*https://doi.org/10.31925/farmacia.2026.2.22* **ORIGINAL ARTICLE**

# NEURODEVELOPMENTAL DISRUPTION INDUCED BY LONG-TERM LOW-DOSE HERBICIDE EXPOSURE: BEHAVIORAL AND NEUROPATHOLOGICAL EVIDENCE FROM A RAT MODEL

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## Abstract

This study evaluated the effects of prolonged exposure to environmentally relevant levels of glyphosate, administered alone or in combination with dicamba and 2,4-dichlorophenoxyacetic acid (2,4-D), on behavioral outcomes and brain integrity in a rat model. Exposure was initiated during gestation and continued through postnatal development until early adulthood. Animals subjected to herbicide exposure exhibited alterations in locomotor activity, anxiety-related behavior, stress responses, and motor coordination, with more pronounced effects observed following combined exposure. These functional changes were associated with histopathological evidence of neuronal degeneration and vascular alterations, alongside biochemical indicators of oxidative imbalance in brain tissue. Our findings indicate that chronic exposure to low-dose herbicides during critical neurodevelopmental windows may induce persistent neurobehavioral and structural brain changes. The enhanced effects observed under mixture exposure conditions highlight the importance of considering combined environmental exposures in toxicological risk assessment.

## Rezumat

Acest studiu a evaluat efectele expunerii prelungite la niveluri relevante din punct de vedere ambiental de glifosat, administrat singur sau în combinație cu dicamba și acid 2,4-diclorofenoxiacetic (2,4-D), asupra comportamentului și integrității cerebrale într-un model experimental pe șobolan. Expunerea a fost inițiată în perioada gestațională și a continuat pe parcursul dezvoltării postnatale până la vârsta adultă timpurie. Animalele expuse la erbicide au prezentat modificări ale activității locomotorii, ale comportamentului de tip anxios, ale răspunsului la stres și ale coordonării motorii, efectele fiind mai pronunțate în cazul expunerii combinate. Aceste modificări funcționale au fost asociate cu dovezi histopatologice de degenerare neuronală și alterări vasculare, precum și cu dezechilibre oxidative la nivel cerebral. Rezultatele indică faptul că expunerea cronică la doze reduse de erbicide în perioade critice de neurodezvoltare poate induce modificări persistente neurocomportamentale și structurale.

**Keywords**: developmental toxicity, glyphosate, dicamba, 2,4-D, mixtures

## Introduction

Environmental chemical exposure has become an intrinsic component of modern life, with agricultural practices representing a major source of biologically active compounds released into ecosystems. Among these, herbicides are extensively used to sustain crop productivity, resulting in their continuous presence in soil, water, and food chains (1-3). Consequently, both wildlife and human populations are chronically exposed to low concentrations of these compounds, often throughout the entire lifespan (4-6).

The central nervous system represents a particularly vulnerable target for environmental toxicants, especially during early developmental stages (7, 8). Brain maturation involves highly coordinated processes such as neuronal proliferation, migration, synaptogenesis, and circuit refinement (9). Disruption of these tightly regulated events, even by subtle chemical perturbations, may lead to long-lasting alterations in neural function and behavior (10).

In recent years, increasing attention has been directed toward the potential neurotoxic effects of widely used herbicides, including glyphosate and auxinic compounds such as 2,4-dichlorophenoxyacetic acid (2,4-D) and dicamba (7, 8, 11). Although traditionally considered to have relatively low toxicity, emerging experimental data suggest that prolonged exposure may interfere with neuronal homeostasis through mechanisms involving oxidative stress, neuroinflammation, and mitochondrial dysfunction (12). Moreover, certain herbicides have been shown to cross biological barriers, including the blood–brain barrier, raising concerns regarding direct effects on neural tissue (13).

Importantly, real-world exposure scenarios rarely involve a single compound. Instead, organisms are simultaneously exposed to complex mixtures of agrochemicals, which may interact at multiple biological levels. Experimental studies have indicated that such combinations can amplify toxic responses beyond those observed for individual substances, potentially through additive or synergistic mechanisms affecting shared molecular pathways (14).

From a developmental perspective, the concept of early-life programming highlights the long-term impact of environmental exposures occurring during critical windows of susceptibility (15). According to the Developmental Origins of Health and Disease (DOHaD) paradigm, perturbations during prenatal and early postnatal life may predispose individuals to neurological dysfunction later in life, even when exposure levels fall within regulatory safety limits (16).

Despite these concerns, significant gaps remain in understanding how chronic exposure to low-dose herbicides—particularly under mixture conditions—affects brain development and function. Most available studies focus on high-dose toxicity or short-term exposure, limiting their relevance to real-life environmental conditions.

Therefore, the present study aimed to investigate whether prolonged exposure to environmentally relevant doses of glyphosate, administered alone or in combination with dicamba and 2,4-D, can induce behavioral alterations and structural brain changes in a rat model. By integrating neurobehavioral assessment with histopathological analysis, this study provides insight into the potential neurodevelopmental consequences of chronic low-dose herbicide exposure and contributes to a more realistic evaluation of environmental neurotoxicity.

## Materials and Methods

*Animals and housing conditions*

Experimental animals consisted of nulliparous female Wistar rats (3 months old) maintained in a controlled research environment. Prior to study initiation, animals were allowed an adaptation interval to laboratory conditions, ensuring stabilization of physiological and behavioral parameters. Animals were housed in ventilated cages under monitored environmental settings, with controlled photoperiod (12-hour light/dark cycle) and stable microclimate conditions. Temperature and humidity were maintained within standard laboratory ranges to avoid confounding effects related to environmental stressors. Standardized pelleted diet (Cantacuzino Institute, Bucharest, Romania) and purified water were provided ad libitum throughout the study period. To obtain timed pregnancies, females were cohabited with males, and reproductive status was verified by routine examination the following day, establishing the onset of gestation (gestational day 0). After confirmation, animals were individually allocated to prevent external influences on gestational progression.

The overall exposure design has been described previously for related experimental endpoints (17, 18) and is briefly summarized here.

*Experimental design and herbicide exposure*

A controlled developmental exposure protocol was applied to evaluate the effects of herbicides administered across pre- and postnatal stages. Following confirmation of gestation, female Wistar rats (n = 20) were randomly assigned to four experimental groups (n = 5 dams/group).

Herbicide exposure was conducted *via* drinking water, starting from gestational day 6 and continuing throughout lactation. The control group received untreated water. The treatment groups were administered glyphosate at 0.5 mg/kg body weight/day (European Union acceptable daily intake, ADI), glyphosate at 50 mg/kg body weight/day (no-observed-adverse-effect level, NOAEL), or a herbicide mixture consisting 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), each corresponding to their EU ADI values.

After weaning, offspring were retained within the corresponding experimental groups. A representative subset was selected from each litter, resulting in 10 males and 10 females *per* group, which were further maintained under the same exposure conditions for an additional 13-week period. Thus, the exposure interval extended from gestational day 6 to postnatal day 111.

The experimental design allowed the assessment of chronic exposure effects under conditions reflecting regulatory-relevant dose levels and combined herbicide exposure.

*Herbicides*

Glyphosate (PRESTANAL®, analytical standard, purity ≥ 98.0%), dicamba (PRESTANAL®, analytical standard, purity ≥ 98.0%), and 2,4-dichlorophenoxyacetic acid (2,4-D, PRESTANAL®, analytical standard, purity 97%) were obtained from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany).

*Neurobehavioral assessment*

To investigate potential functional alterations of the central nervous system induced by herbicide exposure, animals were subjected to a battery of behavioral tests designed to evaluate ambulation through locomotor activity and motor coordination. Animals were also assessed for emotional state through emotional reactivity, anxiety-related responses, and stress coping behavior.

All behavioral experiments were conducted at the end of the exposure period in a dedicated testing room under controlled environmental conditions during the light phase of the day cycle. Animals were allowed to acclimatize to the testing environment for at least 30 minutes prior to testing. All animals from each group (10 males and 10 females *per* group) undertake the behavioral tests. The order of testing was open field test, followed by elevated plus maze test, followed by rotarod test and followed by force swim test, with 3 days of rest between the tests, starting from the least stressful test to the most stressful test. Behavioral observations were performed by an investigator blinded to the experimental groups in order to minimize potential bias. The experimental apparatus was cleaned with 70% ethanol between animals to remove potential olfactory cues.

