https://doi.org/10.31925/farmacia.2025.1.8 **ORIGINAL ARTICLE**

LONG-TERM EFFECTS OF PRENATAL AND POSTNATAL EXPOSURE TO REGULATORY RELEVANT LEVELS OF GLYPHOSATE AND ITS MIXTURE WITH DICAMBA AND 2,4-D ON KIDNEY HEALTH

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Abstract

This study investigated the long-term effects of prenatal and postnatal exposure to regulatory-relevant levels of glyphosate and its mixture with dicamba and 2,4-D on kidney health in rats. Pregnant Wistar rats were exposed to glyphosate at acceptable daily intake (ADI) and no-observed-adverse-effect level (NOAEL) doses or a mixture of glyphosate, dicamba, and 2,4-D at their respective ADI doses from gestational day 6 through lactation and 13 weeks post-weaning. Results showed dose-dependent glomerular and tubular dysfunction in both male and female offspring, with the herbicide mixture producing effects similar to the glyphosate NOAEL dose. Histopathological changes and alterations in kidney injury biomarkers were observed in all treatment groups. The study highlights potential risks of low-dose, long-term exposure to these herbicides, particularly during critical developmental periods, and suggests a need to revise current chemical assessment methods to account for mixture effects and developmental toxicity.

Rezumat

Acest studiu a investigat efectele pe termen lung ale expunerii prenatale și postnatale la a doze permise din punct de vedere a agentiilor de reglementare de glifosat și amestecul acestuia cu dicamba și 2,4-D asupra sănătății rinichilor la șobolani. Femele de șobolan Wistar gestante au fost expuse la glifosat la doze zilnice acceptabile (DZA) și la doze fără efecte adverse observate (NOAEL) sau la un amestec de glifosat, dicamba și 2,4-D la dozele DZA respective din ziua gestațională 6 până la alăptare și 13 săptămâni după înțărcare. Rezultatele au arătat disfuncție glomerulară și tubulară dependentă de doză atât la descendenții masculini, cât și la femele, amestecul de erbicid producând efecte similare cu doza NOAEL de glifosat. Modificări histopatologice și modificări ale biomarkerilor leziunii renale au fost observate în toate grupurile de tratament. Studiul evidențiază riscurile potențiale ale expunerii în doze mici și pe termen lung la aceste erbicide, în special în perioadele critice de dezvoltare, și sugerează necesitatea revizuirii metodelor actuale de evaluare chimică pentru a lua în considerare efectele amestecului și toxicitatea asupra dezvoltării.

Keywords: Glyphosate, dicamba, 2-4-D, low-dose toxicity, kidney toxicity, pesticide mixtures, prenatal exposure, mixtures

Introduction

In recent years, the widespread use of pesticides and their potential effects on human health and the environment have raised many concerns. Exposure to pesticides can occur through various routes, but the primary source of human exposure is the consumption of contaminated food over a lifetime [1-4]. Chemical exposures pose the greatest threat to human health when they occur

in the earliest years of life [5]. Following the formulation of the Developmental Origins of Health and Disease (DOHaD) hypothesis in the early 1990s, a substantial body of research has accumulated, providing substantial evidence for the critical role of early life environments in determining lifelong health and the risk of non-communicable diseases (NCDs) [6, 7]. It is widely accepted that the structure and function of the organ system are largely determined during the developmental windows of the prenatal and early postnatal periods. It, therefore, follows that the early years set the course for lifelong health. It is important to note that doses of toxic chemicals that are harmless in adulthood can have marked effects if they coincide with the rapid cell proliferation, differentiation and tissue formation that occurs during organ development [8]. The immaturity of prenatal organs and detoxification defences, as well as the unique physiological characteristics and behaviours of infants and children, render them more vulnerable to environmental exposures during the early developmental period. During the first two months of life, the metabolism and renal clearance of toxic substances are lower than in adults, resulting in higher rates of toxic bioaccumulation [9]. In comparison to an adult kidney, the developing kidney exhibits a heightened sensitivity to environmental insults. The formation of the kidney commences as early as the fourth week of gestation and is largely complete by birth in full-term infants [10, 11]. Exposure to environmental toxins can result in two main consequences for renal health: reduced nephron number, defined as the number of mature nephrons at birth, and altered filtration and reabsorption processes. These occur during the vulnerable prenatal and postnatal periods and have been identified as risk factors for chronic renal disease and high blood pressure [12].

Glyphosate, an organophosphorus compound, is the active ingredient in the most widely used herbicides (*e.g.* Roundup®). Glyphosate-based herbicides (GBHs) have long been recognised for their role in agricultural and domestic settings. However, recent studies indicate potential nephrotoxic effects from GBH exposure that necessitate closer scrutiny [13, 14]. Research utilising animal model systems has demonstrated notable alterations in kidney function and histopathological changes after exposure to glyphosate-based herbicides at doses that mimic environmental exposure levels. Specifically, findings have revealed a decrease in kidney weight, increased markers of renal impairment, and histological changes indicative of oxidative stress and cellular damage [15]. A comprehensive transcriptomics analysis of renal tissue from rats exposed to an ultra-low dose of Roundup GBH revealed significant tissue damage, including fibrosis, necrosis, phospholipidosis, mitochondrial membrane dysfunction and ischaemia [16]. The findings of this study carry significant implications, particularly within the context of glyphosate's extensive utilisation, which gives rise to concerns regarding potential long-term health implications for humans, notably among agricultural workers and communities in proximity to treated fields [17, 18].

