Acute glufosinate-based herbicide treatment in rats leads to increased ocular interleukin-1β and c-Fos protein levels, as well as intraocular pressure

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ABSTRACT

Glufosinate is a common herbicide with neurotoxic effects, leading to seizures, convulsions and memory loss. Glufosinate indirectly induces glutamate toxicity by inhibiting glutamine synthesis in astrocytes. Here, we studied the acute toxic effects of a glufosinate-based herbicide in rat optic nerve at three doses (40, 80 or 120 μM, equal to 714 or 21 mg/kg bw/day). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, glucose, calcium, as well as creatinine concentrations were analyzed after 24, 48 and 72 h treatment. Intraocular pressure (IOP) (expressed as the average of both eyes) was measured with a rebound tonometer. Interleukin-1β (IL-1β) and c-Fos expression were determined by immunohistochemistry. The results established that the glufosinate-based herbicide significantly increased IL-1β and c-Fos immunopositivity in the optic nerve ($p < 0.05$), concomitant with increased IOP. These results suggest that commercial formulations of glufosinate acutely affect the optic nerve.

1. Introduction

Amino acid phosphinotricin (D,L-homoalanin-4-[methyl] phosphinate) is the active component of the broad-spectrum herbicide, glufosinate [1]. Glufosinate is a common herbicide that is widely used for agricultural control of a broad range of weeds [2]. This herbicide causes plant death by the irreversible inhibition of glutamine synthetase (GlnS), an enzyme with important role in glutamate metabolism. Glutamate is a major excitatory neurotransmitter and its taken up by astrocytes, where GlnS converts it to glutamine [3–6]. Inhibition of GlnS causes increased synaptic glutamate levels and excitotoxicity [7–9].

c-Fos is a protooncogene, which is rapidly induced upon neurotransmitter stimulation and is referred to as an immediate early gene [10,11]. During the neuronal activity, increased c-Fos gene and protein levels protect neurons from injury [12]. However, c-Fos expression may also promote delayed neuronal apoptosis [13,14]. Upon injury, c-Fos gene and protein expression increase secondary to IL-1β, a prototypic pro-inflammatory cytokine that plays a central role in mediating neuroinflammation [15–20].

Glufosinate-ammonium (GLA), the active component of glufosinate-based herbicide has been shown to cause increased brain IL-1β protein expression. Exposure of unprotected eyes to pesticides results in their absorption into ocular tissue with potential ocular toxicity [21,22]. The optic nerve's head is a region of amplified intraocular pressure (IOP)-related mechanical stress [23,24], and studies both in dogs and rats have corroborated the ability of organophosphorus pesticides to increase IOP after oral application [22]. Pesticide exposure occurs via three common routes: skin (contact), mouth (ingestion), and lungs (inhalation). The pharmacokinetics of intraperitoneally (i.p.) injection of glufosinate-based herbicide is analogous to the other routes [25,26] and was used herein experimentally.

The aim of the present study was to examine the effects of glufosinate-based herbicide on optic nerve degeneration and IOP. We aimed to investigate whether glufosinate-based herbicide acts to stimulate the

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IL1β and c-Fos pathways in an acute pesticide toxicity model in the rat optic nerve and to determine whether these mediators modulate IOP.

2. Materials and methods

2.1. Chemical and reagents

Glufosinate-based herbicide was purchased from Agrobest LTD, Turkey and contained pure glufosinate (CAS Number: 51276-47-2, Product code: FP16278) was used in the experiments. 0.9% sodium chloride, formaldehyde (%37) and phosphate buffer solution (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Ethics

The study was approved by Atatürk University Local Board of Ethics Committee for Animal Experiments, Erzurum, Turkey (decision no: 36643897-169). The study was in compliance with the Organisation for Economic Co-operation and Development (OECD) principles of good laboratory practice (GLP), guidelines for testing of chemicals no. 407, and in accordance with standard operating procedures (SOP) established by the institution.