*Open field test*

Spontaneous locomotor activity, exploratory behavior, and emotional reactivity were assessed using the open field paradigm. The test was performed in a square arena (100 × 100 cm, wall height 40 cm), divided into central and peripheral zones to allow differentiation between exploratory drive and anxiety-related responses.

Each animal was individually placed in the central area of the apparatus and allowed to explore freely for 5 minutes. Behavioral activity was recorded and subsequently analyzed. The latency to leave the central zone was used as an indicator of initial exploratory response. Locomotor activity was quantified by the number of crossings between zones, while vertical exploration was evaluated based on rearing frequency. Indices of emotionality were also recorded, including grooming behavior and the number of fecal boli produced during the test session. These parameters were used as markers of stress-related responses.

All measurements were collected both as time-resolved values and as cumulative scores over the entire testing period. To eliminate potential olfactory cues, the arena was cleaned between successive trials. Behavioral assessment was performed under standardized environmental conditions by an observer blinded to group allocation.

*Elevated plus maze*

Behavioral assessment was performed using the elevated plus maze to evaluate ambulation and anxiety-like behavior. The apparatus consisted of two open arms and two enclosed arms arranged in a cross-shaped configuration and elevated above the floor.

Each animal was placed individually on the central platform, facing one of the open arms, and allowed to explore the maze for a period of 5 minutes. Behavioral activity was continuously monitored during the test session.

Ambulation was quantified by recording exploratory movements, including rearing and head-directed exploratory activity, reflecting the general locomotor and investigative drive of the animals.

Anxiety-like behavior was assessed by analyzing the distribution of time spent in the open arms, closed arms, and central platform. Increased time spent in the closed arms was considered indicative of heightened anxiety-like behavior, whereas greater exploration of the open arms suggested reduced anxiety levels. Grooming activity was additionally recorded as an indicator of emotional reactivity.

All experiments were conducted under standardized environmental conditions, and the apparatus was cleaned between trials to eliminate residual olfactory cues. Behavioral evaluation was performed by an observer blinded to the experimental groups.

*Forced swim test*

Stress-related behavioral responses were evaluated using the forced swim paradigm. Animals were individually placed in a transparent cylindrical container containing water maintained at a controlled temperature (25 ± 1°C) and of sufficient depth to prevent contact with the bottom, thereby ensuring continuous swimming or floating activity. The duration of each testing session was 5 minutes.

The primary parameter recorded was the total time of immobility, defined as the minimal movement required to maintain the head above the water surface. This parameter was used as an indicator of stress-coping behavior rather than a direct measure of behavioral despair.

The force swim test was used in this study to assess stress-coping strategies (19, 20) and not as a direct

model of behavioral despair that is widely debated in the behavioral neuroscience literature. Moreover, this test is included as part of a multidimensional neurobehavioral battery, together with the open field test, elevated plus maze, and rotarod test. In this context, the force swim test provides additional information regarding stress-related behavioral adaptation, which may be altered following chronic developmental exposure to herbicides.

All experiments were conducted under standardized environmental conditions, and the apparatus was cleaned between trials to eliminate residual olfactory cues. Behavioral evaluation was performed by an observer blinded to the experimental groups.

*Rotarod test*

Motor coordination, balance, and motor performance were assessed using the rotarod test. The apparatus consisted of a horizontally rotating cylindrical rod (approximately 6 cm in diameter) elevated about 30 cm above the base platform and divided into individual lanes to allow simultaneous testing of multiple animals while preventing interaction between them. Prior to behavioral evaluation, animals underwent three consecutive days of training sessions to familiarize them with the apparatus. During the training phase, the rod was operated at a constant speed of 30 rpm/min. Following the training period, the rotarod test was performed. Each animal was placed on the rotating rod, and the latency to fall from the rod was recorded as an indicator of motor coordination and balance. Animals were subjected to three consecutive trials, and the latency to fall was measured separately for the first, second, and third trials. An inter-trial interval of approximately 10 - 15 minutes was allowed between trials to minimize fatigue. Differences in latency between trials were used to evaluate motor coordination and motor learning, while body weight was also recorded to account for potential influences on motor performance.

*Histopathological examination of brain tissue*

At the end of the exposure period, animals were anesthetized using a ketamine–xylazine combination, followed by euthanasia through exsanguination. Brain tissues were promptly collected, gently cleared of residual blood, and fixed in buffered formalin to preserve structural integrity. Subsequently, samples were processed using standard paraffin-embedding procedures.

Brain weight was recorded and expressed relative to body weight (g *per* 100 g body weight) to allow normalization across experimental groups.

Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin (H&E) for general morphological assessment. Microscopic examination was performed using light microscopy, with particular attention to brain regions involved in behavioral regulation. For semi-quantitative evaluation, a systematic sampling approach was applied, analyzing five non-overlapping high-power fields (×400) *per* section. The mean score *per* sample was calculated to ensure a representative assessment. Structural changes, including neuronal degeneration, vascular alterations, and tissue disorganization, were documented.

Histopathological evaluation was conducted independently by two experienced pathologists blinded to the experimental groups. In cases of discordance, a consensus diagnosis was established through joint review. Lesions were classified according to standardized diagnostic criteria consistent with INHAND guidelines for rodent nervous system pathology (21). The severity of histopathological alterations was graded using a semi-quantitative scoring system (22) as follows: absent (–), mild (+), moderate (++), and severe (+++), based on the extent and intensity of the observed changes.

*Oxidative stress assessment*

*Tissue processing*

Brain oxidative status was investigated by quantifying a panel of redox-related biomarkers using spectrophotometric techniques. Brain samples were harvested from offspring (n = 10 *per* sex), immediately rinsed in cold isotonic saline to eliminate residual blood, rapidly cryopreserved in liquid nitrogen, and maintained at -80°C until analysis.

For sample preparation, brain tissue was accurately weighed and homogenized in phosphate-buffered saline (PBS) supplemented with protease inhibitors (Roche cOmplete™ Protease Inhibitor Cocktail), using a 1:4 (w/v) ratio. Mechanical disruption was performed with a Minilys Personal Homogenizer (Bertin Technologies) at maximum speed for 30 s. The homogenates were subsequently clarified by centrifugation (15,000 × g, 5 min, 5°C), and the resulting supernatants were collected for biochemical assays and stored at -80°C.

*Determination of oxidative stress biomarkers*

Protein content in the samples was quantified using the Bradford colorimetric method with bovine serum albumin as a calibration standard (23).

Reduced glutathione (GSH) levels were assessed based on the 5,5-dithiobis-2-nitrobenzoate (DTNB) reaction, following the procedure described by Reddy *et al*. (24) with later modifications (25). In brief, proteins were precipitated using trichloroacetic acid (TCA), and after centrifugation, the supernatant was reacted with DTNB in phosphate buffer. Following incubation under light-protected conditions, absorbance was measured at 412 nm, and GSH concentrations were calculated using the molar absorptivity of the 5-thio-2-nitrobenzoic acid (TNB) chromophore.

Catalase activity was indirectly evaluated by monitoring hydrogen peroxide degradation, according to Aebi (26), as previously applied (25). Tissue homogenate was incubated in phosphate buffer at physiological temperature, and the reaction was initiated by

adding hydrogen peroxide. The decrease in absorbance at 240 nm was continuously recorded, and the rate of decomposition was calculated using the corresponding extinction coefficient.

Total antioxidant capacity (TAC) was determined through a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, following the methodology of Janaszewska and Bartosz (27). Diluted homogenates were incubated with DPPH solution in buffered conditions, protected from light. After incubation and centrifugation, absorbance was measured at 520 nm, and results were expressed as the amount of DPPH radical reduced by endogenous antioxidants.

Lipid peroxidation was estimated by quantifying thiobarbituric acid reactive substances (TBARS), using a modified protocol derived from Keles *et al*. (28) and further adapted (29). Briefly, homogenates were subjected to acidic conditions and heat in the presence of thiobarbituric acid, promoting the formation of a chromogenic complex. After cooling and removal of precipitated material, absorbance was recorded at 530 nm, and TBARS concentrations were calculated using the molar extinction coefficient of malondialdehyde.