Dicamba (3,6-dichloro-2-methoxybenzoic acid) and 2,4-D (2,4-dichloro-phenoxyacetic acid) are systemic herbicides that have been utilised for the purpose of weed control for a period exceeding half a century [19]. In the face of the substantial infestation of agricultural fields with glyphosate-resistant weeds [20, 21], measures have been implemented to address this issue. These include the introduction of genetically modified (GM) food crops that are tolerant to glyphosate, as well as 2,4-D and dicamba [22]. This has resulted in a considerable increase in the utilisation of 2,4-D and dicamba, which has consequently led to an escalating degree of human exposure [23]. Dicamba has given rise to a number of concerns regarding its potential health implications. A notable finding from the Agricultural Health Study suggests a potential association between dicamba use and an increased risk of developing colon and lung cancer among agricultural workers [24]. 2,4-D is a systemic phenoxy herbicide that was first developed in the 1940s and remains in widespread use at the present time [25]. Research has identified 2,4-D as a developmental toxicant, with reported effects including prolonged gestation periods, skeletal abnormalities, and potential impacts on thyroid function and reproductive organs [26]. The findings of research studies conducted over an extended period of time have indicated that there is a possibility of significant health concerns arising from chronic exposure to 2,4-D, which may include the potential for damage to the liver and kidneys [27-30]. The increased exposure to glyphosate in combination with glyphosate, 2,4-D and dicamba has given rise to novel health concerns. In a previous study, it was demonstrated that exposure to a glyphosate, 2,4-D and dicamba mixture at each regulatory-permitted dose resulted in redox imbalance in the liver of rats [31]. This finding already indicates the potential for unanticipated health consequences to arise from exposure to this combination of herbicides.

It is acknowledged that infants and children exhibit increased susceptibility to the effects of environmental pollutants due to their elevated rate of food and water consumption relative to their body weight, in conjunction with their developing organ systems. A paucity of research has been conducted on the potential health implications of exposure to environmental pollutants, particularly pesticides, during early life stages. The present study was conducted to evaluate the impact of maternal exposure, followed by exposure through the lactation period and 3 months post-weaning to low doses of glyphosate and its mixture with dicamba and 2,4-D, on adult kidney health in a rat model system.

Materials and Methods

In vivo experiment in a rat model system

The protocol to be followed during the animal treatment phase of the experiment has previously been described in detail [32]. In summary, the experiment utilised twenty-three-month-old Wistar rat females (n = 20) from the University of Medicine and Pharmacy of Craiova, Romania Animal House. Following a 14-day acclimatisation period to the new laboratory environment (temperature: 21 ± 2°C; humidity: 55 ± 10%; light cycle: 12 h light/12 h dark), the females were mated. On the day that the gestation was confirmed (day 0), the presence of copulation plugs was used to identify the pregnant females. The pregnant females were then separated from the males and placed in separate cages (two or three animals *per* cage). Filtered tap water and standard animal chow (Cantacuzino Institute, Bucharest, Romania) were provided to the animals ad libitum. The experiment involved the allocation of dams to one of four distinct groups, with a total of five animals assigned to each group, commencing from gestation day (GD) 6. The treatment regimen for each group is outlined below. The control group received unrestricted access to water and food, with no treatment administered. The ADI group was administered a dose of 0.5 mg/kg bw/day glyphosate in drinking water, which is the European Union (EU) acceptable daily intake for glyphosate. The NOAEL group was administered 50 mg/kg bw/day in drinking water, equivalent to the EU, and no adverse effect level was observed for glyphosate. The Mixture group was administered 0.5 mg/kg bw/day glyphosate in combination with 0.3 mg/kg bw/day dicamba and 0.02 mg/kg bw/day 2,4-D in drinking water, corresponding to the acceptable daily intake levels for each of the herbicides as set out by the EU.

Subsequent to weaning, the pups were separated from the dams and grouped into cohorts of 10 males and 10 females *per* group (approximately 2 males and 2 females from each litter). These groups were then exposed to the same chemicals for a further 13 weeks. The total exposure period extended from GD6 to 13 weeks after weaning, as illustrated in Figure 1. The herbicides were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, Germany as Glyphosate PRESTANAL®, analytical standard, ≥ 98.0% purity, Dicamba PRESTANAL®, analytical standard, ≥ 98.0% purity and 2,4-D, 97% purity.

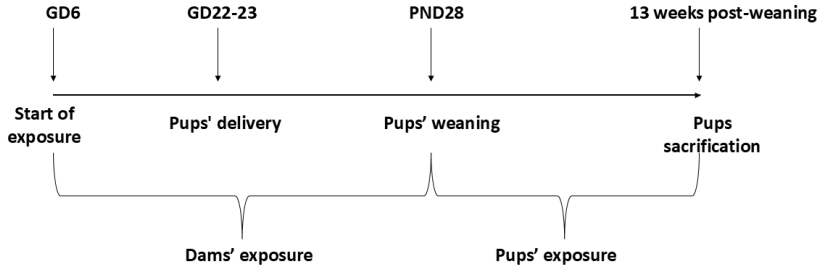

Figure 1.
The timeline of the exposure period during the experiment. GD – gestational day, PND- post-natal day

The *in vivo* study was conducted in accordance with the prevailing guidelines for the utilisation of laboratory animals and was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania. Following the conclusion of the designated exposure period, the animals were euthanised by exsanguination following the administration of anaesthetics. The anaesthetics, xylazine (9.1 mg/kg bw) and ketamine (9.1 mg/kg bw) were procured from Alfasan Int., the Netherlands.