2.3. Animals

Sixty male Sprague-Dawley rats (mean weight 250 ± 10 g SD) were used. Animals were randomly selected and divided into 10 goups (n = 6/group), including control, three low (40 μM ; 24 h, 48 h, 72 h), three middle (80 μM ; 24 h, 48 h, 72 h) and three high dose groups (120 μM ; 24 h, 48 h, 72 h). All doses were calculated based on the LOAEL doses from reports of risk assessment. After a 7-day adaptation period, the glufosinate-based herbicide was mixed with 0.9% isotonic sodium chloride to allow administration of a 7, 14 and 21 mg/kg bw glufosinate equivalent dose of 40 80 and 120 μM. Rats were injected with 3 different doses of glufosinate-based herbicide and sacrificed at 24, 48 or 72 h, respectively. After the injection, blood samples were taken from the heart into vacuum tubes with no anticoagulant (Vacutainer, BD-Plymouth, UK) for serum analyses. Serum samples were separated by centrifugation at 3000 g for 10 min at room temperature and stored at −20 °C until analyses. Rats were decapitated rapidly under deep anesthesia (sevoflurane, USA), and the optic nerves were fixed in 10% formaldehyde (Sigma, USA).

2.4. Biochemical assays

Serum enzyme activities [alanine phosphatase (ALT), aspartate aminotransferase (AST), urea, creatinine, glucose, and calcium concentrations were determined with commercial test kits by a biochemistry autoanalyzer (Cobas 6000/Roche Diagnostics, Germany).

2.5. Measurement of intraocular pressure (IOP)

IOP was measured in both eyes (baseline, 24, 48, and 72 h after injection) with a rebound tonometer (Tonovet, Icare, Vantaa, Finland), and is reported as the average IOP for both eyes. Handling of the rats was accomplished with minimal head and neck restraint. Each animal was placed in sternal recumbency, and the measurements were taken after tonometer calibration. No anesthetic eye drops were used.

2.6. Immunofluorescence assay

Optic nerve tissue was fixed in 10% neutral formalin. After 72 h fixation, tissues were washed with tap water prior to routine serial treatment of the samples with graded alcohol and xylene in Shandon Citadel 2000 tissue system (USA). After routine histopathology processing, samples were poured into paraffin for blocking and microtome sectioned at 5 μm (Leicia RM 2255). Sections were dipped in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Then the slides were immersed in an antigen retrieval solution (pH 6.0) and heated in a microwave for 15 min to unmask antigens. Protein block was dripped onto the tissues to prevent non-specific binding. Sections were incubated with anti-IL1β polyclonal (Santa cruz, Cat. No: ab9722) and c-Fos monoclonal antibodies (Santa Cruz, Cat. No: sc-166940, USA) at a dilution of 1/100 and incubated for 30 min. at 37 °C. Next, sections were incubated in goat anti-mouse IgG Heavy and Light chains (H&L) - Fluorescein Isothiocyanate (FITC) (Cat. No: 6785, Abcam, UK) and goat anti-rabbit IgG H&L- Texas Red (TR) (Cat. no. ab6719, Abcam, UK) at a dilution of 1/ 50 and kept in the dark, and washed with water. Sections were examined with a fluorescence microscope (Zeiss Scope A1). IL-1β and c-Fos immunopositivity were scored as follows: none = -; slight = +; moderate = + +; intense = + + +

2.7. Statistical analysis

All statistical analyses were carried out with SPSS statistical software (SPSS for windows, version 20.0). Data are presented as means (±) standard deviations (S.D.). For biochemical analyses, mean differences were assessed with one-way analysis of variance (One-way ANOVA). For immunofluorescence analysis, differences were analyzed with a nonparametric test (Kruskal–Wallis) followed by Mann–Whitney U test ($P < 0.05$).

Table 1
Effect of glufosinate-based herbicide on alanine phosphatase (ALT), aspartate aminotransferase (AST), and creatinine levels (IU/L). Following 24, 48 and 72 h of exposure to three different doses (40 μM, 80μM, and 120μM) of glufosinate-based herbicide.

Time (hours)	Dose groups	Time (hours)	No. of animals	ALT (IU/L)	AST (IU/L)	CREATININE (IU/L)
24 h	Control	24	5	50.2 ± 11.21	140.8 ± 42.41	0.53 ± 0.06
	40 μM	24	5	50.4 ± 6.34	116.6 ± 1.67	0.49 ± 0.04
	80 μM	24	5	63 ± 7.84	160.8 ± 22.48	0.52 ± 0.05
	120 μM	24	5	58.2 ± 5.63	138 ± 12.04	0.50 ± 0.06
48 h	Control	48	5	50.2 ± 11.21	140.8 ± 42.41	0.53 ± 0.06
	40 μM	48	5	57.2 ± 10.28	119 ± 29.19	0.51 ± 0.03
	80 μM	48	5	69.8 ± 15.51	119.6 ± 11.54	0.48 ± 0.02
	120 μM	48	5	75 ± 21.22	115.8 ± 20.09	0.46 ± 0.09
72 h	Control	72	5	50.2 ± 11.21	140.8 ± 42.41	0.53 ± 0.06
	40 μM	72	5	54.4 ± 6.02	136.2 ± 30.54	0.49 ± 0.06
	80 μM	72	5	69.8 ± 11.12[*]	115.4 ± 64.16	0.50 ± 0.07[*]
	120 μM	72	5	56.2 ± 3.34	135 ± 26.48	0.41 ± 0.05[*]