Protein carbonyl content (CARBS), as an indicator of protein oxidation, was measured using a 2,4-dinitrophenylhydrazine (DNPH)-based derivatization assay (25, 30). Protein fractions were initially precipitated and subsequently reacted with DNPH under acidic conditions. After incubation in the dark and multiple washing steps to remove excess reagent, the derivatized proteins were solubilized, and absorbance was measured at 370 nm. Carbonyl levels were calculated using the appropriate extinction coefficient.

*Statistical analysis*

Data analysis was performed using dedicated statistical software packages. Neurobehavioral outcomes were processed using STATA (StataCorp, USA), while biochemical parameters related to oxidative stress were analyzed using GraphPad Prism (version 8.0.1).

Prior to inferential testing, datasets were examined for distribution characteristics and homogeneity of variance to confirm the applicability of parametric methods. Group comparisons were conducted using one-way analysis of variance (ANOVA), applied independently for each evaluated parameter. When significant differences were identified, post hoc comparisons were carried out using Dunnett's test to assess treatment effects relative to the control group. For oxidative stress biomarkers, multiple group comparisons were performed using ANOVA followed by Tukey's post hoc test, with analyses conducted separately for male and female subgroups.

For behavioral assessments, cumulative indices were generated at the individual level by integrating the total number of recorded events across the entire 5-minute testing interval. These included locomotor and exploratory parameters, as well as grooming activity. Variability within groups was expressed based on individual animal responses.

All results are presented as mean ± standard deviation (SD) for behavioral data and mean ± standard error of the mean (SEM) for biochemical parameters. Statistical significance was defined as a probability value of less than 0.05.

*Ethical approval*

All experimental procedures involving animals were conducted in accordance with the ARRIVE guidelines for reporting animal research (31). The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania. All efforts were made to minimize animal suffering and to reduce the number of animals used.

## Results and Discussion

*Neurobehavioral test results*

*Open field test*

*Ambulation parameters evaluation*

Alterations in locomotor and exploratory behavior were observed in the open field test, with more pronounced effects in female offspring (Table I). The latency to leave the central zone was significantly reduced in females from the ADI and Mixture groups ($p < 0.01$), indicating an increased tendency toward rapid exploratory activity. Exploratory locomotion, assessed by central zone crossings, showed a dose-dependent reduction in females, with significant decreases observed in the ADI ($p < 0.05$) and NOAEL ($p < 0.01$) groups compared with controls. This reduction was more pronounced in the Mixture group ($p < 0.01$), suggesting a possible additive effect of the herbicides when administered together. Analysis of individual time intervals revealed a similar pattern, with significantly fewer central zone crossings recorded during the first, second, third, and fifth minutes in females from ADI, NOAEL, and Mixture groups compared with controls ($p < 0.05$) (Table I).

Peripheral locomotion, measured as peripheral square crossings, was significantly decreased in females from the NOAEL group over the entire 5-min session ($p < 0.01$). Minute-by-minute analysis showed a comparable reduction during the first, second, and fourth minutes of testing ($p < 0.05$). In contrast, females from the ADI group displayed a transient increase in peripheral zone crossings during the first minute ($p < 0.05$), which remained evident when the cumulative 5-min values were analyzed ($p < 0.05$). A similar increase in peripheral zone crossings was also observed in males from the ADI group, both during the first minute ($p < 0.05$) and across the entire testing period ($p < 0.05$) (Table I).

Vertical exploratory activity, assessed by rearing frequency, was significantly reduced in females from the NOAEL group during the first ($p < 0.01$), second

($p < 0.01$), and fifth minutes ($p < 0.05$), as well as for the total 5-min session ($p < 0.05$). In males, a significant reduction in rearing was observed in the NOAEL group during the third minute ($p < 0.05$). When cumulative values were considered, total rearing frequency decreased in a dose-dependent manner in females from the ADI and NOAEL groups ($p < 0.05$) (Table I).

*Emotional state evaluation*

Behavioral indicators associated with emotional reactivity were also affected by herbicide exposure. Total grooming behavior was significantly reduced in females from the NOAEL ($p < 0.05$) and from the Mixture ($p < 0.05$) groups. In contrast, males from the Mixture group exhibited increased grooming activity during the first and second minutes of testing (Table I).

Stress-related autonomic responses, evaluated by defecation (number of fecal boluses), were significantly elevated in both female and male offspring from the Mixture group ($p < 0.01$), suggesting enhanced stress responsiveness following exposure to the herbicide combination (Table I).

**Table I**

Open Field test results in male and female rats exposed from GD6 till PND111 to glyphosate (ADI and NOAEL groups) and glyphosate/2,4-D/dicamba mixture (Mixture group)

| Variable Definition | Control | | ADI | | NOAEL | | Mixture | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | |
| | Female | Male | Female | Male | Female | Male | Female | Male |
| **LOCOMOTOR ACTIVITY** | | | | | | | | |
| Latency to leave from the central zone | 4.5 ± 2.9 | 9 ± 4.9 | 0.3 ± 0.7** | 4.6 ± 3.8 | 6.3 ± 4.1 | 4.2 ± 4.7 | 0.2 ± 0.4** | 10.4 ± 8.3 |
| No crossing over central zone squares - min 1 | 5 ± 1.5 | 5.3 ± 1.8 | 1.7 ± 0.8** | 3.3 ± 1.2 | 2.8 ± 1.9* | 4.1 ± 0.9 | 2.2 ± 1.1** | 2.3 ± 1.1** |
| Number of crossings over central zone squares – min 2 | 2.5 ± 0.9 | 0.7 ± 0.7 | 0.7 ± 1.3** | 0.5 ± 0.5 | 0.3 ± 0.5** | 0.5 ± 1.3 | 0.4 ± 0.5** | 0.3 ± 0.7 |
| Number crossings over central zone squares – min 3 | 2 ± 1.2 | 0.6 ± 0.8 | 0.7 ± 1.1* | 2 ± 3 | 0.2 ± 0.4** | 0 ± 0 | 0.2 ± 0.4** | 0.9 ± 1.4 |
| Number crossings over central zone squares – min 4 | 1.5 ± 0.7 | 0.2 ± 0.4 | 3.2 ± 2.5 | 0.75 ± 0.9 | 0.8 ± 1.5 | 0 ± 0 | 0.3 ± 0.5* | 0 ± 0 |
| Number crossings over central zone squares – min 5 | 1.7 ± 0.8 | 0.1 ± 0.3 | 0 ± 0** | 0.5 ± 0.9 | 0.5 ± 0.9** | 0.4 ± 1.3 | 0 ± 0** | 0 ± 0 |
| Number crossings over central zone squares – all 5 min | 12.7 ± 2.4 | 6.9 ± 2.8 | 6.3 ± 4.7* | 7 ± 3.6 | 4.6 ± 2.6** | 5 ± 1.9 | 3.1 ± 1.3** | 3.5 ± 2.0* |
| Number crossings over peripheral zone squares - min 1 | 22.3 ± 2.5 | 7 ± 3.9 | 27.2 ± 2.3* | 22.6 ± 7.4** | 13.1 ± 6.5* | 9 ± 5.2 | 20.8 ± 3.7 | 15.4 ± 8.1* |
| Number crossings over peripheral zone squares – min 2 | 27.5 ± 3.4 | 27 ± 8.3 | 21.6 ± 4.1* | 20.3 ± 9.1 | 17 ± 6.1** | 13.3 ± 8.9** | 21.4 ± 9.3 | 13.4 ± 7.8** |
| Number crossings over peripheral zone squares – min 3 | 18.1 ± 1.9 | 19.1 ± 6 | 18.5 ± 5.3 | 17.4 ± 6.6 | 12.5 ± 5.2 | 10.1 ± 8.3 | 19.7 ± 8.8 | 11.2 ± 6.6 |
| Number crossings over peripheral zone squares – min 4 | 26.7 ± 3.2 | 12.8 ± 4.6 | 22.1 ± 3.5 | 18.375 ± 8.1 | 14.1 ± 7.2** | 16.6 ± 9.5 | 15.4 ± 7.7** | 13.3 ± 6.2 |
| Number crossings over peripheral zone squares – min 5 | 14.6 ± 3.1 | 11 ± 4.1 | 20.1 ± 2.8* | 17.3 ± 7.6* | 14.9 ± 6.8 | 12.7 ± 8.6 | 18.4 ± 4.9 | 15.2 ± 4.2 |
| Number crossings over peripheral zone squares – all 5 min | 109.2 ± 8.1 | 76.9 ± 20.3 | 109.5 ± 6.8 | 95.9 ± 34.3 | 71.6 ± 26.5** | 61.7 ± 32.5 | 95.7 ± 24.1 | 68.5 ± 25.7 |
| Number rearing movements– min 1 | 8.8 ± 1.3 | 3.3 ± 2.1 | 6.9 ± 3.2 | 5.5 ± 2 | 3 ± 1.9** | 4.5 ± 1.6 | 6.5 ± 1.7 | 3.6 ± 1.8 |
| Number rearing movements – min 2 | 9.1 ± 1.2 | 6 ± 3.8 | 6.3 ± 1.3* | 4.375 ± 2.2 | 2.5 ± 1.4** | 4.4 ± 1.9 | 6.4 ± 2.7 | 5.3 ± 2.5 |
| Number rearing movements – min 3 | 3.5 ± 1.4 | 5.4 ± 1.4 | 4.2 ± 1.7 | 3 ± 0.7 | 2.7 ± 1.3 | 2.4 ± 1.5** | 6.4 ± 3.1* | 3.7 ± 2.4 |
| Number of rearing movements – min 4 | 4.9 ± 1.2 | 3.5 ± 1.7 | 3.1 ± 1.7 | 3.5 ± 1.8 | 3.9 ± 1.9 | 2.3 ± 2.1 | 5.1 ± 1.5 | 3.6 ± 1.6 |
| Number rearing movements – min 5 | 4.8 ± 1.7 | 2.5 ± 1.2 | 3 ± 1.2 | 4.5 ± 2.7 | 2.6 ± 1.9* | 2.4 ± 1.4 | 4.5 ± 1.8 | 4.1 ± 2.8 |
| Number rearing movements – in all 5 min | 31.1 ± 2.4 | 20.7 ± 7.2 | 23.5 ± 7.1* | 20.9 ± 6.6 | 14.7 ± 6.4* | 16 ± 3.5 | 28.9 ± 8.9 | 20.3 ± 8.3 |
| **STRESS LEVEL** | | | | | | | | |

