

Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate

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[Received in February 2018; Similarity Check in February 2018; Accepted in June 2018]

In this 28 day-study, we evaluated the effects of herbicide glyphosate administered by gavage to Wistar rats at daily doses equivalent to 0.1 of the acceptable operator exposure level (AOEL), 0.5 of the consumer acceptable daily intake (ADI), 1.75 (corresponding to the chronic population-adjusted dose, cPAD), and 10 mg kg⁻¹ body weight (bw) (corresponding to 100 times the AOEL). At the end of each treatment, the body and liver weights were measured and compared with their baseline values. DNA damage in leukocytes and liver tissue was estimated with the alkaline comet assay. Oxidative stress was evaluated using a battery of endpoints to establish lipid peroxidation via thiobarbituric reactive substances (TBARS) level, level of reactive oxygen species (ROS), glutathione (GSH) level, and the activity of glutathione peroxidase (GSH-Px). Total cholinesterase activity and the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were also measured. The exposed animals gained less weight than control. Treatment resulted in significantly higher primary DNA damage in the liver cells and leukocytes. Glyphosate exposure significantly lowered TBARS in the liver of the AOEL, ADI, and cPAD groups, and in plasma in the AOEL and cPAD group. AChE was inhibited with all treatments, but the AOEL and ADI groups significantly differed from control. Total ChE and plasma/liver ROS/GSH levels did not significantly differ from control, except for the 35 % decrease in ChE in the AOEL and ADI groups and a significant drop in liver GSH in the cPAD and 100xAOEL groups. AOEL and ADI blood GSH-Px activity dropped significantly, but in the liver it significantly increased in the ADI, cPAD, and 100xAOEL groups vs. control. All these findings show that even exposure to low glyphosate levels can have serious adverse effects and points to a need to change the approach to risk assessment of low-level chronic/sub-chronic glyphosate exposure, where oxidative stress is not necessarily related to the genetic damage and AChE inhibition.

KEY WORDS: ADI; alkaline comet assay; AOEL; cholinesterase activity; cPAD; glutathione; glutathione peroxidase; lipid peroxidation; organophosphate pesticide; ROS

Organophosphorus herbicide glyphosate is a non-selective, pre- and post-emergence herbicide widely used in various agricultural and non-agricultural settings, greenhouses, aquatic and residential areas, and plant treatment plans for genetically modified, glyphosate-resistant crops. As a chemical substance, this *N*-(phosphonomethyl) glycine used alone or as an active ingredient in glyphosate surfactant herbicides acts as a competitor in the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, a rate-limiting step in the synthesis of aromatic amino acids in the shikimate pathway in plants (1). The shikimate pathway exists in algae, archaea, bacteria, fungi, prokaryotes, and unicellular eukaryotic

organisms (2–4), but is absent in animals (1), which makes glyphosate selectively toxic (5).

Glyphosate's acute oral rat LD₅₀ is ~5.6 g kg⁻¹ of body weight per day (6). The proposed doses for general exposure were set up from hepatorenal toxicity measurements of chronic rat exposure (5, 7). In the European Union (EU), the acceptable daily intake (ADI) is 0.5 mg kg⁻¹ bw per day. (8), and the allowed concentration in the drinking water is <0.1 µg L⁻¹ (9) and in food 0.01–5 mg kg⁻¹ (8). For fish tissue consumed by humans (10) there are no recommended limits, as glyphosate degrades quickly in soil or water and poorly bioaccumulates in fish. In the USA, the ADI is 1.75 mg kg⁻¹ (11), whereas the allowed concentration in drinking water is 700 µg L⁻¹ (12). A big debate about its safe use has been going on since 2010 due to these considerable regulatory discrepancies about possible

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harmful effects of glyphosate. Some regulatory bodies reclassified glyphosate as probably carcinogenic to humans (Group 2A) (World Health Organization – International Agency for Research on Cancer, WHO-IARC) (13, 14), but also as unlikely to be carcinogenic (European Food Safety Authority, EFSA) (15) or pose carcinogenic risk to humans from exposure through diet (Food and Agriculture Organization, FAO-WHO) (16). European Chemicals Agency (ECHA) classified glyphosate as causing serious eye damage and being toxic to aquatic life with long-lasting effects (17). In contrast, the US Environmental Protection Agency (EPA) reclassified glyphosate from the *least toxic* (category IV) substrate for animals (18) into *practically non-toxic and not an irritant*. According to the EU assessment (8), glyphosate is among the 10 % of herbicides with a higher ADI in long-term dietary exposure and slightly more toxic in short-term dietary exposure (45th percentile) than is the average for herbicides. Information about glyphosate ranking and percentile among about 150 herbicides assessed in the EU gives an indication of glyphosate toxicity to humans relative to other herbicides and is based on acute risk assessment (Acute Reference Dose, ARfD) in addition to ADI, which are in this case the same values of 0.5 mg kg⁻¹ bw per day (19).

A growing number of studies has demonstrated non-target effects on mammalian metabolism at low, environmentally relevant levels. *In vitro* research evidences that glyphosate levels in the range of human population exposure can affect mammalian mitochondrial function by disrupting liver mitochondrial oxidative phosphorylation (20–22), by increasing mitochondrial membrane permeability for protons and calcium ions (22), and by inhibiting succinate dehydrogenase (23). Glyphosate can also trigger oxidative stress and cause oxidative damage to lipids, proteins, and DNA (24), especially in erythrocyte and lymphocyte cell membranes (25). It can affect glutathione, aromatase (26) and antioxidant enzyme levels, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), and glutathione reductase (27–30), and can inhibit acetylcholinesterase (AChE) activity (28, 29, 31).

Short-term studies in rodents did not demonstrate apparent toxic effect (32). Lifelong exposure demonstrated liver and kidney dysfunction and toxicity (33), deficiencies in foetal ossification in pregnant rats (34), a greatly increased risk of cancer, and shorter lifespan (13). A two-year rat study (35) demonstrated changes in urine and organ biochemical parameters as well as in proteomic and metabolomic profile (35). Although the toxicity of glyphosate can be both dose- and species-dependent (36) – aquatic organisms seem to be more sensitive – new studies shift their concern from acute to chronic, sub-chronic, and reproductive toxicity, as more relevant (37).

Our knowledge of glyphosate toxicokinetics is based on rat studies by the Monsanto Company (38–40), the US National Toxicology Program (41), glyphosate-derived

radioactivity tissue studies (10 mg kg⁻¹ or higher dose treatment) (42, 43), and reviews by Williams et al. (9, 14, 15, 19, 44, 45) of the carcinogenicity studies conducted by the IARC, EFSA, and EPA. With knowledge this limited, we need to go further to investigate the toxicokinetic profile of glyphosate with multiple doses, ranging between low and high for mechanistic understanding and key events in the biological pathways as well as time- or dose-dependencies (45). The reason for looking into low doses is the new evidence of harmful effects (2, 35, 46, 47), which gets worse with sub-chronic and chronic exposure (35, 37). Glyphosate traces found in human urine and blood of agricultural and non-agricultural workers, pregnant women and children (2, 14, 48–52) point to a higher risk of long-term environmental exposure (53).

Based on the evidence from these new studies and our own evidence (46), we decided to look further into the toxicological mechanisms and effects of sublethal, environmentally relevant (yet allegedly human-safe) glyphosate doses in rats to see how they affect: (a) body weight and liver weight, (b) cholinesterase (ChE), AChE, and butyrylcholinesterase (BChE) activities, (c) oxidative stress markers (lipid peroxidation, reactive oxygen species, GSH, and GSH-Px), and (d) the levels of primary DNA damage in leukocytes and small and medium-sized liver cells, all of which are the established markers of glyphosate mechanisms and effects.