Values are expressed as mean ± SD.
 * $p < .05$ is significant compared to control.

Table 2
Effect of glufosinate-based herbicide on urea, glucose and calcium levels (mg/dL). Following 24, 48 and 72 h of exposure to three different doses (40 μM, 80μM, and 120μM) of glufosinate-based herbicide.

Time (hours)	Dose groups	Time (hours)	No. of animals	UREA (mg/dL)	GLUCOSE (mg/dL)	CALCIUM (mg/dL)
24 h	Control	24	5	42 ± 6.52	137.2 ± 44.08	8.96 ± 2.81
	40 μM	24	5	36.24 ± 3.54	137 ± 17.84	7.78 ± 0.12
	80 μM	24	5	32.62 ± 1.40	143.48 ± 11.71	8.05 ± 0.42
	120 μM	24	5	40.84 ± 10.13	124.2 ± 7.46	8.45 ± 1.04
48 h	Control	48	5	42 ± 6.52	137.2 ± 44.08	8.96 ± 2.81
	40 μM	48	5	44.64 ± 8.18	128.4 ± 12.21	9.81 ± 0.42
	80 μM	48	5	42.78 ± 4.61	145.4 ± 7.70	9.29 ± 0.21
	120 μM	48	5	45.6 ± 5.12	185.8 ± 70.65	9.93 ± 0.73
72 h	Control	72	5	42 ± 6.52	137.2 ± 44.08	8.96 ± 2.81
	40 μM	72	5	48.42 ± 8.11	151.2 ± 58.31	9.86 ± 0.50
	80 μM	72	5	42.44 ± 10.17	133.4 ± 34.23	9.31 ± 0.22
	120 μM	72	5	45.12 ± 6.25	135.4 ± 25.54	9.10 ± 0.20

3. Results

We evaluated the ocular toxicity of a glufosinate-based herbicide. We assessed serum levels of ALT, AST, creatinine, urea, glucose, and calcium (Tables 1 and 2). The significant difference was noted in creatinine levels between control and treatment groups (80 μM for 72 h and 120 μM for 72 h, P < 0.05). Creatinine levels in response to 24 h 40 μM glufosinate-based herbicide treatment were statistically indistinguishable from controls.

ALT level significantly increased in the 72 h 80 μM glufosinate-based herbicide treated rats compared to controls (Table 1). No statistically significant differences in AST, urea, glucose, or calcium levels were noted in all experimental groups (Tables 1 and 2).

The mean baseline of both IOP values was 9 ± 1.277 mmHg (n = 60 rats). Glufosinate-based herbicide increased IOP in a dose-dependent manner. Following 24 h treatment, statistically significant increases in IOP were noted in the 24 h 40 μM and 120 μM glufosinate-based herbicide treated groups compared to controls ($P = 0.02$ and $P = 0.071$, respectively). IOP trended higher upon 24 h 80μM glufosinate-based herbicide treatment but was statistically indistinguishable from the controls (Table 3, Fig. 1). Following 48 h 40 μM, 80 μM, and 120 μM glufosinate-based herbicide treatment increased IOP in all groups ($P < 0.05$) (Table 3, Fig. 1). Following 72 h 40 μM, 80 μM and 120 μM glufosinate-based herbicide treatment further increased the IOP compared to controls ($P < 0.05$).

IL-1β and c-Fos protein immunopositivity in the optic nerve of the 72 h 40 μM and 120 μM glufosinate-based herbicide treated groups significantly increased vs. controls. A significant increase in the 72 h 40 μM and 120 μM glufosinate-based herbicide treated groups compared to the other groups (Fig. 2, p < 0.05) was also noted.