| Variable Definition | Control | | ADI | | NOAEL | | Mixture | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | |
| | Female | Male | Female | Male | Female | Male | Female | Male |
| Number grooming activities - min 1 | 0.5 ± 0.5 | 0 ± 0 | 0.1 ± 0.3 | 0 ± 0 | 0.1 ± 0.3 | 0.2 ± 0.4 | 0.2 ± 0.4 | 0.4 ± 0.5* |
| Number of grooming activities - min 2 | 1.2 ± 0.6 | 0 ± 0 | 0.3 ± 0.5* | 1.25 ± 0.5** | 0.3 ± 0.7** | 0.4 ± 0.5 | 0.6 ± 0.7 | 0.7 ± 0.7* |
| Number of grooming activities - min 3 | 0.4 ± 0.5 | 0.6 ± 0.8 | 0.3 ± 0.5 | 0.25 ± 0.5 | 0.6 ± 0.5 | 1.3 ± 1.4 | 0.6 ± 0.5 | 0.9 ± 0.7 |
| Number of grooming activities - min 4 | 0.5 ± 0.5 | 0.4 ± 0.5 | 0.6 ± 0.5 | 0.5 ± 0.8 | 0.6 ± 0.5 | 0.5 ± 0.7 | 0 ± 0 | 0.2 ± 0.4 |
| Number of grooming activities - min 5 | 0.9 ± 0.9 | 0.8 ± 0.9 | 0.6 ± 0.7 | 0.4 ± 0.5 | 0.4 ± 0.5 | 0.6 ± 0.7 | 0.7 ± 0.8 | 0.6 ± 0.8 |
| Number of grooming activities – in all 5 min | 3.5 ± 1.8 | 1.8 ± 2.1 | 1.9 ± 0.9 | 2.4 ± 1.1 | 2 ± 0.7* | 3 ± 1.6 | 2.1 ± 0.9* | 2.8 ± 1.9 |
| Number of fecal boluses | 0.3 ± 0.5 | 0 ± 0 | 1 ± 1.5 | 1.75 ± 2.1 | 0.6 ± 0.7 | 2.3 ± 2.9 | 3.6 ± 1.6** | 4.2 ± 2.4** |

Statistically significant differences between the exposed and control groups are indicated in bold. *p < 0.05; **p < 0.01 compared with controls (n = 10 males/females *per* group) (Dunnett's test).

*Elevated Plus Maze test*

*Ambulation parameter evaluation*

Behavioral performance in the elevated plus maze revealed significant treatment-related alterations in exploratory activity (Table II). The frequency of rearing behavior was significantly reduced in males from the NOAEL group ($p < 0.01$), whereas females from the Mixture group showed an increase in rearing activity ($p < 0.01$) compared to the control group.

Similarly, bending movements, another indicator of exploratory activity, were significantly reduced in males in a dose-dependent manner, with significant decreases observed in the ADI ($p < 0.05$) and the NOAEL ($p < 0.05$) groups relative to controls (Table II).

*Emotional state evaluation*

Exposure-related changes were also observed in parameters associated with emotional reactivity and anxiety-like behavior. The time spent in the open arms was significantly reduced in males from the NOAEL ($p < 0.01$) group and in females from the Mixture group ($p < 0.05$) compared with controls (Table II). Alterations in grooming behavior were also evident. Grooming frequency increased significantly in females from the NOAEL group ($p < 0.05$) and was markedly elevated in both females and males from the Mixture group ($p < 0.01$) (Table II).

Consistent with these findings, males from the NOAEL group ($p < 0.01$) and females from the Mixture group ($p < 0.05$) spent significantly more time in the closed arms (dark compartment) of the maze compared with controls. In contrast, females from the ADI group exhibited a significant reduction in the time spent in the closed arms ($p < 0.05$) (Table II).

**Table II**

Elevated Plus Maze test results in male and female rats exposed from GD6 till PND111 to glyphosate (ADI and NOAEL groups) and glyphosate/2,4-D/dicamba mixture (Mixture group)

| | Control | | ADI | | NOAEL | | Mixture | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | |
| | Female | Male | Female | Male | Female | Male | Female | Male |
| **EXPLORATORY ACTIVITY** | | | | | | | | |
| Number of bending movements | 4 ± 0.8 | 2.4 ± 1.3 | 2.5 ± 1.6 | 0.7 ± 0.9* | 2.8 ± 1.8 | 0.4 ± 0.5** | 1.5 ± 3.1 | 1.9 ± 1.3 |
| Number of rearing movements | 7.6 ± 2.1 | 6.8 ± 1.9 | 9.8 ± 1.4 | 6.125 ± 1.5 | 10.1 ± 1.4 | 2.3 ± 1.4** | 12.1 ± 4.2** | 10 ± 2.8 |
| **STRESS LEVEL** | | | | | | | | |
| Time spent in the open arms (seconds) | 18.4 ± 7.6 | 18.6 ± 6.0 | 32.9 ± 20.9 | 17.125 ± 2.9 | 8.1 ± 8.9 | 0.9 ± 1.3** | 4.8 ± 10.7* | 12.6 ± 15.2 |
| Time spent in the center (seconds) | 14.2 ± 6.7 | 12.6 ± 2.9 | 29.6 ± 20.4 | 14.9 ± 3.4 | 18.4 ± 7.1 | 7.1 ± 6.9 | 3 ± 6.1 | 10.9 ± 12.2 |
| Number of grooming activities | 1.3 ± 1.6 | 2.1 ± 1.7 | 1.6 ± 0.7 | 2.125 ± 1 | 3.7 ± 1.2* | 2.8 ± 3.2 | 8.1 ± 3.6** | 5.7 ± 2.4** |
| Time spent in the closed arms (dark compartment) | 264.5 ± 11.9 | 268.8 ± 8.1 | 239 ± 13.1* | 268 ± 2.1 | 273.5 ± 12.1 | 292 ± 6.7** | 292.2 ± 16.4* | 270.1 ± 28.8 |

Statistically significant differences between the exposure and control groups are indicated in bold. *p < 0.05; **p < 0.01 compared with controls (n = 10 males/females *per* group) (Dunnett's test).

*Force Swim test* *Emotional state evaluation*

In the forced swim test, the duration of immobility was significantly increased in males from the NOAEL males ($p < 0.05$) compared to controls (Table III). No significant differences were observed in females or in animals from the Mixture group (Table III).