Serum biochemical analysis

Blood samples were collected from the tail vein of each rat. A total of 2 or 2.5 millilitres of blood was collected from each animal, after which the serum was separated using serum separation tubes manufactured by Arkay (Japan). Serum creatinine and urea levels were evaluated immediately using an automated analyser, the SPOTCHEM EZ SP-4430 (Arkay, Japan). The residual serum was stored at a temperature of -70°C, pending the subsequent measurement of other markers.

Measurement of serum thyroid hormone levels

The levels of total triiodothyronine (T3), total thyroxine (T4), and thyroid-stimulating hormone (TSH) were determined in serum using an ELISA (DRG Instruments GmbH, Germany) according to the manufacturer's instructions. In this study, a range of commercially available kits was utilised, including those for measuring total T3 (catalogue no. EIA-4569), total T4 (cat. no. EIA-4568), and TSH (cat. no. EIA-1782).

Kidney collection and histopathological analysis

Following the removal of the left and right kidneys, they were meticulously cleansed with physiological saline, thoroughly dried with paper towels, and weighed. The ratio of renal weight to body weight was subsequently calculated and expressed in grams *per* 100 grams of body weight. Half of the kidneys were then preserved for a 24-hour period in a 4%

paraformaldehyde solution, following a thorough gross inspection for the presence of cysts. Subsequent to this, the tissues were dehydrated with increasing ethanolic concentrations (70% for 1 h, 90% for 1 h, 100% for 5 h). Paraffin embedding of the tissue was then performed, after which the blocks were cut into 25 µm sections using a microtome and stained with haematoxylin/eosin. An experienced pathologist, unaware of the experimental arm, examined the tissues using Panthera L light microscopy (Motic Europe, SLU) and graded the pathological changes as sub-lethal/reversible and lethal/irreversible injury in accordance with standard pathological guidelines [33]. Histopathologic changes were scored on the basis of their intensity and the number of animals affected. Intensity scores were (-) none, (+) mild, (++) moderate, and (+++) severe.

ELISA determination of kidney injury biomarkers in tissue homogenates

The right and left kidneys were weighed and homogenised on ice in a 1:1 ratio (100 mg tissue: 1 mL PBS) before being stored at -20°C overnight. The homogenates were then subjected to two freeze-thaw cycles in order to break down the cell membranes. The final step was centrifugation at 2-5°C, 5000xg for 5 min. The resultant supernates were frozen until use. The level of matrix metalloproteinase-9 (MMP-9) in the tissue homogenate was then determined by ELISA (Antibodies-online GmbH, Germany) (cat. no. ABIN 6730943) in accordance with the manufacturer's instructions. Levels of cystatine-c (CYS-C) (cat. no. CSB-E08385r), kidney injury molecule-1 (KIM-1) (cat no. CSB-E08808r), and matrix metalloproteinase-2 (MMP-2) (cat. no. CSB-E07411r) were measured in tissue homogenates by ELISA (Cusabio, Wuhan, China) in accordance with the manufacturer's instructions.

Statistical analysis

The statistical software program SPSS 20 (SPSS Inc., Chicago, USA) was utilised for the execution of all statistical analyses. In the case of continuous data, the mean ± standard deviation of the mean was employed. One-way ANOVA was utilised for data analysis, while Dunnett's Test was employed to compare multiple data sets. A level of $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Serum creatinine and urea levels in male and female rats

Serum creatinine and urea values were found to be significantly elevated in male subjects, while urea levels were significantly elevated in female subjects ($p < 0.01$) when compared to the control group (Figure 2). Following exposure to the glyphosate, 2,4-D and dicamba herbicide mixture, a significant increase in serum creatinine and urea levels was observed in both males and females compared to the control group ($p < 0.001$ and $p < 0.05$ for urea in females) (Figure 2).

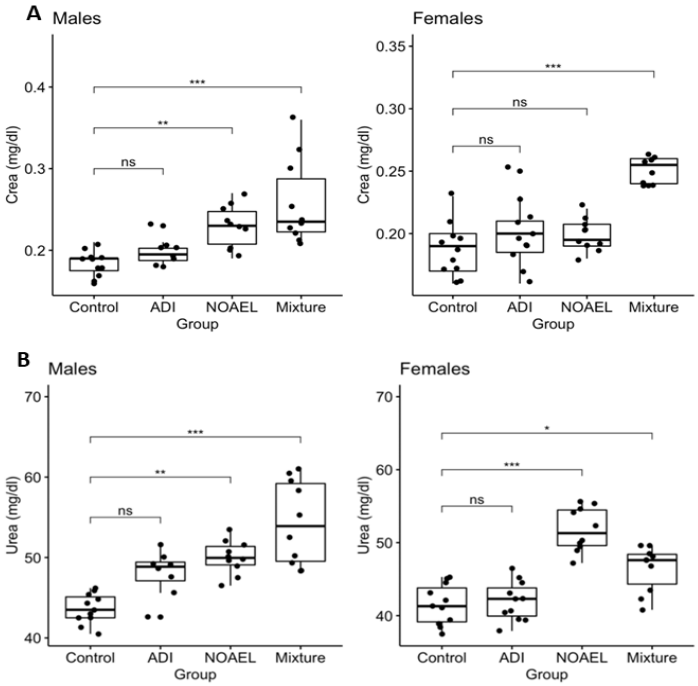

Figure 2.