In the 40 μM, 80 μM and 120 μM (at 24 h and 48 h post injection) glufosinate-based herbicide treated groups, slight and moderate increases in immunopositivity of IL-1β and c-Fos were noted. The most intense immunopositivity was noted in the 72 h 80 μM and 120 μM glufosinate-based herbicide treated groups (Fig. 1). Immunopositivity for c-Fos was noted in the cytoplasm of neurons in the optic nerve of the glufosinate-based herbicide treated groups. IL-1β immunopositivity was predominantly observed in neuropil.

Table 3
IOP in control and glufosinate-based herbicide treated rats (40 μM, 80μM, and 120μM) represented as the average IOP values in both eyes.

| Groups	0 h	24 h	48 h	72 h
Control	8.2 ± 1.03	8.2 ± 1.03	8.2 ± 1.03	8.2 ± 1.03
40μM	9.1 ± 1.74	$11.7 \pm 1.2^*$	$10 \pm 0.79^*$	$10.3 \pm 1.15^*$
80μM	9.8 ± 0.75	10.3 ± 0.90	$10 \pm 0.70^*$	$10 \pm 0.93^*$
120μM	8.9 ± 1.19	$11.7 \pm 1.68^*$	$11.3 \pm 0.57^*$	$10.1 \pm 0.82^*$

* P < 0.05 as compared to control.

4. Discussion

The present study demonstrates a toxic effect of glufosinate-based herbicide on the rat optic nerve, establishing that herbicide exposure leads to increased IOP (Fig. 1), which is associated with activation of c-Fos and IL-1β (Fig. 2). All doses of glufosinate-based herbicide resulted in elevation of IOP (Fig. 1), however, treatment with only high doses of glufosinate-based herbicide led to increased c-Fos and IL-1β (Fig. 2).

Glufosinate, a methyl phosphonate analog of glutamate, causes excitotoxic cell death and moderate to severe neurotoxicity, including convulsions and memory loss in the brain [27,28].

The toxic effect of pesticides on ocular structures has been previously documented in both epidemiological and experimental studies. Exposure of the ocular surface to pesticides may lead to damaged cornea, referred to as corneal ectasia [21]. Furthermore, exposure to organophosphate and organochloride can damage intraocular structures, leading to cataract, crystalline lens and retinal or optic nerve damage [21,29]. Toxic effect of pesticides to the optic nerve has been demonstrated in Saku disease, secondary to exposure to the organophosphate malathion characterized by optic neuropathy, narrowing of the visual fields, abnormal pupillary responses and other effects not related to the optic nerve, such as high myopia progression and retinal degeneration. Optic neuropathy has also been observed in response to organophosphate (OP) exposures, combined with other systemic adverse reactions, such as arteriosclerotic changes in heart, brain and retinal vessels [21,30]. The optic nerve is especially sensitive to several nutritional and environmental factors. Toxic optic neuropathy clinically manifests by visual loss, central or cecocentral scotoma, and loss of color vision. Agents triggering toxic neuropathy include antibiotics and antituberculosis drugs, methanol, cyanides, phosphodiesterase type 5 inhibitors, ethambutol, chloramphenicol, linezolid, aminoglycosides antiretroviral drugs as well as pesticides [31].