**Table III**

Force Swim test results in male and female rats exposed from GD6 till PND111 to glyphosate (ADI and NOAEL groups) and glyphosate/2,4-D/dicamba mixture (Mixture group)

| | **Control** | | **ADI** | | **NOAEL** | | **Mixture** | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | |
| | Female | Male | Female | Male | Female | Male | Female | Male |
| Time of immobility | 84.8 ± 18.5 | 76.7 ± 18.8 | 84.4 ± 15.9 | 75.75 ± 13.6 | 97.6 ± 16.5 | 103 ± 16.8* | 82.6 ± 26.6 | 70.4 ± 18.9 |

Statistically significant differences between exposure and control groups are indicated in bold. *p < 0.05; **p < 0.01 compared with controls (n = 10 males/females *per* group) (Dunnett's test).

*Rotarod test*

*Ambulation parameters evaluation*

Performance in the rotarod test revealed sex- and dose-dependent differences in motor coordination and balance (Table IV). In females, exposure to the NOAEL-glyphosate dose resulted in a significant reduction in latency to fall during the first (T1; $p < 0.01$) and second (T2; $p < 0.05$) trials compared with controls (Table IV).

In contrast, males from the herbicide Mixture group exhibited a significant increase in latency to fall across all testing sessions (T1 - T3; $p < 0.01$) relative to the control group. Additionally, body weight gain (G0 - G3) was significantly greater in males exposed to the herbicide Mixture ($p < 0.01$) (Table IV).

**Table IV**

Rotarod test results in male and female rats exposed from GD6 till PND111 to the ADI-glyphosate, NOAEL-glyphosate, and glyphosate/2,4-D/dicamba Mixture

| | **Control** | | **ADI** | | **NOAEL** | | **Mixture** | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | | Mean ± Std. Dev. | |
| | Female | Male | Female | Male | Female | Male | Female | Male |
| Rotarod Latency to fall T1 | 107.4 ± 12.2 | 68.5 ± 12.2 | 91.6 ± 17.9 | 87.25 ± 25.5 | 79.1 ± 6.5** | 67.1 ± 12.5 | 106.7 ± 6.1 | 123.3 ± 12.2** |
| Rotarod Latency to fall T2 | 108.8 ± 11.4 | 74 ± 16.8 | 102.1 ± 20.2 | 102.125 ± 21.4* | 89.5 ± 12.4* | 72.2 ± 13.6 | 108.6 ± 4.8 | 117.3 ± 16** |
| Rotarod Latency to fall T3 | 107.8 ± 14.1 | 81.8 ± 17.4 | 108.5 ± 16 | 107.5 ± 19.9 | 111.3 ± 14.4 | 76.2 ± 13.2 | 113.9 ± 6.7 | 124.9 ± 21.8** |
| Rotarod Weight difference G0-G3 | 1.7 ± 0.6 | 1.4 ± 0.5 | 1.5 ± 0.7 | 1.87 ± 0.6 | 1.5 ± 0.5 | 1.2 ± 0.4 | 2.4 ± 0.5 | 2.4 ± 0.5** |

Statistically significant differences between exposure and control groups are indicated in bold. *p < 0.05; **p < 0.01 compared with controls (n = 10 males/females *per* group) (Dunnett's test).

*Histopathological evaluation of brain tissue in male and female rats*

Relative brain weight did not differ significantly between the control and exposure groups.

Histopathological evaluation was performed on brain tissue sections from 10 female and 10 male rats from each control and herbicide exposure group. The examined samples came from four experimental groups with the main pathologies being inflammation, hyperaemia, or neuronal cell degeneration.

*Histopathological evaluation of female brain tissue*

Histopathological evaluation of H&E-stained brain sections revealed clear differences in the incidence and severity of lesions among the experimental groups (Table V). No histopathological alterations were observed in female animals from the control group, where neuronal morphology, vascular structures, and inflammatory status appeared normal in all examined specimens (0 out of 10 specimens) (Figure 1A; Table V).

In females from the ADI group, only mild alterations were detected. Focal neuronal degenerative changes were observed (6 out of 10 specimens), characterized by neurons of reduced size compared with those in the control group and graded as moderate (++) severity. The predominant alterations involved the vascular compartment, with enlarged and hyperemic vessels representing the most common finding (6 out of 10 specimens) and graded with moderate intensity (++). In addition, chronic inflammatory infiltrates were identified but were limited to focal areas, occurring either within the brain parenchyma or in perivascular regions (3 out of 10 specimens) and graded as mild (+) severity. Overall, the lesions were mild and focal in distribution (Figure 1B, Table V).

In the NOAEL group, the lesions observed in female brain tissue were similar to those observed in the

ADI group, but were more pronounced in severity. Neuronal degeneration was more extensive (6 out of 10) and was frequently associated with perineuronal oedema and graded as moderate intensity (++). Vascular hyperemia was slightly more frequent compared with the ADI group (7 out of 10 specimens) and graded as moderate (++) severity. Chronic inflammatory infiltrates were maintained and showed a tendency toward a more diffuse distribution compared with the ADI group (2 out of 10 specimens) and remained mild in severity (+) (Figure 1C, Table V).

In females from the herbicide Mixture group, more severe histopathological alterations were evident. Neuronal degeneration was widespread throughout the brain parenchyma (detected in 8 out of 10 specimens) and graded as moderate severity (++) and was focally associated with inflammatory infiltrates (5 out of 10 specimens) and graded as moderate (++) severity. Vascular congestion was more pronounced and diffusely distributed (8 out of 10 specimens) and graded as intense (+++) severity, with enlarged vessels and occasional deposition of hyaline material within the vascular walls (Figure 1D, Table V).

Overall, these findings indicate a progressive increase in the extent and severity of histopathological alterations across the exposure groups, with the most pronounced lesions observed in the Mixture group, primarily affecting neuronal integrity and the cerebral vascular compartment.

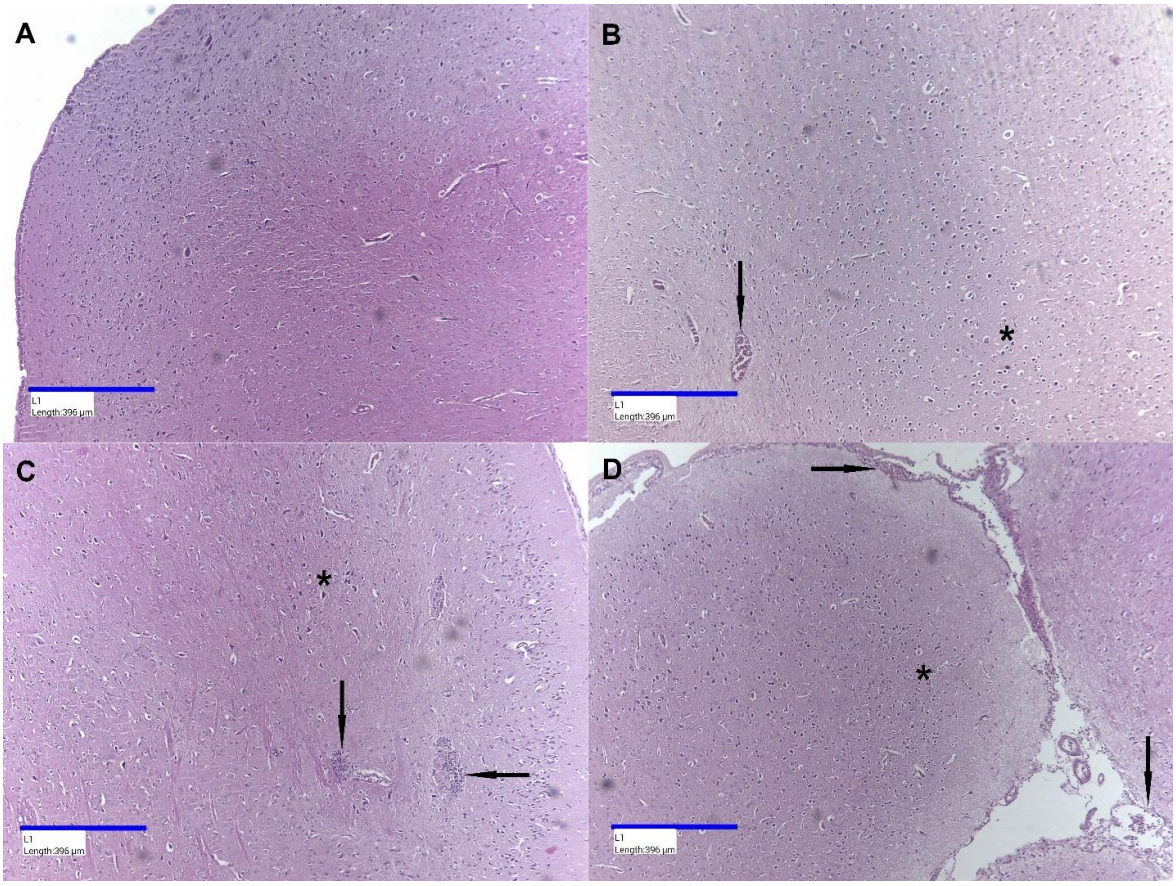


**Figure 1.**

Histopathological analysis of female rat brain tissue by H&E staining following exposure to glyphosate alone (ADI and NOAEL groups) and the glyphosate/2,4-D/dicamba herbicide mixture (Mixture group)