Serum levels of: A. Creatinine in male and female rats; B. urea in male and female rats.

*p < 0.05; **p < 0.01; *** p < 0.001 compared with control; ns- nonsignificant. Crea – blood creatinine levels at the fine of the experiment; urea – blood urea levels at the fine of the experiment

Serum thyroid hormone levels

In the glyphosate, 2,4-D and dicamba herbicide mixture exposure groups, a significant increase in serum T3 levels was observed in both males and females, without any change in T4 and TSH concentration, compared to the control group ($p < 0.01$) (Figure 3). In the glyphosate ADI dose group, a significant decrease in T3 levels was observed in females without any alterations in T3 and TSH compared to the control group ($p < 0.001$) (Figure 3). In the male subjects from the glyphosate ADI dose group, a significant increase in T4 levels was observed, with no changes in T3 and TSH levels compared to the control group ($p < 0.05$) (Figure 3). In the glyphosate NOAEL dose group, a significant decrease in TSH levels was observed in female subjects compared to the control group, with no changes observed in the other two biomarkers ($p < 0.05$) (Figure 3).

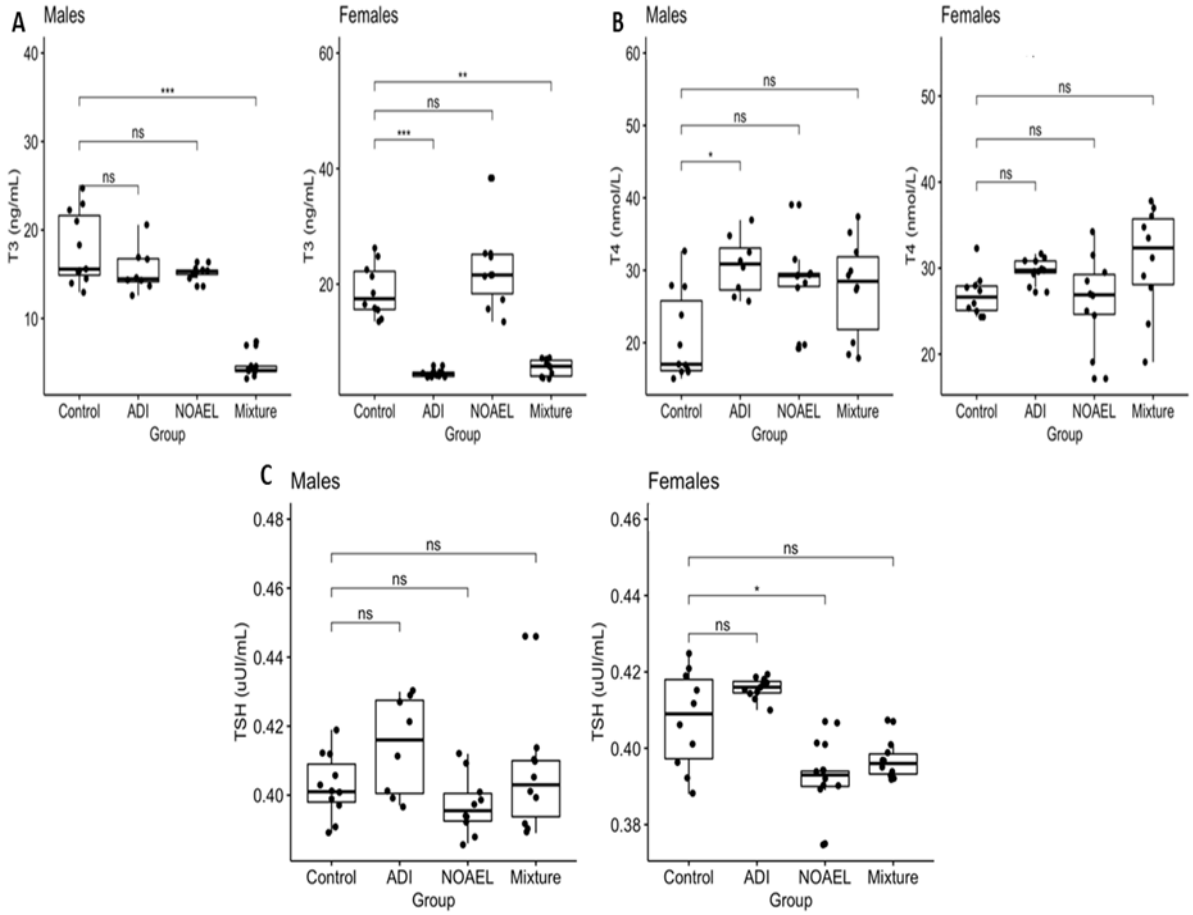

Figure 3.

Serum levels of: A. T3; B: T4; C. TSH in male and female rats.