Intense immunopositivity for IL-1β was found in the 72 h 80 μM and 120 μM glufosinate-based herbicide treated groups compared to the control group, while c-Fos immunopositivity increased in a dose-dependent manner. The most intense c-Fos immunopositivity was found in the 72 h 80 μM and 120 μM glufosinate treated groups. IL-1β is a proinflammatory cytokine released by several cell types, acting in autocrine and/or paracrine manner, thereby stimulating and/or modifying a variety of signaling pathways. In the hippocampus, IL-1β is associated with spatial memory [32,33], whereas in other brain areas IL-1β exerts effects ranging from epileptogenesis [34] to neurodegenerative [35] and neurotoxic [36], or excitotoxic neuronal damage [37–39]. One of the intracellular target molecules of IL-1 is the transcription factor c-Fos, which is a potent activator of glutamate receptors that induce neuroapoptosis [40–43]. Özdemir et al. reported that the acute toxicity of various pesticides (Cypermethrin, Chlorpyrifos, Deltamethrin, and Imidacloprid) in zebrafish brain increased the expression of the c-Fos gene [44]. Rogers et al. proposed that overexpression
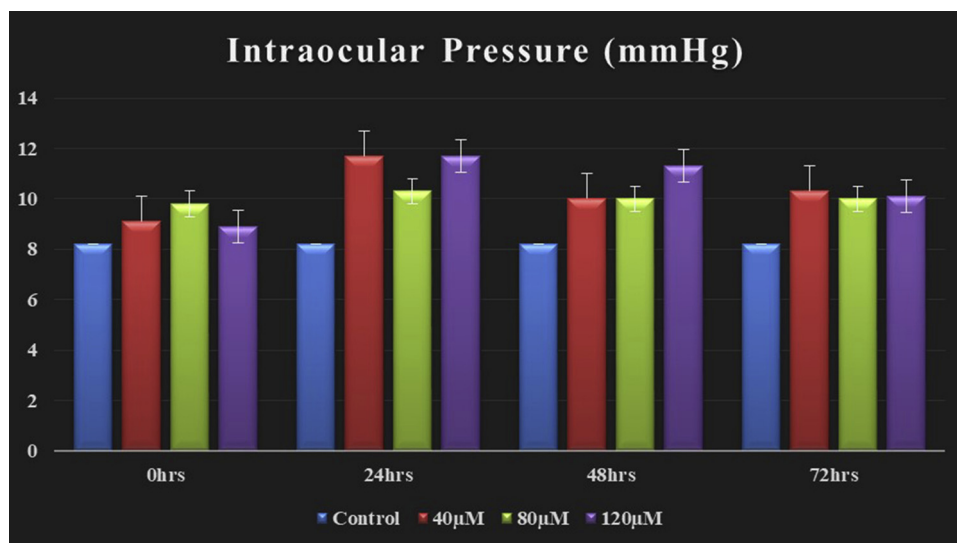
Fig. 1. Average intraocular pressure values in both eyes of rats exposed to a glufosinate-based herbicide. Data are means ± S.D. (n = 6 rats in each group). *p < 0.05, *vs* control group.

of c-Fos mRNA might be used as an indicator of excitotoxicity in *in vitro* neuronal cell systems [45]. Taking all these observations into consideration, we may assume that the increased IL-1β and c-Fos positivity in the optic nerve, along with the increased IOP noted in response to the glufosinate-based herbicide treatment, concomitant with optic nerve damage are indications of toxicity. This is because an intense inflammatory process triggered by glufosinate likely induces the production of c-Fos/MAPK/IL-1β [46]. The glufosinate-based herbicide treatment at 80 μM and 120 μM for 72 h is deemed sufficiently high and of sufficient duration, respectively, to exert an inflammatory reaction culminating in increased expression of IL-1β (potentially through the

activation of NFκB). IL-1β, in turn, *via* its receptor IL-1R induced the production of MAPK, which in turn induced increased c-Fos activity. Finally, c-Fos induced the production of glutamate, that in addition to the glufosinate-inhibited GlnS, increased excitotoxic injury.