Animals were euthanized and tissues analysed at the end of the exposure period (PND111). A: Control group: normal architecture. B: ADI group: neuronal degeneration -black asterix, vascular hyperemia -black arrow. C: NOAEL group: neuronal degeneration -black asterix, chronic inflammation-black arrow. D: Mixture group: neuronal degeneration -black asterix, enlarged vessels with hyperemia -black arrow. Magnification: x100.

**Table V**

Incidence and severity of brain histopathological alterations in female rats from control and herbicide exposure groups determined by microscopic evaluation of H&E-stained tissue sections. Brain tissue was excised and analysed at the end of the period of exposure (PND111) to the glyphosate (ADI and NOAEL groups) and mixture of glyphosate/2,4-D/dicamba (Mixture group)

| Parameters | Female experimental groups | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n = 10) | | ADI (n = 10) | | NOAEL (n = 10) | | Mixture (n = 10) | |
| | Intensity | Specimens Number | Intensity | Specimens Number | Intensity | Specimens Number | Intensity | Specimens Number |
| Neuronal cell degeneration | - | 0/10 | ++ | 6/10 | ++ | 6/10 | ++ | 8/10 |
| Inflammation | - | 0/10 | + | 3/10 | + | 2/10 | ++ | 5/10 |
| Vascular congestion | - | 0/10 | ++ | 6/10 | ++ | 7/10 | +++ | 8/10 |

Pathological severity scoring: (-) none, (+) mild, (++) moderate, (+++) intense

*Histopathological evaluation of male brain tissue*

Histopathological examination of H&E-stained brain sections from male animals revealed normal brain architecture in the control group, with no evidence of neuronal degeneration, inflammation, or vascular congestion (0 out of 10 specimens for all evaluated parameters) (Figure 2A, Table VI).

In the male from the ADI group, neuronal degeneration was observed in 4 out of 10 specimens and was graded as mild (+) severity (Figure 2B, Table VI), and was less evident in comparison with the females from the same exposure group. Inflammatory changes were not detected in this group (0 out of 10 specimens) (Table VI). The most notable alteration involved the vascular compartment, where vascular congestion was observed in 6 out of 10 specimens and graded as moderate intensity (++), characterized by vessels distended and packed with erythrocytes.

In males from the NOAEL group, neuronal degeneration became more frequent and severe, being detected in 7 out of 10 specimens and rated as moderate intensity (++) (Figure 2C and Table VI). Inflammatory infiltrates were not observed in this group (0 out of 10 specimens). Vascular congestion remained a consistent finding, present in 6 out of 10 specimens and graded as moderate (++) severity, with enlarged vessels occasionally showing luminal accumulation of neutrophils (Figure 2C and Table VI).

In males from the Mixture group, neuronal degeneration with changes in shapes and sizes was detected in 7 out of 10 specimens and was graded as moderate (++) severity (Figure 2D, Table VI). In contrast to the other exposure groups, inflammatory changes were also present, being observed in 4 out of 10 specimens and graded as moderate intensity (++), appearing as focal intraparenchymal inflammatory infiltrates (Figure 2D, Table VI). Vascular congestion persisted and was the most frequent alteration, occurring in 8 out of 10 specimens and graded as moderate (++) severity, with widespread vascular dilation (Figure 2D, Table VI).

Overall, the histopathological findings in male rats demonstrate an increase in the incidence and severity of neuronal and vascular alterations across the exposure groups, with the Mixture group showing the most consistent combination of neuronal degeneration, inflammation, and vascular congestion.

**Table VI**

Incidence and severity of brain histopathological alterations in male rats from control and herbicide exposure groups were determined by microscopic evaluation of H&E-stained tissue sections. Brain tissue was excised and analyzed at the end of the period of exposure (PND111) to the glyphosate (ADI and NOAEL groups) and mixture of glyphosate/2,4-D/dicamba (Mixture group)

| Parameters | Male experimental groups | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n = 10) | | ADI (n = 10) | | NOAEL (n = 10) | | Mixture (n = 10) | |
| | Intensity | Specimens Number | Intensity | Specimens Number | Intensity | Specimens Number | Intensity | Specimens Number |
| neuronal cell degeneration | - | 0/10 | + | 4/10 | ++ | 7/10 | ++ | 7/10 |
| inflammation | - | 0/10 | - | 0/10 | - | 0/10 | ++ | 4/10 |
| vascular congestion | - | 0/10 | ++ | 6/10 | ++ | 6/10 | ++ | 8/10 |

Pathological severity scoring: (-) none, (+) mild, (++) moderate, (+++) intense.

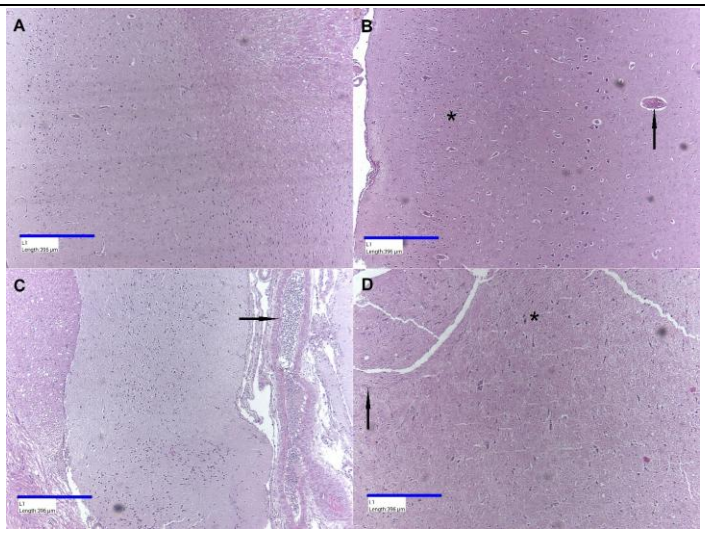


**Figure 2.**

Histopathological analysis of male rat brain tissue by H&E staining following exposure to glyphosate alone (ADI AND NOAEL groups) and the glyphosate/2,4-D/dicamba herbicide mixture (Mixture group)

Animals were euthanised and tissues analysed at the end of the exposure period (PND111). A: Control group: normal architecture. B: ADI group: neuronal degeneration -black asterix, vascular hyperemia -black arrow. C: NOAEL group: enlarged vessels with neutrophils -black arrow. D: Mixture group: neuronal degeneration-black asterix, acute inflammation-black arrow. Magnification: x100.

*Oxidative stress evaluation*

We assessed the redox status of the brain tissues, as this could provide mechanistic insight into the observed pathological outcomes. The results for oxidative stress responses in female and male rats are provided in Figures 3 and 4, respectively.

A statistically significant 50% increase in reduced GSH levels was observed in females following exposure to the herbicide Mixture compared to controls, as well as a 56% increase relative to the ADI group (Figure 3A). A slight but statistically significant increase in lipid peroxidation levels (TBARS) was also detected in the same set of animals between all other female groups, showing a 30% increase compared to controls, 22% compared to ADI, and 23% compared to the NOAEL group (Figure 3D). No significant differences were observed in $H_2O_2$ decomposition rate, TAC, and CARBS levels (Figures 3B-C-E).