MATERIALS AND METHODS

Chemicals and reagents

All of the chemicals were of analytical grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise specified. Glyphosate (CAS No. 1071-83-6) was of analytical standard purity grade (≤100 %), purchased under the brand name PESTANAL[®], a registered trademark of Sigma-Aldrich Laborchemikalien GmbH (Germany).

Animals

Animal procedures and protocols were carried according to internationally accepted animal welfare guidelines, and the study was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health (IMROH), Zagreb, Croatia. For the experiments we used 30 male Wistar rats obtained from the breeding unit at IMROH. They were kept under pathogen-free, steady-state microenvironmental conditions in clear polycarbonate cages with 40–60 % humidity at 22 °C and normal 12-hour light/dark cycle. The animals had free access to standard Good Laboratory Practice-certified food (Mucedola, 4RF21, Italy) and tap water. When they were three months old, the rats were weighted, inspected, and

judged to be healthy and fit for the experiment by a licensed veterinarian at IMROH.

Experimental design

Stock glyphosate solution was prepared in phosphate-buffered saline (PBS). The tested doses included environmentally relevant exposure levels usually not harmful to humans, more specifically, the EFSA's acceptable operator exposure level (AOEL) of 0.1 mg kg⁻¹ bw per day (54) and the acceptable daily intake (ADI) for consumers of 0.5 mg kg⁻¹ bw per day (9), the EPA's chronic population-adjusted dose (cPAD) of 1.75 mg kg⁻¹ bw per day (11), and the 100 times the AOEL, which is 10 mg kg⁻¹ bw per day (100xAOEL).

Ethyl methanesulphonate (EMS) served as positive control, being a well-established genotoxicant recommended for *in vivo* comet assay in rodents (55), which was also dissolved in PBS.

Male Wistar rats were randomly divided into six groups of five animals receiving 1 mL of PBS (with or without glyphosate) by oral gavage. The control group received 1 mL of PBS at room temperature throughout the experiment. The positive control group received EMS (300 mg kg⁻¹ bw) over the last three days of the experiment. The AOEL, ADI, cPAD, and 100xAOEL groups received glyphosate in the above described doses.

Body weight was monitored once a week and the glyphosate doses adjusted accordingly. Survival and clinical signs of poisoning were also monitored on a daily basis.

The treatment lasted 28 days. All animals were humanely euthanised and dissected on day 29, 24 h after the last dose. Euthanasia was performed by exsanguination under Xylapan/Narketan anaesthesia (Xylapan, Vetoquinol UK Ltd., 12 mg kg⁻¹ bw, *i. p.*/Narketan, Vetoquinol UK Ltd., 80 mg kg⁻¹ bw) directly from the heart. Immediately after euthanasia, all animals were examined for gross pathological changes of the internal organs. To calculate relative liver weight, we used the following formula:

$$\text{ROW (relative organ weight)} = \frac{\text{absolute organ weight (g)}}{\text{BW (body weight) (g)}} * 100 \%$$

where body weight was measured moments before and the absolute liver weight after euthanasia.

Sample collection and preparation

All samples were taken immediately after euthanasia. Liver was removed from the abdomen, rinsed with cold PBS at pH 7.4, and weighed. Liver was then washed in cold TBS buffer (50 mmol L⁻¹ Tris-Cl, 150 mmol L⁻¹ NaCl, pH 7.5) (56) to remove as much blood as possible, homogenised in a 50 mmol L⁻¹ potassium PBS pH 7.4 with 1 mmol L⁻¹ EDTA (1 mL of buffer to 100 mg of tissue), and centrifuged at 20,000 x g at 4 °C for 30 min to obtain the supernatant.

In a separate procedure, a small portion of the liver tissue was minced in a chilled mincing solution (75 mmol L⁻¹

NaCl, 24 mmol L⁻¹ Na₂EDTA, pH 7.5) (56) to obtain cellular suspension. Individual cells were separated with a pair of fine scissors. The cell suspension was left a few seconds for large clumps to settle, and the supernatant was used to prepare comet slides. All this was performed within 60 min from death to avoid confounding necrotic changes.

Blood samples were collected directly from the heart into heparinised vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA) with an extra addition of Li-heparin, mixed vigorously to prevent clumping. Samples were then refrigerated at 4 °C until further processing. For biochemical assays, heparinised blood was centrifuged at 976 x g and 4 °C for 10 min to remove plasma and then frozen at -20 °C until further processing.

Assessment of primary DNA damage with alkaline comet assay in blood and small and medium sized liver cells

Two microgels were prepared per tissue per animal. Slides were marked with a randomly generated code. For each slide, an aliquot of 10 µL of the cell suspension was mixed with low melting point agarose (LMPA) (0.5 %) dissolved in TBS buffer. "Sandwich" agarose microgels made of four layers were prepared on microscopic slides. Slides were pre-coated with 1 % normal melting point agarose (NMPA) (Sigma) and air-dried. The second gel layer of 0.6 % NMP agarose was then applied. The third layer consisted of a 0.5 % LMPA mixed with heparinised whole blood (10 µL per slide) or 10 µL of liver cell suspension per slide. Finally, 0.5 % LMPA was applied as the top layer over the gel-embedded cells.

After solidification of the gel on ice-cold metal tray, the slides were submerged in freshly prepared cold lysing solution (100 mmol L⁻¹ EDTA, 2.5 mol L⁻¹ NaCl, 10 mmol L⁻¹ Tris-Cl, pH 10, 1 % of Triton-X 100 and 10 % DMSO) at 4 °C overnight. The slides were quickly washed with distilled water and left in a vertical Coplin jar with chilled electrophoresis buffer (300 mmol L⁻¹ NaOH, 1 mmol L⁻¹ Na₂EDTA, pH >13) at 4 °C for 10 min. The slides were then transferred into a horizontal electrophoresis unit. The liver samples were electrophoresed at 1 V cm⁻¹ and constant current of 300 mA for 10 min (56), and the blood samples at 0.86 V cm⁻¹ and constant current of 300 mA for 20 min (57). After electrophoresis, the slides were washed three times with neutralisation buffer (0.4 mol L⁻¹ Tris-HCl, pH 7.5). All gels were dehydrated with 70 % and 96 % ethanol, respectively, air dried, and stored at room temperature.

Before scoring, the slides were stained with ethidium bromide (20 µg mL⁻¹) and analysed with a fluorescent microscope under 200x magnification (Olympus BX50, Olympus, Tokyo, Japan) using the Comet Assay IV image analysis system (Instem-Perceptive Instruments Ltd., Suffolk, Halstead, UK) equipped with appropriate filters. Three hundred cells (2 x 150 nucleoids) were scored in total for each animal and sample. Medium-sized cells (parenchymal cells or hepatocytes, between 30 and 40 µm

of head length) and small-sized cells (non-parenchymal cells, <30 μm of head length) were recorded separately (58).

Areas near slide margins were not scored. DNA damage was measured as comet DNA tail intensity (% of DNA in tail) and tail length (TL, expressed in μm , measured from the estimated edge of the comet head).

Comets with small or non-existing head and large, diffuse tails (cells with >80 % DNA in the tail) were excluded from analysis. The frequency of such comets ("hedgehogs" or "clouds") was determined based on visual scoring among 100 nucleoids per sample. According to literature data, they may represent DNA damage resulting from cytotoxicity (59). We did not measure the abnormal-sized tail parameters, as we find them inappropriate for this study.