*p < 0.05; **p < 0.01; *** p < 0.001 compared with control; ns- nonsignificant. T3 - total triiodothyronine; T4 - total thyroxine; TSH-thyroid stimulating hormone

Kidney injury biomarker measures in tissue homogenates

In both male and female rats, the glyphosate ADI dose resulted in a significant elevation in Cys-C ($p < 0.001$) and a substantial reduction in MMP-2 ($p < 0.001$) when compared to the control groups. In the glyphosate NOAEL dose and mixture groups, significant increases in Cys-C levels ($p < 0.001$) and significant decreases in MMP-2 and MMP-9 levels ($p < 0.001$) were observed in both males and females compared with controls. However, KIM-1 levels did not differ between the treated and control groups. The evaluation of relative kidney weight showed a significant increase in both the right and left kidney in males exposed to a mixture of herbicides. No other changes were observed in other groups (Figure 4).

In the present study, a preliminary examination of the kidneys from rat specimens in the control group revealed no overt abnormalities. Both the renal cortex and medulla exhibited no indications of pathological changes in both female (see Figure 5A, Table II) and male (see Figure 6A, Table II) subjects.

Table I

Kidney injury biomarkers in tissue homogenates in male and female rats at the fine of the exposure period

Biomarker	Control Group		ADI Group		NOAEL Group		Mixture group	
	Female	Male	Female	Male	Female	Male	Female	Male
Cys-C (ng/mL)	9.36 ± 0.89	9.72 ± 1.38	11.53 ± 0.65*	11.96 ± 0.65*	12.23 ± 1.08*	10.35 ± 0.8*	11.94 ± 0.9*	12.26 ± 0.44*
KIM-1 (ng/mL)	23.52 ± 1.98	23.2 ± 2.18	23.8 ± 0.92	23.96 ± 1.94	23.04 ± 01.57	23.53 ± 1.92	23.28 ± 0.97	23.67 ± 0.97
MMP-2 (ng/mL)	10.95 ± 1.54	10.01 ± 0.65	8.6 ± 0.7*	9.44 ± 0.59*	6.82 ± 1.14*	6.41 ± 0.37*	3.08 ± 0.21*	2.72 ± 0.52*
MMP-9 (ng/mL)	380.67 ± 17.31	383.10 ± 18.95	452.42 ± 174.13	391.48 ± 17.47	290.43 ± 10.91*	298.2 ± 11.93*	270.31 ± 17.26*	274.62 ± 12.13*

CYS-C- cystatin-c; KIM-1-kidney injury molecule-1; MMP-2 - matrix metalloproteinase-2; MMP-9 - matrix metalloproteinase-9.
* $p < 0.001$ compared with respective sex controls (n = 10 animals *per* group).

Kidney histopathological analysis

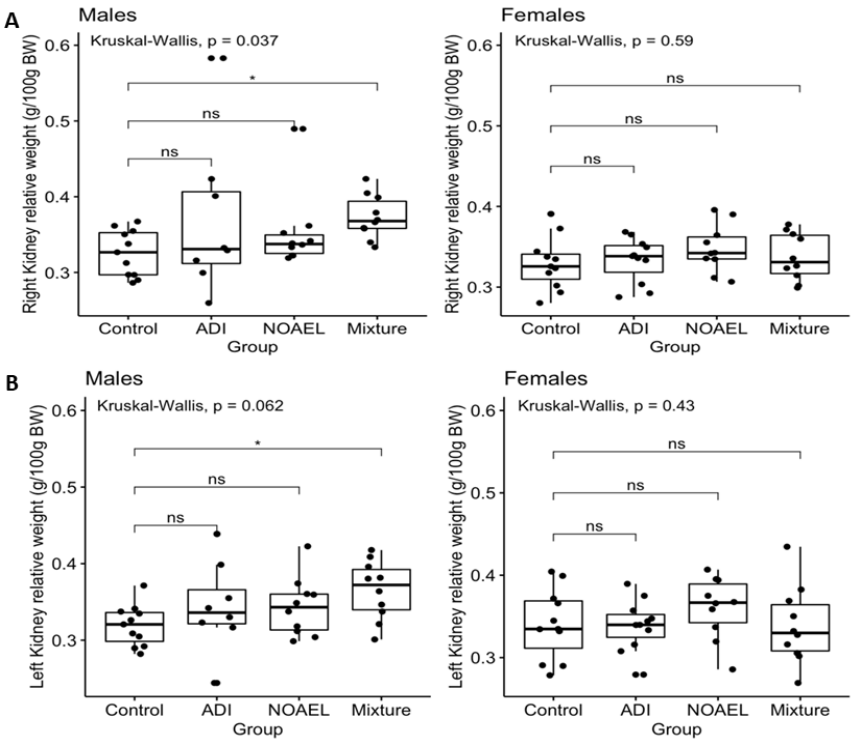

Figure 4.
Relative weight of: A. Right kidney in male and female; B. Left kidney in male and female rats

Conversely, rats exposed to the NOAEL doses of glyphosate exhibited significant histopathological alterations in the tubules of female rats (Figure 5C, Table II) and the glomerular structures of male rats (Figure 6C, Table II). A reduction in the subcapsular space and nuclear swelling in the parietal lamina was observed in both female (Figure 5C, Table II) and male (Figure 6C, Table II) rats. Glomerular hyperaemia was evident, along with occasional areas of haemorrhagic necrosis. Furthermore, dilatation of blood vessels and the presence of minor haemorrhagic foci were observed in both female (Figure 5C, Table II) and male (Figure 6C, Table II) subjects. In the ADI group, histopathological alterations were present in tubules and were similar for female (Figure 5B, Table II) and male (Figure 6B, Table II) rats. The predominant reversible change identified was hydropic degeneration. Multinucleate, intensely eosinophilic masses, likely representing tubular remnants, were observed alongside basophilic, anucleate masses indicative of microcalcifications. Glomerular alterations were minimal, with only slight to moderate enlargement of the subcapsular space observed in both female (Figure 5B, Table II) and male (Figure 6B, Table II) rats.