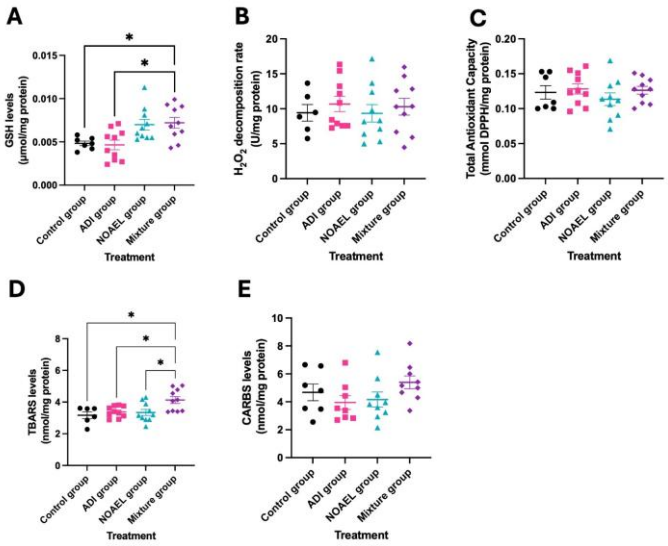


**Figure 3.**

Effects on redox biomarkers in female rat brain tissue following exposure to glyphosate alone (ADI and NOAEL groups) and the glyphosate/2,4-D/dicamba herbicide mixture (Mixture group)

A: GSH concentration, B: $H_2O_2$ decomposition rate, C: TAC levels, D: TBARS levels, E: CARBS levels. *: Statistically significant difference compared to the control group, the ADI-glyphosate group, and the NOAEL-glyphosate group ($p < 0.05$)

Although no significant antioxidant adaptations were observed in male rats (Figures 4A-C), the levels of oxidative damage indicators were elevated in the NOAEL and Mixture groups. Specifically, the most pronounced toxic effects in males were observed in the NOAEL group, causing an increase by 50% and 56% in TBARS and CARBS levels, respectively, in comparison to the control group (Figures 4D, E). Interestingly, exposure to the herbicide Mixture also induced a 45% increase in TBARS levels in males relative to the untreated group (Figure 4D).

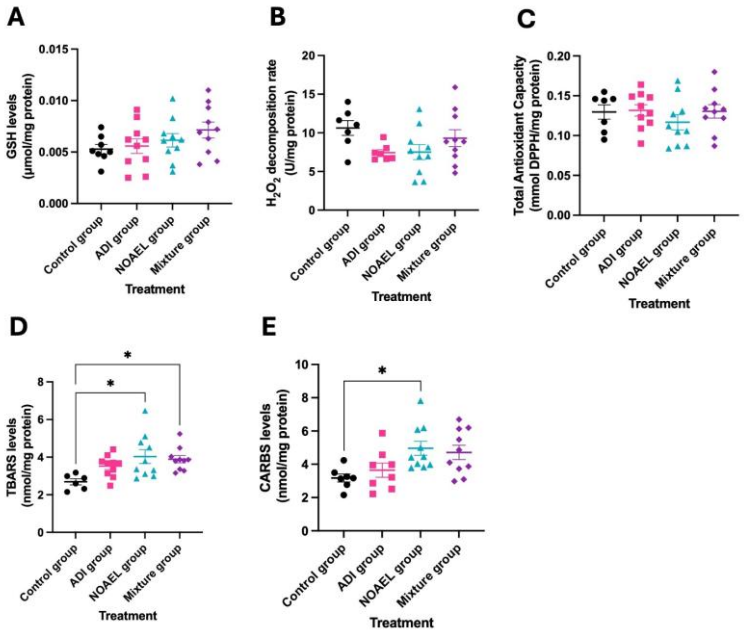


**Figure 4.**

Effects on redox biomarkers in male rat brain tissue following exposure to glyphosate alone (ADI and NOAEL groups) and the glyphosate/2,4-D/dicamba herbicide mixture (Mixture group)

A: GSH concentration, B: $H_2O_2$ decomposition rate, C: TAC levels, D: TBARS levels, E: CARBS levels. *: Statistically significant difference compared to the control group ($p < 0.05$).

Taken together, these observations suggest that exposure to NOAEL-glyphosate and herbicide Mixture is associated with detrimental effects of oxidative stress in males, as indicated by the elevated levels of lipid and protein oxidation. Contrastingly, exposure of females to the herbicide Mixture induced lipid peroxidation, along with an increase in GSH pools, suggesting an attempted compensation response to oxidative damage to cell membrane lipids.

The present study investigated the neurobehavioral and neuropathological consequences of prolonged developmental exposure to glyphosate administered at the EU ADI and NOAEL, as well as to a mixture of glyphosate, dicamba, and 2,4-D at their respective EU ADI-equivalent doses. Because exposure extended from GD6 through early adulthood, it overlapped with critical processes of brain maturation, including neuronal proliferation, synaptogenesis, and circuit refinement (32). Within this framework, the present novel findings indicate that these herbicide active ingredients at doses anchored to current regulatory reference values can still produce measurable neurobehavioral and histopathological alterations when exposure occurs during vulnerable developmental windows. Our results corroborate previous observations involving glyphosate and GBHs. Cattani *et al*. demonstrated that developmental exposure to GBHs during both prenatal and early postnatal stages, at doses below those associated with maternal toxicity, can induce oxidative stress and disrupt neurotransmission within the hippocampus of rat offspring. In particular, alterations in both cholinergic and glutamatergic signaling pathways were reported in immature as well as adult animals. These findings suggest that early-life GBH exposure may interfere with hippocampal neurochemical homeostasis and produce persistent neurobiological alterations that extend into later stages of life (33). Cresto *et al*. reported that continuous exposure to glyphosate at doses corresponding to the ADI and NOAEL levels, from gestation until postnatal day 60 in C57BL/6J mice, induced synaptic alterations and adaptive responses in microglial cells. These effects were particularly evident in male offspring, suggesting a sex-dependent susceptibility to developmental glyphosate exposure (34).

A central finding of the study is that glyphosate alone was not inert in inducing behavioral changes, even at the EU ADI dose. Although the changes at the ADI-glyphosate dose were less pronounced than

those observed at the NOAEL-glyphosate, they still indicate altered exploratory patterning. In the open field test (Table I), ADI-glyphosate-exposed animals showed changes in latency to leave the center and in peripheral activity, while in the elevated plus maze, ADI-glyphosate group males showed reduced bending behavior (Table II). Rather than reflecting overt motor suppression, this pattern is more consistent with altered risk assessment and emotional reactivity. This interpretation agrees with the growing view that low-dose glyphosate exposure may modify neurobehavioral endpoints that are not readily captured by standard systemic-toxicity studies or short-duration adult assays (35, 36).

The NOAEL-glyphosate dose produced the clearest glyphosate-only phenotype in the present study. Females from the NOAEL group present reduced internal crossings and rearing in the open field test (Table I) and reduced latency to fall on the rotarod (Table IV), indicating impairment across both exploratory and motor domains. Males from the NOAEL group spent less time in the open arms and increased time spent in the closed arms of the elevated plus maze (Table II), together with increased immobility in the forced swim test (Table III). This profile is compatible with a combination of reduced exploration, increased anxiety-like responding, and altered stress coping. Similar behavioral abnormalities have been described after glyphosate exposure in other experimental systems, including recent work showing anxiety-like behavior together with hippocampal microglial activation and NLRP3-mediated pyroptosis in mice (35).

The biological plausibility of NOAEL-glyphosate exposure-related effects is strengthened by studies investigating mechanisms of neurotoxicity induced by this compound. Glyphosate has been shown to impair hippocampal long-term potentiation and learning through activation of pro-inflammatory signaling, providing a direct synaptic correlate for later behavioral dysfunction (37). In developmental models, glyphosate and GBHs have also been shown to alter hippocampal neurogenesis, supporting the idea that early-life exposure may produce persistent effects on circuits involved in exploration, emotional behavior, and cognition (33, 38-40). More recent work also indicates that chronic GBH exposure can alter the N-glycome profile of the hippocampus and prefrontal cortex, suggesting additional molecular disruption in regions directly relevant to the behavioral changes observed here (41-44).