ROS detection

ROS levels in blood plasma and liver homogenates were measured using 2',7'-dichlorofluorescein diacetate (DCF-DA). The acetate group of 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) gets DCFH-DA into the cells or organelles, and once inside, it is removed by cellular esterases, producing reduced DCFH which then can be oxidised by peroxides to form fluorescent oxidised DCF that can be measured spectrophotometrically. Plasma samples and 1 % liver tissue homogenate were prepared by dilution with ice-cold PBS (pH 7.4). Black 96-well plates were filled with 0.07 mL of PBS, and 0.03 mL of 1 % liver tissue homogenate or with 0.1 mL of 10 % blood plasma in quintuplicate for each glyphosate concentration and sample type. Each well was then added 20 μL of 0.12 mmol L^{-1} DCFH-DA dye in PBS, and the plates were incubated at 37 °C for 30 min. Control for dye autofluorescence was prepared without the addition of dye. Control samples were included in each experiment. Samples were analysed using a Victor3™ (PerkinElmer, Inc., Waltham, MA, USA) multilabel plate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The readings were expressed as relative fluorescence units (RFU).

Determination of lipid peroxidation in plasma and liver

The concentrations of thiobarbituric reactive substances (TBARS), as a measure of lipid peroxidation, were determined using a modified method by Drury et al. (1997) (60). Butylated hydroxytoluene (BHT; 5 μL ; 0.2 %; w/v) and phosphoric acid (750 μL ; 1 %; v/v) were added to 50 μL of sample. After mixing, 250 μL of 0.6 % (w/w) thiobarbituric acid (TBA) and 445 μL of H_2O were added, and the reaction mixture was incubated in a water bath at 90 °C for 30 min. The mixture was cooled, and absorbance measured at 532 nm on a Shimadzu UV probe spectrophotometer (Shimadzu Corporation, Kyoto, Japan). TBARS concentrations were calculated using the standard curves

for 1,1,3,3-tetramethoxypropane, obtained by increasing its concentrations, and expressed as $\mu\text{mol L}^{-1}$.

Quantification of GSH

GSH levels were analysed with a fluorogenic bimine probe using monochlorobimane (MBCl), which reacts specifically with GSH to form a fluorescent adduct (61). Plasma samples and liver tissue homogenates were prepared as previously described for ROS measurement and then added 20 μL of 0.24 mmol L^{-1} MBCl dye in PBS to react at 37 °C for 20 min. The amount of GSH in the samples was analysed using a Victor3™ multilabel plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Control samples were included in each experiment. The readings were expressed as relative fluorescence units (RFU). Each sample analysis was performed in quintuplicate.

Determination of GSH-Px activity in whole blood and liver

GSH-Px activity in whole blood and the supernatant of liver homogenate were determined in accordance with the European standardized method (62). The amount of GSH oxidised by t-butyl hydroperoxide was determined based on decrease in β -NADPH absorbance at 340 nm, measured by spectrophotometry (Cary 50 UV-Vis, Varian Inc., Santa Clara, CA, USA). One unit of GSH-Px was the number of micromoles of β -NADPH oxidised per minute. Its activity in whole blood was expressed per gram of haemoglobin ($\text{U g}_{\text{Hb}}^{-1}$), and in the liver homogenate per gram of total protein ($\text{U g}_{\text{protein}}^{-1}$).

Protein quantification

Proteins were quantified according to the method of Bradford (1976), using bovine serum albumin as standard (63).

Determination of plasma cholinesterase activity

Plasma samples were analysed for total ChE, AChE, and BChE activities in a 0.1 mol L^{-1} sodium phosphate buffer, pH 7.4, at 25 °C using ATCh (1.0 mmol L^{-1}) and DTNB (0.3 mmol L^{-1}) as described by Ellman et al. (64). To distinguish between AChE and BChE activities we used the BChE-selective inhibitor ethopropazine (20 $\mu\text{mol L}^{-1}$). Increase in absorbance was monitored at 412 nm over 4 min. All of the measurements were performed on a Cecil 9000 spectrophotometer (Cecil Instruments Limited, Cambridge, UK). Enzyme activities were expressed as $\text{IU g}_{\text{protein}}^{-1}$.

Statistical analysis

Statistical analysis was run on Dell Statistica software STATISTICA, version 13.2 (Dell Inc., Round Rock, TX, USA). The data were first evaluated with descriptive statistics. The results were expressed as means \pm standard

deviation, and for the comet assay we also used medians and ranges (min-max).

Relative liver weights were logarithmically transformed [$\log_{10}(N+2)$] and analysed with one-way ANOVA. For pairwise organ comparison we used the *post-hoc* Tukey's HSD test.

Normality of (Gaussian) distribution was tested with the Levene's test. Since the results of the alkaline comet assay were not normally distributed even after logarithmic transformation, we used the non-parametric Mann-Whitney U test.

For multiple comparisons of cholinesterase activities, TBARS, and GSH-Px activities between the glyphosate and control groups we used the Kruskal-Wallis test. ROS and GSH levels were compared between the groups using the non-parametric Mann-Whitney U test. P values ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Changes in body and liver weight

The 28-day treatment with different glyphosate doses did not cause premature death or any sign of systemic toxicity in the male adult Wistar rats. Throughout the treatment, and especially on weighting days, our trained veterinary observed the animals for the presence of miosis, mouthsmacking, salivation, or lacrimation, tremors, or gait abnormalities, as described elsewhere (65), and found no significant differences between controls and treated animals, except in body weight gain. Gross necropsy did not reveal any treatment-related findings.

Figure 1 shows body weight changes and Figure 2 demonstrates relative liver weight across the groups over

the 28 days of treatment. All glyphosate-treated animals had similar weight gain through the 28-day treatment, with significant difference between the body mass on the day before the treatment and the end of treatment. On the last day, only the cPAD and the 100xAOEL group showed gain. However, it did not differ significantly between the groups.

The existing literature suggests that glyphosate treatment affects the growth of rats. Tang et al. (66) observed lower body weight gain in adult male rats after 35 days of treatment with glyphosate at the daily doses of 5-500 mg kg⁻¹ bw. Liver weight changes upon glyphosate treatment have also been demonstrated in many studies (as reviewed in 13). The US EPA suggested that they depended on glyphosate concentration and rodent species (5). However, there are also studies that demonstrated no changes (67, 68) or even pointed to an increase in liver weight (41). This increase could be connected with the non-alcoholic fatty liver disease and its progression to non-alcoholic steatohepatosis, as reported by Mesnage et al. (35) and Samsel and Senef (2).

Alkaline comet assay

Table 1 shows the results of the alkaline comet assay in leukocytes and liver cells with small and medium-sized nuclei.

Glyphosate-treated rats had higher primary DNA damage in leukocytes compared to control in both comet assay parameters. While tail length was significantly greater after all treatments, only the lowest tested dose resulted in significantly higher mean tail intensity. One reason for that could be high standard deviations.