Organophosphorus pesticides cause oxidative stress [47,48], affect metabolic pathways [49], and lead to dysfunction in several organs [50]. Furthermore, they lead to ultrastructural, biochemical and metabolic effects in the liver, as evidenced by changes in hepatic biomarkers such as serum aminotransferase (ALT, AST) [51–53]. Binukumar et al [54] demonstrated liver dysfunction secondary to organophosphate exposure in a rodent model. Organophosphate
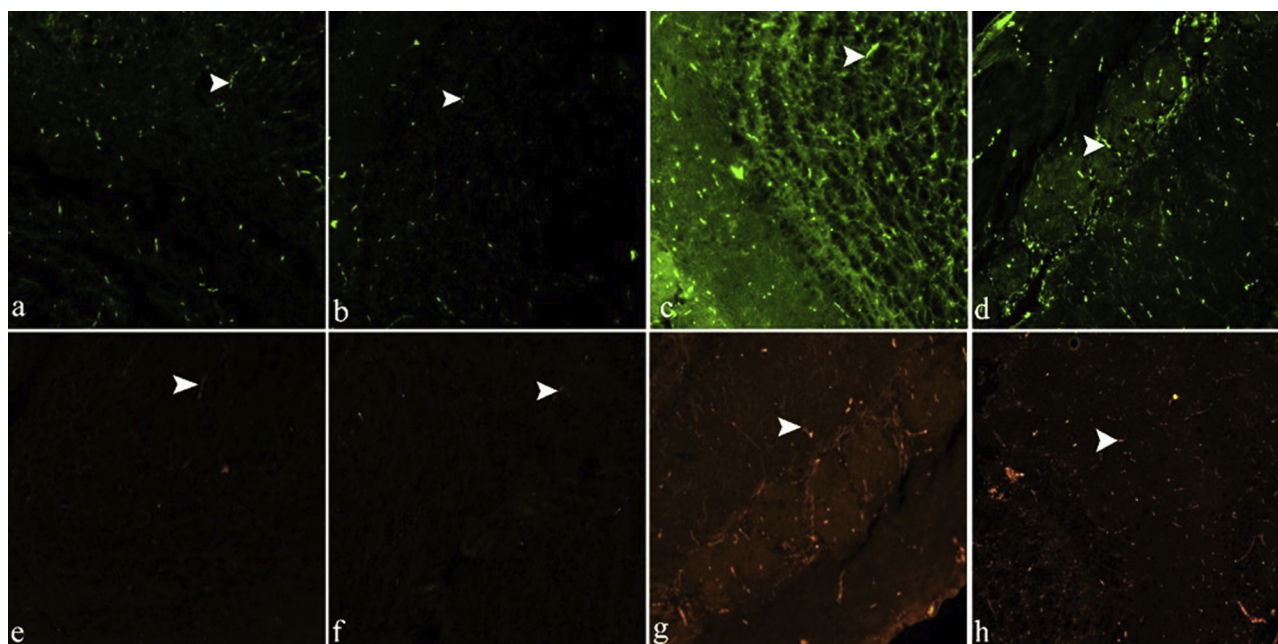
Fig. 2. Effect of glufosinate-based herbicide exposure on optic nerve by immunofluorescence with anti- IL1-β and anti-c-Fos antibodies. **a)** Control group showing very slight level IL1-β immunopositivity, FITC **b)** Glufosinate-based herbicide (40 μM for 24 h, 40 μM for 48 h, 40 μM for 72 h, 80 μM for 24 h, 80 μM for 48 h, 120 μM for 24 h, 120 μM for 48 h) treated animals showing low IL1-β immunopositivity, FITC **c–d)** 80 μM for 72 h and 120 μM for 72 h glufosinate-based herbicide treated animals showing remarkable intense IL-1β staining in glial cells shown by arrowheads, FITC **e)** Control group showing very slight level c-Fos immunopositivity, (Texas-Red labeling) **f)** Glufosinate-based herbicide (40 μM for 24 h, 40 μM for 48 h, 40 μM for 72 h, 80 μM for 24 h, 80 μM for 48 h, 120 μM for 24 h, 120 μM for 48 h) treated animals showing low c-Fos immunopositivity, TR **g–h)** 80 μM for 72 h and 120 μM for 72 h glufosinate-based herbicide treated animals showing intense c-Fos immunopositivity (arrowheads), TR.

exposure increases hepatic reactive oxygen species (ROS) levels, which cause increased expression of liver enzymes (AST, ALT). Contrary to Binukumar's study, Aroonvilairat et al. reported that primary signs of the kidney function (creatinine) and liver function (AST, ALT) did not show difference between healthy and organophosphate exposed groups, and their levels were within the normal range of reference values in a pesticide study in farmers [55]. Furthermore, Neghab et al. did not find any significant difference for the kidney function status (BUN, creatinine) and liver enzyme (ALT and AST) between pesticide retailers and workers [56]. Herein, we did not observe significant changes for several biochemical parameters (ALT, AST, creatinine, ureas, glucose and calcium). Our findings indicate no association exists between glufosinate-based herbicide treatment and early biochemical changes in rats, consistent with previous studies.

5. Conclusions

The current study presents new insights into the mechanism of glufosinate based herbicide induced apoptosis, its toxicity in optic nerve and IOP. Moreover, this is the first study on IOP related to glufosinate based herbicide and changes induced in optic nerve. We showed increased levels of the optic nerve proinflammatory factor, IL-1β, and the apoptotic factor, c-Fos. Our study demonstrates toxic effects of a glufosinate herbicide formulation in the eye, suggesting it should be used with caution. Further research is necessary to ascertain whether changes in IOP are a determining factor in the presence of a possible optic nerve injury in acute pesticide intoxications or not.

Author contributions

ÇS, and SÇ participated in the rat experiment, performed biochemical and pathological analysis, and drafted the manuscript. GK, KD, AT, MÖ, MA and AMT coordinated the investigation and assisted in the drafting of the manuscript.

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