For the Mixture group, the changes were consistent with those observed in the ADI group, particularly at the tubular level. The alterations observed included hydropic degeneration, characterised by a granular

or vacuolar cytoplasm, dilated and hyperemic blood vessels, and a mild reduction in the subcapsular space, observed in both female (Figure 5D, Table II) and male (Figure 6D, Table II) rats.

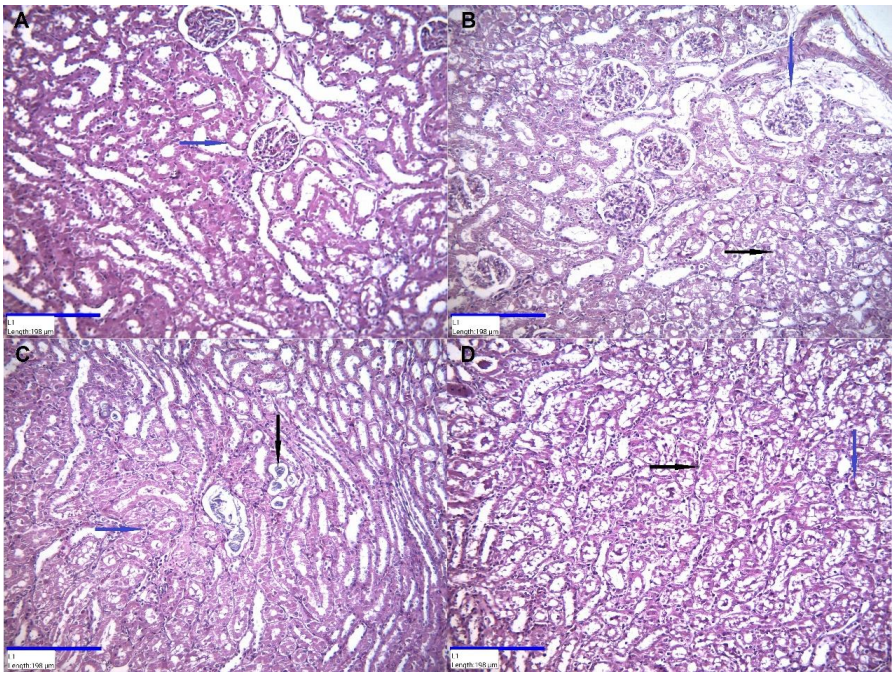

Figure 5.

Female specimen, A. Control group: normal kidney cell structure (blue arrow); B: ADI group: glomerular enlarged subcapsular space (blue arrow), tubular cytoplasmic granular or vacuoles (black arrow); C: NOAEL group: tubular reversible changes (blue arrow), microcalcifications (black arrow); D: MIXED group: tubular reversible changes (black arrow), eosinophilic, multinuclear masses (blue arrow); HE staining, X200.

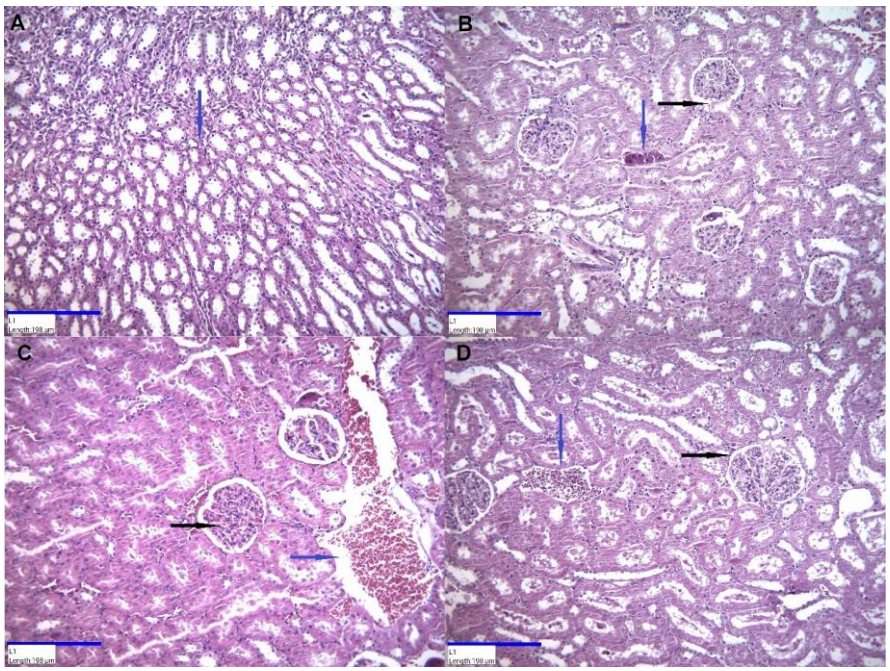

Figure 6.

Male specimen, A. Control group: normal kidney cell structure (blue arrow); B: ADI group: eosinophilic, multinuclear masses (blue arrow), glomerular enlarged subcapsular space (black arrow); C: NOAEL group: hyperaemic vessels (blue arrow), glomerular hyperaemia (black arrow); D: MIXED group: tubular reversible changes and hyperaemic vessels (blue arrow), glomerular reduction subcapsular space (black arrow); HE staining, X200.