Worth noticing is that glyphosate caused greater DNA damage in the liver cells than in the leukocytes (Table 1). Since liver is a complex organ with multiple cells working

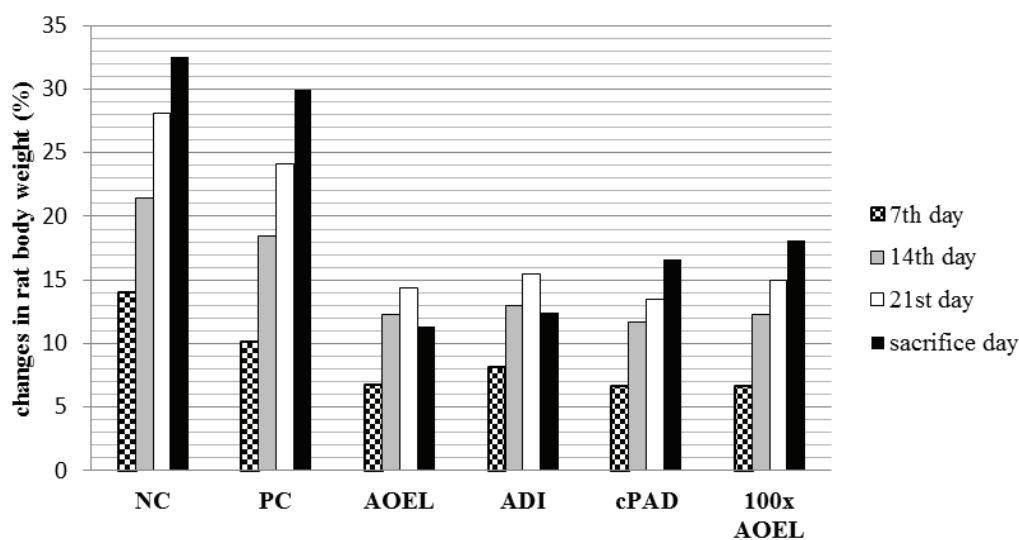


Figure 1 Changes in the percentage of the body weight gain in Wistar rats ($N=5$ per group) on the 7th, 14th, 21st, and 28th day of treatment with different doses of glyphosate in PBS. NC – negative control receiving 1 mL of PBS; PC – positive control receiving 300 mg kg⁻¹ bw day⁻¹ of ethyl methanesulphonate last three days of treatment; AOEL – acceptable operator exposure level (0.1 mg kg⁻¹ bw day⁻¹); ADI – acceptable daily intake for consumers (0.5 mg kg⁻¹ bw day⁻¹); cPAD – chronic population adjusted dose (1.75 mg kg⁻¹ bw day⁻¹); 100xAOEL – 100 times the AOEL (10 mg kg⁻¹ bw day⁻¹)

Table 1 Results of the alkaline comet assay in adult male Wistar rats (N=5 per group) orally treated with different doses of glyphosate for 28 days

Cell type	Mean±SD Median Range	Negative control	Positive control EMS 300 mg kg ⁻¹ bw day ⁻¹	AOEL [#] 0.1 mg kg ⁻¹ bw day ⁻¹	ADI [§] 0.5 mg kg ⁻¹ bw day ⁻¹	cPAD [§] 1.75 mg kg ⁻¹ bw day ⁻¹	100xAOEL 10 mg kg ⁻¹ bw day ⁻¹
Leukocytes	TL	16.92±3.30 ^{PC} 15.83 11.25–35.42	29.84±10.87 ^{NC} 27.92 10.42–75.42	19.49±3.83 ^{PC,NC,*} 18.75 11.67–40	19.06±3.50 ^{NC,PC,*} 17.92 11.25–32.08	19.46±3.47 ^{NC,PC,a} 18.75 11.25–36.67	18.44±4.02 ^{NC,PC,*} 16.67 11.25–36.25
	TI	0.96±1.75 ^{PC} 0.07 0–11.34	7.93±7.69 ^{NC} 5.58 0–39.33	1.04±1.95 ^{NC,PC,*} 0.06 0–10.19	1.23±2.28 ^{PC,*} 0.06 0–12.64	1.17±2.06 ^{PC} 0.09 0–10.87	1.02±1.82 ^{PC} 0.10 0–10.24
Small-sized nuclei	TL	18.34±4.05 ^{PC,a,*} 17.08 5.83–38.33	23.23±3.92 ^{NC,a,*} 22.92 12.5–36.25	23.65±8.01 ^{NC,PC,a,*} 20.83 11.67–52.5	25.36±7.32 ^{NC,PC,a,*} 23.75 10–54.17	25.27±8.54 ^{NC,PC,a,b} 23.33 11.67–65.42	28.17±9.40 ^{NC,PC,*} 27.08 7.92–54.58
	TI	0.87±1.69 ^{PC,a,*} 0.07 0–14.74	4.10±3.26 ^{NC,a,*} 3.26 0–16.19	4.37±6.26 ^{NC,PC,a,*} 1.03 0–32.28	2.89±3.70 ^{NC,PC,a,*} 1.31 0–21.39	2.47±3.39 ^{NC,PC,a,b} 0.77 0–19.03	4.25±4.48 ^{NC,PC,*} 2.64 0–24.32
Medium-sized nuclei	TL	21.26±4.07 ^{PC,a,*} 20.42 11.67–48.33	23.13±4.07 ^{NC,a,*} 25.42 12.5–39.17	26.69±11.24 ^{NC,PC,a,b} 21.67 11.25–61.25	29.27±8.59 ^{NC,PC,a,*} 27.92 10.83–54.58	28.74±8.30 ^{NC,PC,a,b} 27.5 15–60.83	32.59±10.16 ^{NC,PC,*} 32.08 12.92–63.75
	TI	0.90±1.71 ^{PC,a,*} 0.05 0–9.81	3.10±2.99 ^{NC,a,*} 1.96 0–16.32	4.97±7.13 ^{NC,PC,a,b} 0.65 0–37.45	3.06±3.82 ^{NC,PC,a,*} 1.38 0–21.51	2.17±2.89 ^{NC,PC,a,b} 0.77 0–15.89	4.54±4.65 ^{NC,PC,*} 3.16 0–21.89

[#]EFSA (2011); [§]EFSA (2015); [§]US EPA (2006). A non-parametric Mann-Whitney U test was used for comparison between different treatment groups. Results are presented as mean ±S.D., median and range (min-max). TL – tail length in µm; TI – tail intensity (%DNA in tail); NC – significantly different from negative control; PC – significantly different from positive control; + – significantly different from AOEL; * – significantly different from cPAD; a – significantly different from 100x AOEL; b – significantly different from ADI. Statistical significance was set at p<0.05. Negative control receiving 1 mL of PBS; positive control receiving 300 mg kg⁻¹ bw day⁻¹ of ethyl methanesulphonate last three days of treatment; AOEL – acceptable operator exposure level (0.1 mg kg⁻¹ bw day⁻¹); ADI – acceptable daily intake for consumers (0.5 mg kg⁻¹ bw day⁻¹); cPAD – chronic population adjusted dose (1.75 mg kg⁻¹ bw day⁻¹); 100xAOEL – 100 times the AOEL (10 mg kg⁻¹ bw day⁻¹)