The Mixture groups displayed the broadest behavioral disturbances. In females, the exposure to the herbicide mixture was associated with markedly reduced internal crossings, altered grooming, increased defecation, reduced open-arm time, and increased closed-arm time (Table I). In males, the exposure to the herbicide mixture increased early grooming and defecation in the open field test (Table I) and altered rotarod performance (Table IV) across all trials. Although not every endpoint changed in the same direction across sexes, the observed overall pattern indicates that exposure to the herbicide mixture affected emotional reactivity, exploratory behavior, and motor output more extensively than glyphosate alone. These findings are consistent with the concept that combined herbicide exposure can yield additive or more-than-additive neurobehavioral effects (45-47), particularly when exposure occurs during neurodevelopment (48, 49).

A plausible contributor to the heightened herbicide Mixture effect is 2,4-D, for which evidence of experimental neurotoxicity is stronger than for dicamba. Previous work has shown that 2,4-D alters open-field test behavior and neurochemical parameters in rats, implicating central monoaminergic pathways (49). More recent studies reported that chronic 2,4-D exposure in rats produced neurobehavioral deficits together with reduced cortical thickness and dysregulation of apoptotic signaling (48). Data from *in vitro* studies have also shown that 2,4-D can induce degeneration of midbrain dopaminergic neurons, with toxicity enhanced in the presence of glia (50). Although the literature describing dicamba neurotoxicity is less extensive than that for either glyphosate or 2,4-D, experiments in zebrafish indicate that dicamba exposure can induce neurotoxic effects related to the induction of oxidative stress (51). Taken together, these data support the interpretation that 2,4-D and dicamba may amplify mixture-related neurotoxicity through oxidative, inflammatory, apoptotic, and neurotransmitter-related mechanisms.

The histopathological findings reinforce the observed behavioral outcomes. Neuronal degeneration and vascular alterations were detected across exposed groups, with the greatest lesion burden in the Mixture groups (Figures 1, 2; Tables V, VI). These structural findings are consistent with the findings of others showing that glyphosate exposure can affect neural tissue through oxidative stress, inflammatory activation, and molecular changes in brain regions involved in cognition and behavior (36, 44, 52). The prominence of vascular congestion observed in the present study is also notable because neurovascular dysfunction may exacerbate neuronal injury by impairing metabolic support and facilitating inflammatory signaling within the brain (53, 54). Importantly, our previous analysis of tissues from the same animals investigated here has demonstrated that chronic exposure to glyphosate and the glyphosate/2,4-D/dicamba mixture induces systemic toxicological effects beyond the nervous system. These include glomerular and tubular dysfunction in both male and female offspring (18), as well as dose-dependent hepatic alterations, including centrilobular vein dilation, granular degeneration, micro-vacuolar changes, and focal necrosis accompanied by increased liver enzyme

levels (55). In addition, increased oxidative stress markers in hepatic tissue were also found (56). Taken together, these findings suggest that systemic oxidative stress and inflammatory responses may represent common mechanisms linking the multi-organ toxicity associated with chronic herbicide exposure, including the neurobehavioral and neuropathological alterations observed in the present study.

Redox analyses (Figures 3, 4) also suggest an oxidative stress outcome that could at least in part contribute to the observed brain histopathology and consequent changes in behavior. Our redox analysis showed that exposure to the NOAEL-glyphosate and herbicide Mixture induced alterations in redox state in both sexes. In females, the Mixture had a broader impact on redox status, significantly increasing GSH and TBARS levels. The observed elevated GSH concentration could be attributed to the activation of the body's defence mechanism to provide protection against oxidative stress. In males, both the NOAEL-glyphosate and Mixture doses caused severe oxidative damage to critical cellular components, as evidenced by the increased TBARS and CARBS levels. Consistent with our findings, perinatal exposure to GBHs has been associated with oxidative stress responses in the brain tissue of rat models (57-59), highlighting the susceptibility of the tissue to chemical insult, probably due to its high perfusion. Evidence from *in vivo* studies demonstrating that glyphosate crosses the blood-brain barrier provides a mechanism for how the redox imbalance observed here could occur (60, 61). However, the relevant data investigating sexually dimorphic responses to glyphosate formulations is scarce, and further investigation is required (44).

The present results are particularly relevant because the effects emerged at doses linked to current regulatory exposure thresholds. By definition, the ADI is intended to represent a daily exposure without appreciable risk, and the NOAEL is commonly interpreted as the highest tested dose without adverse effects under the conditions of the original study design (62). However, the findings we present here suggest that when exposure spans gestation, lactation, and post-weaning development, subtle but persistent neurobehavioral alterations may become detectable even at these regulatory reference levels. This point is especially important for developmental neurotoxicology, where outcomes may depend as much on timing and duration of exposure as on nominal dose (42, 43).

The sex-dependent nature of the findings also deserves emphasis. Females were more clearly affected in exploratory-related endpoints in the Mixture group, whereas males in the NOAEL group showed more consistent anxiety-like and forced-swim effects. Such divergence is common in neurotoxicology and may reflect sex differences in stress-axis maturation, synaptic plasticity, antioxidant defence, and neuroimmune responses (63, 64). In the present context, these differences support the view that herbicide exposure does not produce a uniform neurobehavioral phenotype but instead engages partially distinct pathways in male and female offspring.

Taken together, the present data support three main conclusions. First, glyphosate alone produced measurable neurobehavioral changes at both the EU ADI and NOAEL doses, with the NOAEL dose generating the clearest adverse profile. Second, the herbicide mixture produced broader and often more marked alterations, supporting concern for combined neurotoxicity under realistic co-exposure conditions. Third, the behavioral findings are consistent with increasing mechanistic evidence implicating neuroinflammation, microglial activation, altered synaptic plasticity, and disrupted neurodevelopment in glyphosate-related neurotoxicity (35, 41, 42).

Several limitations of the present study should be considered when interpreting the findings. Although the behavioral alterations observed in exposed animals were supported by histopathological changes and oxidative stress responses in brain tissue, the mechanistic pathways linking herbicide exposure to the detected neurobehavioral outcomes remain only partially characterized. The present study provides evidence of structural and biochemical alterations consistent with neurotoxicity. However, the causal molecular mechanisms underlying these effects were not directly investigated. Future studies incorporating molecular approaches, including the assessment of neuroinflammatory signaling pathways, synaptic plasticity markers, and neurotransmitter system alterations, would help clarify the biological processes involved.

In addition, the study did not include measurements of glyphosate or its metabolites in biological matrices such as blood, urine, or brain tissue. Consequently, internal exposure levels could not be directly quantified or correlated with the observed behavioral and neuropathological outcomes. Incorporating biomonitoring of glyphosate and its primary metabolite aminomethylphosphonic acid (AMPA) in future experimental designs would provide important information regarding internal dose–response relationships and strengthen the interpretation of the toxicological findings.

## Conclusions

This study demonstrates that chronic developmental exposure to glyphosate can induce persistent neurobehavioral and neuropathological alterations even when administered at doses corresponding to the EU ADI and NOAEL. The NOAEL-glyphosate dose produced the most consistent glyphosate-only effects, including reduced exploratory behavior, increased anxiety-like responses, altered stress-related

behavior, and impaired motor performance. The ADI-glyphosate dose, although less disruptive, also modified selected exploratory and emotionality-related endpoints, indicating that it was not completely benign under the conditions of prolonged developmental exposure.

The herbicide mixture containing glyphosate, dicamba, and 2,4-D generated a broader pattern of behavioral disturbance and was associated with the greatest histopathological outcomes, supporting the view that combined exposure may enhance neurotoxic risk relative to single-compound assessment. This interpretation is biologically plausible given the established neurobehavioral, cortical, apoptotic, and dopaminergic effects reported for 2,4-D in experimental models.

Overall, the present findings add to the growing body of evidence that regulatory reference doses may not fully capture subtle but persistent effects on the developing nervous system, especially when exposure occurs across sensitive developmental windows and in the context of mixed herbicide exposure. These data support the need to incorporate developmental exposure paradigms, sex as a biological variable, and mixture toxicity into future hazard characterization and risk-assessment strategies for 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**

All experimental procedures were approved by Ethical Committee of University of Medicine and Pharmacy of Craiova, Craiova, Romania, (approval No. 120/10.11.2020).

**AI use disclosure**

During the preparation of this manuscript, the author(s) used ChatGPT for editing assistance and language improvement, specifically to improve spelling, grammar, word choice, readability, and clarity. The author(s) reviewed and revised all outputs from these tools and take(s) full responsibility for the final content of the work.

**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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