Table II

Grade and incidence of renal histopathology lesions in female and male rats exposed to glyphosate in ADI and NOAEL doses and to the herbicide mixture.

Parameters	Experimental groups															
	Control				ADI				NOAEL				MIXT			
	Female		Male		Female		Male		Female		Male		Female		Male	
	I	N	I	N	I	N	I	N	I	N	I	N	I	N	I	N
Changes in subcapsular space	-	10/10	-	10/10	++	7/10	++	7/10	++	7/10	++	6/10	+	7/10	+	7/10
Multinucleate eosinophilic masses	-	10/10	-	10/10	++	7/10	++	6/10	+	5/10	+	4/10	+/-	4/10	+/-	3/10
Hyperemic blood vessels	-	10/10	-	10/10	+	6/10	+	7/10	+	7/10	+	8/10	+	7/10	+	8/10

I - intensity, N - specimens number.
(-) absent, (+) mild, (++) moderate, (+++) severe.

It is evident that crops derived from genetically modified organisms (GMOs) now account for 90% of the maize and soybeans cultivated in North and South America. While the 2000 and 2004 reviews conducted by the US National Academy of Sciences did not identify any health risks associated with the consumption of GM foods, both reports highlighted concerns regarding the potential health risks from the herbicides, particularly glyphosate-based herbicides (GBHs) employed in conjunction with GM food crops [34].

In the context of early glyphosate biomonitoring studies, the presence of glyphosate in farm workers, family members and other individuals was rarely found to be below the EPA reference dose. However, it should be noted that these levels may be subject to change over time [35]. Indeed, the results of more recent human biomonitoring studies of urinary glyphosate and its major metabolite, aminomethyl-phosphonic acid (AMPA), have detected residues of these compounds in virtually 100% of samples from US citizens. Furthermore, these residues have been positively associated with fatty liver disease [35] and pre-term birth [36-38]. Occupational exposure to glyphosate has been found to be associated with oxidative stress [39,40]. A meta-analysis of human biomonitoring data has been conducted, and the results indicate a significantly increased risk of developing non-Hodgkin lymphoma in cases of higher GBH exposure [41]. There is a paucity of data on childhood exposure to glyphosate, particularly for babies and young children [42]. Nonetheless, the findings of a biomonitoring survey have indicated that exposure to glyphosate and dicamba during childhood is associated with an elevated risk of developing fatty liver disease and cardiometabolic disorders in later life [43]. The higher incidence and levels of glyphosate exposure that have been observed are consistent with the increased amounts of the substance that have been detected in food [44].

Many studies involving animal model systems have reported multiple detrimental health effects of GBHs at environmental and regulatory relevant levels of exposure, such as oxidative stress [31,45-47], endocrine disrupting effects [48,49], inflammatory diseases [50], neurological disorders [51], reproductive disruptions [49] and possible carcinogenicity [52] linked with genotoxicity [53].

Recent studies have identified the kidney as being particularly vulnerable to the effects of glyphosate. Previous investigations have established a link between exposure to glyphosate and alterations in kidney function, renal injury, and chronic renal disease of unknown aetiology [54-56]. There is mounting evidence to suggest that agricultural workers in Central America, Sri Lanka and Central India are afflicted with an epidemic of chronic kidney disease of unknown origin. However, it is hypothesised that this may be attributable to exposure to GBH [57]. However, the paucity of studies conducted to date has resulted in a dearth of information. The majority of these studies were of the case-control variety, and none included children. Furthermore, the majority of these studies were conducted in occupational settings with agricultural workers. The majority of these studies did not examine the effects of glyphosate exposure on kidney function in children, nor did they examine the effects of prenatal and postnatal exposure to glyphosate and its mixture with dicamba and 2,4-D on adult kidney health.

In the present study, the rats are exposed from gestational day 6 through the lactation period and extending to 13 weeks post-weaning. It is important to note that breast milk constitutes another significant source of postnatal exposure to a wide range of chemicals [58]. As demonstrated in previous studies, exposure to glyphosate resulted in a dose-dependent manifestation of renal dysfunction, specifically affecting the glomeruli and tubules, as well as an elevation in

thyroid hormone levels [59]. Additionally, there were observed inflammatory changes in breast tissue [60] and a dose-dependent induction of non-alcoholic liver disease in rat dams [61]. Furthermore, exposure to the glyphosate, 2,4-D and dicamba herbicide mixture resulted in renal effects analogous to those observed with glyphosate at the NOAEL dose [59]. This finding indicates the presence of potentially deleterious, synergistic effects of this particular herbicide mixture, even at doses that are considered safe by regulatory authorities. In this study, exposure to the NOAEL dose of glyphosate resulted in increased serum creatinine and urea levels, an increase in kidney Cys-C levels and a decrease in kidney MMP-2 and MMP-9 levels in male animals and an increase in serum urea levels and kidney Cys-C levels and a decrease in serum TSH levels and in kidney MMP-2 and MMP-9 levels in females. These biochemical effects were associated with marked cytoarchitectural changes in the tubular and glomerular regions of the kidneys in both sexes, with males being more affected. In the glyphosate ADI dose group, a significant increase in Cys-C levels and a significant decrease in MMP-2 levels were observed in both male and female rats compared to the control group. Furthermore, a significant decrease in serum T3 levels was observed in females, and a significant increase in serum T4 levels was observed in males. At a tissue histological level, the ADI dose groups exhibited less pronounced pathological effects than the glyphosate NOAEL dose group, with reversible changes, especially in tubules in both males and females. The glyphosate, 2,4-D and dicamba herbicide mixture exposed groups demonstrated a clear additive effect, characterised by a significant increase in serum creatinine, urea and T3 levels, and in kidney Cys-C levels, along with a significant decrease in kidney MMP-2 and MMP-9 levels, in both male and female rats. Furthermore, the herbicide mixture exposed groups displayed cytoarchitectural changes that were comparable with the glyphosate ADI dose in both male and female groups.