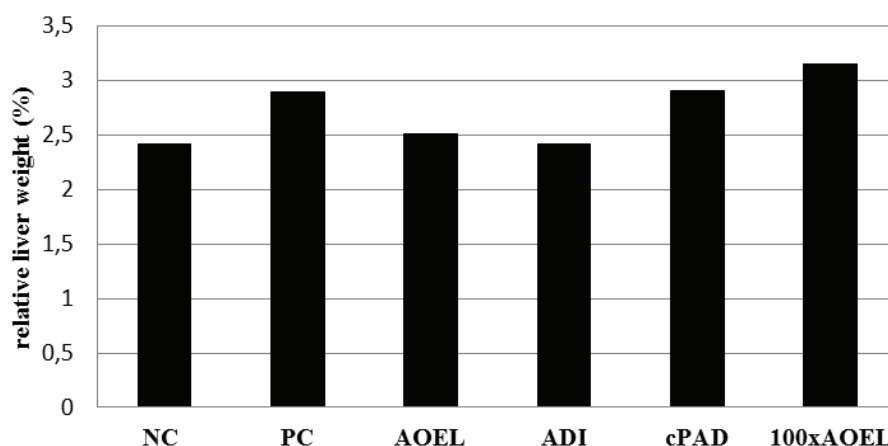


Figure 2 Changes in the relative liver weight of Wistar rats ($N=5$ per group) treated with different doses of glyphosate orally for 28 days according to the ROW formula. NC – negative control receiving 1 mL of PBS; PC – positive control receiving 300 mg kg⁻¹ bw day⁻¹ of ethyl methanesulphonate last three days of treatment; AOEL – acceptable operator exposure level (0.1 mg kg⁻¹ bw day⁻¹); ADI – acceptable daily intake for consumers (0.5 mg kg⁻¹ bw day⁻¹); cPAD – chronic population adjusted dose (1.75 mg kg⁻¹ bw day⁻¹); 100xAOEL – 100 times the AOEL (10 mg kg⁻¹ bw day⁻¹)

together in cohesion, two cell types should be taken into account to obtain reliable information about the toxic effects of the tested compound at the DNA level. Parenchymal cells or hepatocytes (medium-sized nuclei) account for 80 % of the cells in the liver and are primarily responsible for drug metabolism. Non-parenchymal cells (small-sized nuclei) have many specific functions in the liver and contribute to inflammatory responses. Differences in the responses of the two cell populations to treatments (Table 1) could be associated with their intrinsic variations in DNA sensitivity.

Interestingly, the greatest liver cell DNA damage (judged by tail intensity) occurred with the lowest and the highest exposure both in parenchymal and non-parenchymal liver cells. In fact, the 100xAOEL group suffered even greater damage in medium-sized liver cells than positive control.

Our findings are in line with previous comet assay *in vitro* and *in vivo* animal studies with glyphosate, which reported its damaging potential for the DNA. In one study (69), *in vitro* treatment of human lymphocytes with 0.7-700 μmol L⁻¹ of glyphosate led to a significant increase in tail length. In another study (70), exposure of non-dividing human lymphocytes to 0.5-580 μg mL⁻¹ of glyphosate along with metabolic activation resulted in a significant increase in tail intensity only at 580 μg mL⁻¹ (~3.4 mmol L⁻¹), as established with the alkaline comet assay (70). The same authors reported a dose-related increase in tail length measured with the hOGG1-modified comet assay. In a study using Hep-2 cells, Manas et al. (71) reported a significant increase in mean tail length and tail intensity at the concentration range of 3.0-7.5 mmol L⁻¹ of glyphosate.

Rats exposed to glyphosate at the doses of 5-490 mg kg⁻¹, administered every 48 h for 75 days, demonstrated irreversible damage to hepatocytes (34). In another study (68), mild liver damage was reported in rats following sub-chronic exposure to glyphosate (56 and 560 mg kg⁻¹) for 35 and 90 days.

Several comparable studies were conducted in mice. Mice receiving 40 and 400 mg kg⁻¹ bw of glyphosate per day via drinking water demonstrated a significant increase in primary DNA damage in blood and liver cells after a 14-day exposure. Since no effects on oxidative stress parameters were observed, the authors suggested that DNA damage may not be related to oxidative damage.

Bolognesi et al. (73) showed DNA damage in mice exposed intraperitoneally (*i.p.*) to a single 300 mg kg⁻¹ dose of glyphosate. Single strand DNA breaks increased in the liver and kidney 4 h after the injection, but they returned to control levels on hour 24. Similar effects in mice were seen for high *i.p.* doses (41).

El Shenawy et al. (67) showed that cellular injury occurred with millimolar glyphosate doses given over longer time due to cumulative effects.

Speaking about the types of DNA damage induced by glyphosate treatments, Bolognesi et al. (73) demonstrated 8-OHdG in the liver of mice following single *i.p.* injection of 300 mg kg⁻¹ of glyphosate. Peluso et al. (74) reported that technical glyphosate did not form DNA adducts in the liver or kidney of mice injected up to 270 mg kg⁻¹ *i.p.*

Greim et al. in their review article (75) point to the essential role of glycine in glyphosate-mediated DNA damage, more specifically in distinguishing 8-oxoG from guanine at position G42. Glyphosate substitution for glycine can impair the function of OGG1 (75) and thus trigger a cascade of consequences, starting with the accumulation of unrepaired 8-oxoG and clustering of DNA damage and double-strand breaks (visible by the comet assay) and resulting in chromatid deletions and achromatic lesions, as already reported by Monsanto in 1983 (as reviewed in 75).

Oxidative stress markers

Several studies (27-30) have demonstrated that glyphosate or glyphosate-based herbicide exposure affects

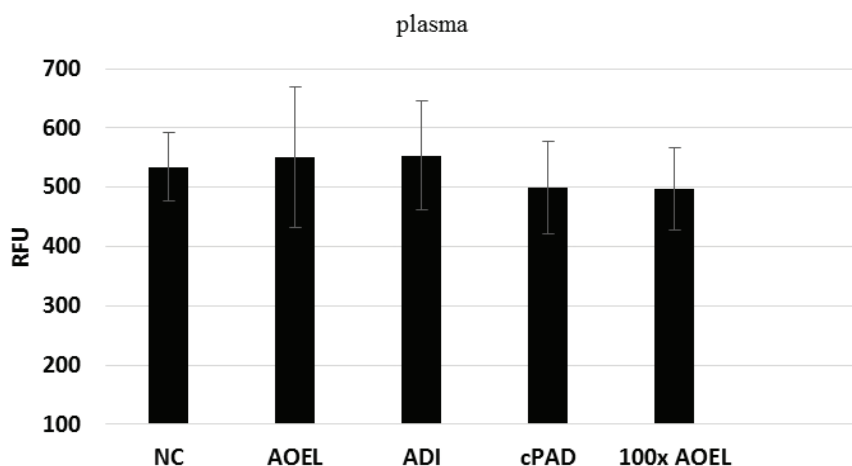


Figure 3a ROS in the plasma of Wistar rats treated with different doses of glyphosate orally for 28 days

RFU – relative fluorescence units; NC – negative control receiving 1 mL of PBS; AOEL – acceptable operator exposure level ($0.1 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); ADI – acceptable daily intake for consumers ($0.5 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); cPAD – chronic population adjusted dose ($1.75 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); 100xAOEL – 100 times the AOEL ($10 \text{ mg kg}^{-1} \text{ bw day}^{-1}$)

indicators of oxidative stress. Our study in general (Figures 3-6) did not reveal significant disturbances of the oxidative stress markers at the tested doses nor has it pointed to dose-dependency.

ROS levels in plasma and liver

Plasma samples (Figure 3a) did not demonstrate any significant difference between the glyphosate groups and control. A small drop in ROS of around 7 % (compared to the control) is still visible in the groups exposed to the highest glyphosate doses. The liver tissue showed a similar pattern (Figure 3b), but ROS levels in the liver were 100 times greater than in the plasma and the drop in 100xAOEL group was significant compared to control.

Lipid peroxidation

Figure 4 shows that the TBARS concentrations in the plasma and liver dropped in all glyphosate-treated groups compared to control. It also details how significant these changes were between groups.

GSH levels in plasma and liver

Plasma samples showed no significant difference between the treated groups and control, even though GSH was higher in the two groups treated with the highest doses (6.82 % and 12.29 %, respectively). In the liver, GSH dropped in all treated groups, but the difference from control was significant only in the cPAD and 100xAOEL group (22.72, and 26.92 %, respectively) (Figure 5).

