular congestion were observed in the glyphosate ADI group, whereas more pronounced lesions, including seminiferous epithelial degeneration and architectural disorganization were present in the glyphosate NOAEL group. The herbicide Mixture group exhibited the most severe alterations,

characterized by extensive interstitial edema, seminiferous epithelial depletion, and focal areas of necrosis. These findings are consistent with previous experimental studies reporting degenerative changes in seminiferous tubules and disruption of spermatogenic cell layers following glyphosate exposure (32). Structural damage to the seminiferous epithelium may compromise spermatogenesis and ultimately impair male fertility (35).

Interestingly, the present study also demonstrated a significant increase in relative testicular weight in animals exposed to the herbicide Mixture. Testicular weight alterations may reflect inflammatory responses, interstitial edema, or compensatory cellular changes occurring in response to toxic insult (36). Similar findings have been reported in toxicological studies evaluating endocrine-disrupting chemicals, where tissue weight changes were associated with histological evidence of cellular degeneration and vascular alterations (37, 38).

The prostate gland also exhibited structural alterations following herbicide exposure. While control animals displayed normal glandular architecture, exposed groups showed epithelial folding, micropapillary intraluminal projections and interacinar edema. As in the case of effects on the testis, these alterations were most pronounced in the herbicide Mixture group, where glandular dilation, epithelial hypertrophy, and vascular changes accompanied by inflammatory elements were observed. Prostatic tissue is highly dependent on androgen signaling, and therefore reductions in testosterone levels may contribute to structural remodeling of prostatic glands (39). Endocrine disruption and oxidative stress have been proposed as mechanisms underlying herbicide-induced reproductive toxicity (40).

An important observation of the present study is that structural and hormonal alterations were detected even at exposure levels corresponding to regulatory permitted reference doses. While traditional toxicological assessments generally consider these levels safe, the present findings suggest that prolonged exposure during sensitive developmental periods may reveal biological effects not evident in either shorter-term studies or those starting exposure at an adult stage. Developmental stages represent critical windows for reproductive organ differentiation and endocrine programming, and disturbances occurring during these periods may result in persistent alterations in reproductive physiology (41).

Moreover, the more pronounced effects observed in the herbicide Mixture group highlight the importance of evaluating combined exposure scenarios. In real-world settings both on an agricultural occupational and general consumer contexts, exposure rarely occurs to a single compound, and individuals are simultaneously exposed to multiple pesticides (42, 43). Experimental studies have shown that mixtures of pesticides may produce additive or synergistic toxicological effects, particularly when compounds share similar mechanisms of action or affect related biological pathways (44-46). The results of the present study support the need to consider mixture toxicity in toxicological risk assessment.

Limitations of the study

Our study has several limitations that should be considered when considering the implications the observed findings. First, although histopathological evaluation and hormonal analysis provide evidence of reproductive alterations, the molecular mechanisms underlying these effects were not investigated. Additional analyses focusing on oxidative stress biomarkers, inflammatory mediators, or gene expression involved in steroidogenesis could provide further insight into the mechanisms of herbicide-induced reproductive toxicity.

Second, the study did not include measurements of internal exposure levels, such as glyphosate or metabolite concentrations in biological tissues or serum. The absence of toxicokinetic data limits the ability to directly correlate internal dose with the observed histopathological and hormonal changes.

Third, functional reproductive parameters, including sperm quality, sperm count, or fertility outcomes, were not evaluated. Such endpoints would provide additional information regarding the potential impact of the observed structural alterations on reproductive capacity.

Despite these limitations, the study provides experimental evidence that developmental exposure to glyphosate and herbicide mixtures at regulatory permitted reference doses may induce structural and endocrine alterations in male reproductive organs.

Conclusions

The present study demonstrates that chronic developmental exposure to glyphosate at regulatory reference (EU ADI and NOAEL) doses, as well as exposure to an EU ADI Mixture of glyphosate, dicamba, and 2,4-D, may induce structural and hormonal alterations in male reproductive organs. Continuous exposure from gestational day 6 through postnatal day 111 resulted in histopathological changes in the testes and prostate, accompanied by a reduction in serum testosterone levels in the herbicide Mixture-exposed animals. The observed lesions, including seminiferous epithelial degeneration, interstitial edema, and prostatic glandular alterations, suggest that prolonged exposure to herbicide mixtures may interfere with normal reproductive tissue architecture and endocrine regulation. These findings highlight the importance of considering developmental exposure and mixture toxicity in the evaluation of potential reproductive effects associated with widely used herbicides.

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Availability of data and materials

All data presented in the manuscript are available from the authors upon request.

Ethics approval and consent to participate

The protocols utilized therein were approved by the Ethical Committee of UMF Craiova, Romania, No. 120/19.11.2020.

AI use disclosure

The authors used an AI-assisted language tool to improve readability and language. The authors take full responsibility for the content of the manuscript.

Conflict of interest

RM and MNA have served as consultants on glyphosate risk assessment issues as part of litigation in the US over glyphosate-based herbicide health effects. The other authors declare no competing interests.

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