It is acknowledged that there are sensitive developmental periods during which exposure to toxicants may programme subclinical nephrotoxicity. This manifests later in life, as renal development and maturation processes begin in utero and continue through early childhood [62]. The period of infancy is also indicative of renal maturation windows, which have been shown to increase susceptibility to environmental insults. This is due to the ongoing evolution of homeostatic processes, including renal blood flow and glomerular filtration [63]. Environmental toxins during critical prenatal and postnatal stages of development cause reduced nephron number, impaired filtration and absorption, and increased risk of chronic kidney disease and hypertension [12, 64]. In the present study, the nephrotoxic effects were initiated at the glyphosate ADI dose and progressed dose-dependently to the NOAEL dose. It is important to note that both dose regimens were considered by regulatory authorities to have no adverse effect on animals and, by extrapolation, on humans.

In instances of acute kidney injury, an elevation in serum creatinine is only indicative of a decrease in glomerular filtration rate, whether such a decrease is moderate or sharp [65]. Cystatin C has been shown to be a superior predictor of mortality and cardiovascular incidents in comparison to creatinine. It is less influenced by factors such as age, race, and muscle mass, thus making it a viable alternative or complementary biomarker to creatinine for the assessment of glomerular function [66]. The present study revealed that cystatin C levels were elevated in all treatment groups and in both male and female rats. This finding indicates that the observed nephrotoxic effects were dose-dependent. Furthermore, an additive effect was observed in the herbicide mixture exposure group. KIM-1 is a transmembrane glycoprotein predominantly expressed by proximal renal tubular cells [67]. As demonstrated in [68], the persistent expression of KIM-1 within the tubules has been observed to induce a state of inflammation and interstitial fibrosis. Furthermore, elevated levels of KIM-1 have been found to be correlated with both acute and chronic kidney diseases [69]. In the present study, the levels of kidney KIM-1 were found to be unaffected by the administration of the herbicide treatments. Matrix metalloproteinases (MMPs), with a particular emphasis on gelatinases, have been identified as pivotal in the process of renal interstitial fibrosis, given their role in regulating the extracellular matrix turnover within glomerular and tubular basement membranes [70]. The present study has demonstrated that there is an increase in the expression of MMP-2 and MMP-9 in renal fibrosis [71]. In all groups that had been treated with herbicides, it was found that serum levels of both gelatinases had been reduced. The effects were found to be dose-dependent and were more pronounced in the group that had been exposed to a mixture of glyphosate, 2,4-D and dicamba, suggesting an additive effect of the three herbicides.

In relation to thyroid hormone levels, a significant reduction in T3 was observed in male and female rats within the herbicide mixture group. Moreover, existing data indicates an elevated prevalence of low serum T3 levels in patients exhibiting impaired renal function [72]. T3 is produced by the enzyme 3 5′-deiodinase in the kidney from its precursor T4 [72]. In conditions external to the thyroid gland, an increase in serum T3 levels is the consequence of an increase in production and a decrease in breakdown [73]. In conditions of hypothyroidism, a decrease in

T4 production generally results in a concomitant decrease in T3 levels. However, in mild hypothyroid conditions, T3 levels may be normal or elevated [74]. In light of the foregoing, the effects observed in relation to T3 levels in the herbicide mixture group of male and female rats are consistent with the expected outcomes of a condition reflecting kidney injury. These outcomes correspond with other kidney disease biomarkers and histopathological changes that were also observed.

To the best of our knowledge, the study presented here is the first to assess how maternal exposure, continued through lactation and extended to three months post-weaning, to regulatory relevant (NOAEL, ADI) doses of glyphosate and its mixture with dicamba and 2,4-D affects adult kidney health in a rat model system. A further strength of the study design is that it simulates a real-life human exposure scenario, namely long-term and low-dose exposure to a chemical mixture.

Conclusions

Exposure to low doses of glyphosate (corresponding to the EU ADI and NOAEL doses) starting prenatally and extending postnatally through lactation and 3 months postweaning resulted in glomerular and tubular dysfunction in both male and female rats in a dose-dependent manner. Furthermore, the combination of glyphosate with dicamba and 2,4-D at the EU ADI dose resulted in effects on serum and tissue kidney biomarkers that were analogous to those observed in the glyphosate NOAEL dose groups. These effects included structural and functional injury to the kidneys, as well as a significant decrease in serum T3 levels. It has been established that decreased kidney conversion of T4 to T3 due to kidney damage is associated with lower serum T3 levels. These findings underscore the necessity for a comprehensive re-evaluation of the chemical assessment framework, which is predicated on toxicological testing with high doses of individual pesticides, without accounting for the potential consequences of exposure to chemical mixtures.

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Conflict of interest

The authors declare no conflict of interest.

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