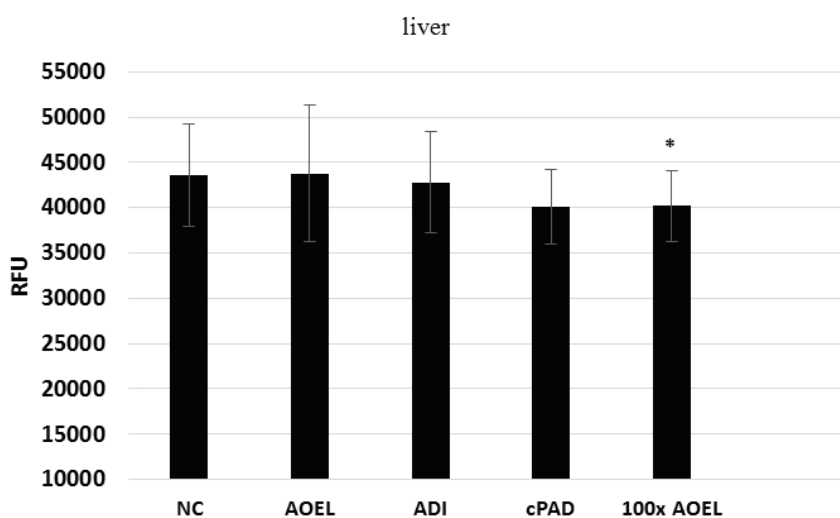


Figure 3b ROS in the liver tissue of Wistar rats treated with different doses of glyphosate orally for 28 days

RFU – relative fluorescence units. Statistical significance was set at $p < 0.05$. *Significantly different from the negative control. NC – negative control receiving 1 mL of PBS; AOEL – acceptable operator exposure level ($0.1 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); ADI – acceptable daily intake for consumers ($0.5 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); cPAD – chronic population adjusted dose ($1.75 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); 100xAOEL – 100 times the AOEL ($10 \text{ mg kg}^{-1} \text{ bw day}^{-1}$)

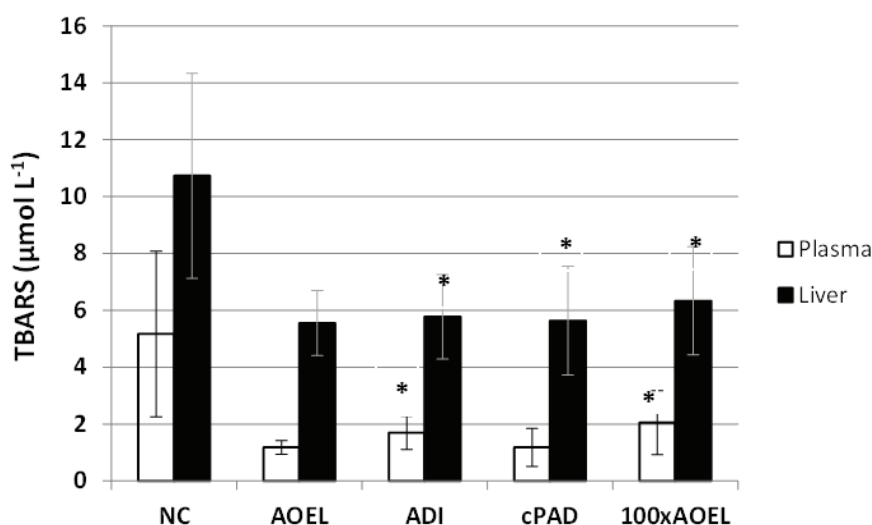


Figure 4 Changes in TBARS concentration in the plasma and liver of Wistar rats after the 28-day treatment with different glyphosate doses. The results are shown as mean and SD values. Significantly different values ($P < 0.05$) were: (*) compared to control. NC – negative control receiving 1 mL of PBS; AOEL – acceptable operator exposure level ($0.1 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); ADI – acceptable daily intake for consumers ($0.5 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); cPAD – chronic population adjusted dose ($1.75 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); 100xAOEL – 100 times the AOEL ($10 \text{ mg kg}^{-1} \text{ bw day}^{-1}$)

GSH-Px activity in whole blood and liver

Blood GSH-Px activity was significantly lower in the AOEL and ADI groups than in control (Figure 6). Its liver activity, in turn, was significantly higher in the ADI, cPAD, and 100xAOEL group.

Literature associates elevated levels of oxidative stress with exposure to high glyphosate doses (71), which means higher bioavailability, relatively high glyphosate plasma concentrations over a short time, slower distribution/elimination from the plasma to the organs, followed by ROS generation that depletes the antioxidants already present in the body and induces additional production of antioxidants and antioxidant enzyme activity. Higher ROS and higher defence enzyme activities would be expected in the liver,

since this organ plays a major role in the biotransformation and detoxification of toxic substances.

Possible reasons why we did not observe increased lipid peroxidation are that glyphosate is not capable of crossing the lipid membrane without the help of carriers or open ion channels (76) and that the route of exposure (oral gavage) and tested doses were too low to ensure enough bioavailability for lipid peroxidation. On the other hand, Astiz et al. (77) administered the compound via *i.p.* route which ensured higher bioavailability and resulted in increased lipid peroxidation.

As demonstrated by Mesnage et al. (35), El Shenawy et al. (67), and Slaninova et al. (78), liver glutathione is often depleted after short-term oxidative stress but elevated

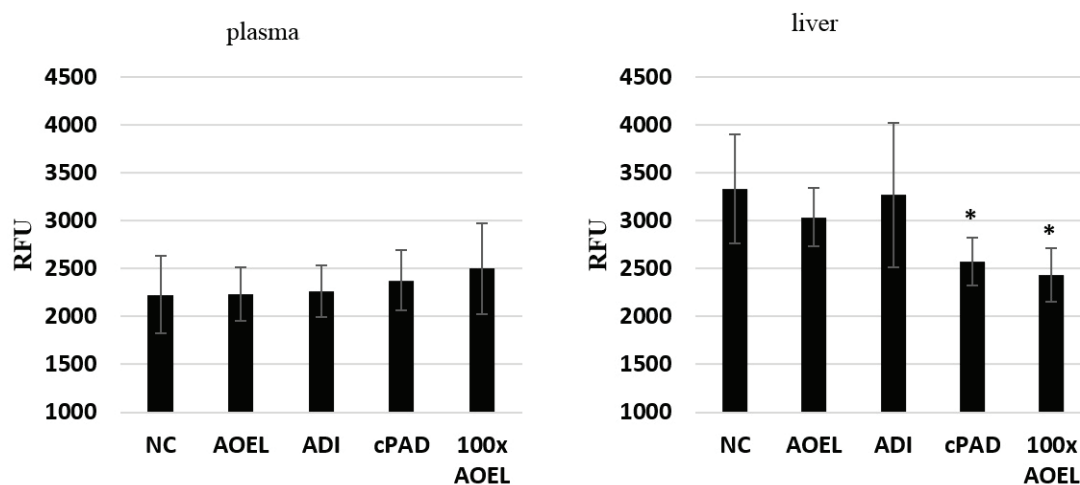


Figure 5 GSH activity in the plasma and liver tissue of Wistar rats receiving different glyphosate doses over 28 days. Statistical significance was set at $p < 0.05$. *Significantly different from the negative control. NC – negative control receiving 1 mL of PBS; AOEL – acceptable operator exposure level ($0.1 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); ADI – acceptable daily intake for consumers ($0.5 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); cPAD – chronic population adjusted dose ($1.75 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); 100xAOEL – 100 times the AOEL ($10 \text{ mg kg}^{-1} \text{ bw day}^{-1}$)

after long-term exposure to oxidants. Low glyphosate doses we tested did not produce significant changes in the GSH level. In the study of El Shenawy et al. (67), higher glyphosate availability after one and two weeks of *i.p.* treatment with 135 and 270 mg kg⁻¹ in rats every other day caused a decrease in liver GSH vs. control samples. Mesnage et al. (35) also found slightly decreased liver GSH in a two-year study of female Sprague-Dawley rats receiving 4 ng kg⁻¹ bw of a glyphosate equivalent in a pesticide formulation Roundup through drinking water a day. They also observed lower levels of cysteine and increased levels of gamma-glutamyl dipeptides, which suggests that the two-year exposure led to a redistribution of cellular cysteine stores toward glutathione synthesis.

In our study, glyphosate at higher doses stimulated the antioxidant defence system by increasing the activity of GSH-Px in the liver. This is in line with several other studies reporting increased GSH-Px (20, 79, 80) after glyphosate treatment. Our findings are also in line with Alp et al. (81), who demonstrated that daily exposure of Wistar rats to lower glyphosate doses (4 mg kg⁻¹ bw) lowered the total antioxidant status. Larsen et al. (24), in turn, demonstrated enhanced metabolic activity of selenium-independent GPx in kidneys and small intestines, but not in the liver after 90 days exposure to glyphosate.

Cholinesterase activity in plasma

Figure 7 shows the effects of glyphosate on rat plasma ChE activities. Glyphosate did not significantly affect total ChE, even though it dropped by about 35 % in the AOEL and ADI groups compared to control. The only significant difference was between the ADI and the 100xAOEL group.

AChE activity in turn, did drop significantly in the AOEL and ADI group compared to control.

BChE varied across the groups, but the only significant difference was between the ADI and the 100xAOEL group.

Inhibition of acetylcholinesterase (AChE) activity as a toxicological endpoint of glyphosate has been a matter of debate. Although it is structurally related to organophosphates, glyphosate lacks a specific chemical group such as a halide, sulphur, or thiocyanate group on the phosphorus atom to bind to the active centre of AChE. For the US EPA (5), this fact was a sufficient reason not to assess the neurotoxicological effects of glyphosate. But in 2009, the US EPA changed their mind (45) and called for acute and subchronic neurotoxicity studies, whose findings are due this year. Several independent reports, however, have shown AChE inhibition in non-mammalian species by environmentally relevant doses of glyphosate (28, 82-88). At low concentrations, glyphosate seems to cause neurotoxic effects indirectly, that is, through the glycine in its chemical structure, which is also part of different proteins, enzymes, and mechanisms in the body. As an analogue to glycine, glyphosate can affect AChE in two ways: by interfering with the synthesis of glycogen synthase kinase 3, whose overexpression can inhibit acetylcholine synthesis (2, 89) or by decreasing the synthesis of hormone-sensitive lipases (again glycine substituents) that are distantly related to AChE levels (2, 89). So far, only our study and the study of Larsen et al. (89) have demonstrated AChE inhibition in rats, with the difference that the inhibition in our study was significant with the two lowest doses.

Our study suggests that sub-chronic exposure to glyphosate mostly affects DNA in the liver and white blood cells. We have not confirmed general oxidative stress, while total cholinesterase activity showed some, yet inconsistent, deviations from control. Consistent, however, was significantly diminished AChE activity with all tested doses.

The discrepancy between our and other reported findings may primarily be owed to different exposure routes, treatment duration, and glyphosate doses used. Still, some

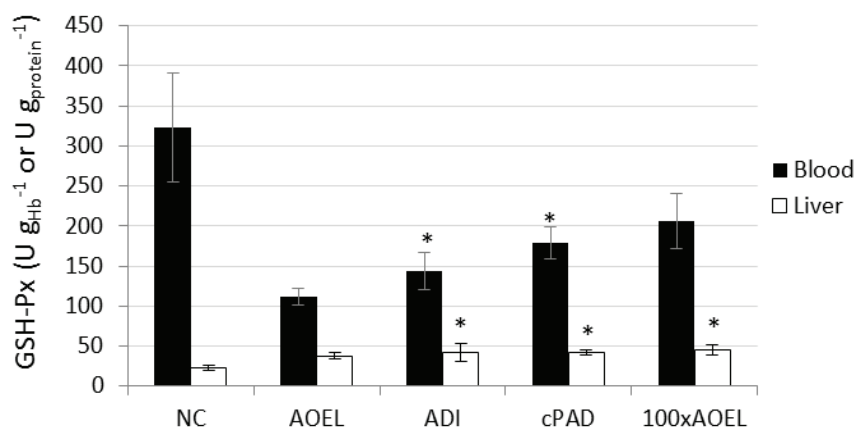


Figure 6 GSH-Px activity in the whole blood and liver tissue of Wistar rats receiving different glyphosate doses over 28 days. Statistical significance was set at $p < 0.05$. *Significantly different from the negative control. NC – negative control receiving 1 mL of PBS; AOEL – acceptable operator exposure level (0.1 mg kg⁻¹ bw day⁻¹); ADI – acceptable daily intake for consumers (0.5 mg kg⁻¹ bw day⁻¹); cPAD – chronic population adjusted dose (1.75 mg kg⁻¹ bw day⁻¹); 100xAOEL – 100 times the AOEL (10 mg kg⁻¹ bw day⁻¹)

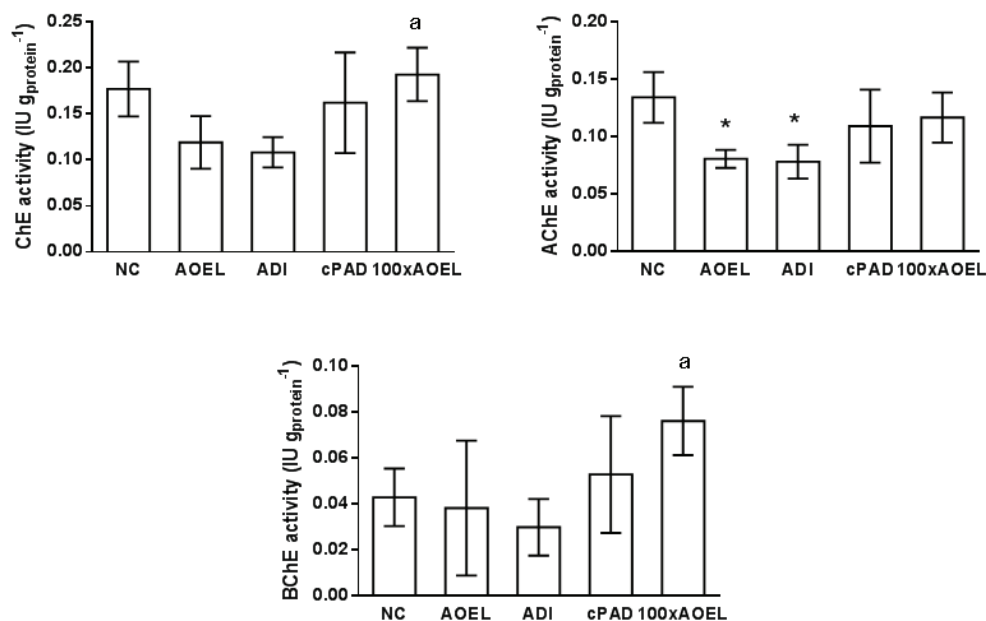


Figure 7 Changes in ChE, AChE, and BChE activity in the plasma of Wistar rats receiving different glyphosate doses over 28 days. The results are shown as mean and SD values. Significantly different values ($P < 0.05$) were: (*) compared to control, (a) compared to ADI. NC – negative control receiving 1 mL of PBS; AOEL – acceptable operator exposure level ($0.1 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); ADI – acceptable daily intake for consumers ($0.5 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); cPAD – chronic population adjusted dose ($1.75 \text{ mg kg}^{-1} \text{ bw day}^{-1}$); 100xAOEL – 100 times the AOEL ($10 \text{ mg kg}^{-1} \text{ bw day}^{-1}$)

reports (2, 35, 46, 83), like ours, suggest that glyphosate may produce harmful effects even at low doses. As for exposure duration, several short-term studies did not demonstrate toxic effects (as reviewed in 32), but lifelong exposure was associated with kidney and liver toxicity in rats (33-35). Higher toxicity was also reported for sub-chronic and chronic exposure (35, 37).

Exposure route entails another set of issues to consider. Intravenous (*i.v.*) and intraperitoneal (*i.p.*) routes used in earlier studies resulted in much higher bioavailability of the tested compound than the oral route (43). We, however, opted for oral exposure as the most likely exposure route in humans. When a tested compound is administered orally, as glyphosate was in our study, its metabolism also has to be taken into account, as it can produce even more reactive and toxic substances.

However, only 6 % of the parent glyphosate compound administered orally is transformed into its first pass metabolites aminomethylphosphonic acid (AMPA), *N*-methylAMPA, and *N*-acetylgllyphosate (42, 43) and both glyphosate and AMPA have a similar half-day elimination rate (43). Another thing to consider is glyphosates poor absorption through the gastrointestinal tract. Almost 90 % of glyphosate is excreted unchanged through faeces or urine in 72 hours, while 1 % persists in the tissues, colon and bone in particular, even after seven days (42). This points to the risk of its bioaccumulation, which could affect the results of some of the analytical methods used, such as the alkaline comet assay. The treatment schedule we applied caused a constant delivery of new amounts of the tested compound. This means that the parent compound and its

metabolites constantly induced primary DNA damage. The level of DNA damage we measured, therefore, represents the sum of both direct and repair-induced DNA lesions, since after 28 days equilibrium between DNA damage infliction and repair might occur.

Our study also has some limitations. Since the comet assay pointed only to the primary DNA damage, further studies are needed before we can draw a general conclusion about glyphosate genotoxicity. These studies should focus on biomarkers that provide more insight into aneugenic effects, epigenetic mechanisms of DNA damage, and cell-cycle disturbances. Furthermore, we did not assess inflammatory processes as possible confounders involved in hepatocellular damage, which were noticed in previous studies. For instance, Kumar et al. (90) and Tang et al. (66) demonstrated that glyphosate exposure increased mRNA expression of inflammatory parameters and concluded that glyphosate-induced liver toxicity is mediated by inflammation, oxidative stress, and lipid-related pathways. All this remains to be proven in future studies.

CONCLUSION

Exposure to environmentally relevant glyphosate levels, presumably not harmful to humans, seems to have different effects from exposure to much higher glyphosate doses (our 100xAOEL and doses reported elsewhere), especially where oxidative stress is concerned. We have demonstrated that, even without oxidative stress, small doses (allowed for human exposure) can produce significant primary DNA

damage and inhibit AChE, which may both be related to indirect action through glycine substitution. In fact, our study suggests that the mechanisms of action depend on the exposure dose and that a new approach is needed to study the effects of small chronic and sub-chronic exposure, which has also been suggested by Mesnage et al. (35). Even though we are far from the conclusion about whether small doses in subchronic and chronic exposures are safe, a risk for human health certainly cannot be excluded.

Acknowledgement

This work was financially supported by Project Organic Pollutants in Environment – Markers and Biomarkers of Toxicity (OPENTOX), funded by the Croatian Science Foundation (grant number 8366).

Conflicts of interest

None to declare.

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Oksidacijski stres, aktivnost kolinesteraza i primarna oštećenja u jetri, krvi i plazmi Wistar štakora nakon 28-dnevnog izlaganja glifosatu

U okviru 28-dnevnog pokusa istražili smo učinke herbicida glifosata na modelu odraslih mužjaka Wistar štakora koji su oralno dobivali testirani spoj u subletalnim dnevnim dozama: 0,1 od prihvatljive razine izloženosti operatera (0,1xAOEL), 0,5 od prihvatljivog dnevnog unosa za potrošače (0,5xADI), 1,75 (odgovara kroničnoj populacijskoj prilagođenoj dozi, cPAD) i 10 mg kg⁻¹ tjelesne težine na dan (odgovara 100xAOEL). Tijekom pokusa praćeni su sistemski toksični učinci. Nakon završetka svih tretmana svakoj je pokusnoj životinji izmjerena tjelesna težina i težina jetre te su uspoređene s polazišnim vrijednostima. Alkalnim komet-testom izmjerena je razina primarnih oštećenja DNA u leukocitima i jetrenim stanicama. Primjenom metoda za procjenu oksidacijskog stresa izmjerene su razine lipidne peroksidacije (TBARs), reaktivnih kisikovih vrsta (ROS) i glutationa (GSH) te aktivnost enzima glutation peroksidaze (GSH-Px). Izmjerene su i aktivnosti ukupnih kolinesteraza (ChE), acetilkolinesteraze (AChE) i butirilkolinesteraze (BChE). Izloženi štakori imali su manje priraste težine od kontrolnih. Izloženost glifosatu uzrokovala je značajne poraste razine primarnih oštećenja DNA u jetrenim stanicama te malo manje u leukocitima. U svim izloženim skupinama izmjerene su niže vrijednosti TBARs u odnosu na kontrolu, sa značajno nižim vrijednostima u AOEL, ADI i cPAD skupinama u uzorcima jetre te u AOEL i cPAD skupinama u uzorcima plazme. Aktivnost AChE bila je smanjena u svim tretmanima, s najnižom stopom nakon izlaganja dozi ADI. Aktivnost BChE blago je smanjena nakon izlaganja ADI, a povećana nakon izlaganja dozama cPAD i 100xAOEL. Ukupna aktivnost ChE te razine ROS/GSH u plazmi / jetri nisu se značajno razlikovale od kontrole, osim značajnog smanjenja jetrenog GSH nakon izlaganja dozama cPAD i 100xAOEL te 35-postotnog smanjenja aktivnosti ChE nakon izlaganja dozama AOEL i ADI. Aktivnost GSH-Px u krvi značajno je smanjena u AOEL i ADI tretmanu, a aktivnost GSH-Px u uzorcima jetre značajno je povećana u skupinama ADI, cPAD i 100xAOEL prema kontroli. Dobiveni rezultati pokazuju da čak i izloženost vrlo niskim dozama glifosata može izazvati mjerljive toksične učinke te upućuje na potrebu za promjenom pristupa procjeni rizika zbog kronične/subkronične izloženosti niskim dozama glifosata gdje oksidacijski stres ne mora nužno korelirati s razinom oštećenja DNA i inhibicijom acetilkolinesteraze.

KLJUČNE RIJEČI: ADI; aktivnost kolinesteraza; alkalni komet test; AOEL; cPAD; glutation; glutationska peroksidaza; lipidna peroksidacija; organofosforni pesticidi; ROS