Chapter

DNA Damage and Glutathione Peroxidase Activity in Liver and Kidney Cells in Wistar Rats Exposed to Terbuthylazine (TERB) for 28 Consecutive Days

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Abstract

The potential of low doses of the chloro-triazine herbicide terbuthylazine to induce DNA damage and impair activity of glutathione peroxidase (GPx) was evaluated in kidney and parenchymal and non-parenchymal liver cells of adult male rats. In a 28-day study, terbuthylazine was applied daily by oral gavage at doses: 0.004, 0.4 and 2.29 mg/kg bw/day. Tail Intensity (T Int) and Tail Length (TL) were used as descriptors of DNA damage. In the kidney, Tail Int was significantly different in all treated groups, while TL was different in 0.4 and 2.29 mg/kg bw/day groups, compared to controls. Significant differences in TL were recorded in parenchymal and non-parenchymal liver cells of all treated groups. Tail Int was significantly different from controls in non-parenchymal liver cells at all applied doses and in parenchymal cells at terbuthylazine doses of 0.004 and 2.29 mg/kg bw/day. A significant increase in GPx activity was observed only in the kidney at doses 0.4 and 2.29 mg/kg bw/day compared to the controls indicating its possible role in the protection of kidney from free radicals. It appears that repeated exposure to low doses of terbuthylazine could cause DNA instability in kidney cells and in parenchymal and non-parenchymal liver cells in rats.

1. Introduction

Terbuthylazine (TERB) is a chloro-s-triazine herbicide, mostly used for the removal of weeds to protect crops [1]. It is also used as an aquatic herbicide to control submerged and free-floating weeds and algae in fish ponds, swimming pools and reservoirs [2, 3]. It is the most frequently used triazine in Europe in the last two decades [4, 5]. The EFSA (2011) pointed out that TERB poses a high risk to non-target plants in the off-field areas, while the risk for soil micro- and macro-organisms and bees is low. Mammals could be exposed to TERB through oral,

dermal or inhalation routes [6]. The acute toxicity of TERB can be low to moderate, causing slight eye and skin irritation and sensitisation. TERB shows adverse effects on the cellular activity of enzymes such as aromatase, an enzyme which converts androgen to oestrogen [6], and leads to cytotoxicity, as well as affects the functions of the kidney and liver [7, 8].

People are exposed to TERB in several ways, occupationally through inhalation and dermal contact at workplaces and at places where TERB is produced or used. The general population is mostly exposed through ingestion of contaminated drinking water or by dermal contacts [8].

This herbicide persists in the environment and easily moves from treated soil to water [9, 10]. The current cancer classification states TERB belongs to Group D "Not classifiable as a human carcinogen" [7].

Literature data shows that herbicides have the potential to induce reactive oxygen species (ROS), leading to oxidative stress on non-target organisms [11]. The first line of defence against the oxidative stress consists of the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which convert superoxide anions (O_2^-) into H_2O_2 and then into H_2O and O_2 [12–14]. Studies performed in experimental animals showed that atrazine and TERB mostly affected antioxidant defence of exposed animals, depending on species, its sex and age, herbicide concentration, and duration of exposure [15].

An US EPA study [8] indicated that TERB is highly toxic to aquatic organisms. It causes dose-dependent morphological changes and damage in gills, intestine, and kidney of fish [16], and disturbances in biochemical and oxidative stress parameters in carp [17]. Recent studies in aquatic organisms indicated that TERB can induce oxidative stress [18–20] and accumulation of ROS in cells [17, 21–23].

As for TERB genotoxicity, data are limited, i.e. no genotoxic effect was found using the bone-marrow micronucleus test both in female and male mice [24]. In an *in vitro* study, Mladinić et al. [25] showed that TERB could produce primary DNA damage in a 14-day extended human lymphocyte culture. Tariba Lovaković et al. [26] in *in vivo* study on rats exposed orally to TERB points to the disturbance of oxidant/antioxidant balance in erythrocytes and plasma. Plasma SOD activity decreased at two of the smallest doses (0.004 and 0.4 mg/kg bw/day), as well as plasma CAT activity at the highest applied dose (2.29 mg/kg bw/day).

The liver is the primary site of the metabolism, detoxification and excretion of potentially toxic substances [27]. The xenobiotic metabolism starts in the liver immediately after absorption from the gastrointestinal tract. One of the reasons for this is the fact that the liver has the highest supply of biotransformation enzymes of all organs in the body. Literature data [28] point to the fact that the liver is a complex organ with multiple cell types. Hepatocytes represent 60–65% of total rat liver cells [29]. They are responsible for the drug metabolism [30]. Their function is dependent on their micro environment, i.e., of direct cell-cell and cell-matrix interactions, and a lot of diffusible factors secreted by nearby non–parenchymal cells [31]. Non-parenchymal cells represent 40% of the total number of liver cells, but only 6.5% of its volume. These cells contribute to inflammatory responses. Several studies highlighted the importance of non-parenchymal liver cells and their responses to drug toxicity [32–36]. The main process of TERB metabolism in plants and animals is side-chain de-alkylation and oxidation to 2-hydroxy derivatives [37].

The aim of the present study was to investigate the impact of TERB on liver and kidney of adult male Wistar rats treated by oral gavage for 28 consecutive days. We determined levels of primary DNA damage by comet assay measuring Tail Length (TL) and Tail Intensity (T Int) in kidney and two types of liver cells: small or non-parenchymal cells (sized <30 μm head length) and medium sized cells or parenchymal cells or hepatocytes (sized between 30 and 40 μm head length).

We also measured the GPx activities in liver and kidney tissue to determine the extent of oxidative stress caused by the treatment. The results of the present study could contribute to understanding TERB's potential toxicity related to its low dose exposure.

2. Materials and methods

This experiment was conducted at the Institute for Medical Research and Occupational Health (IMROH), Zagreb, Croatia, in Animal Breeding Unit, Mutagenesis Unit and Analytical Toxicology and Mineral Metabolism Unit, in spring 2016.

2.1 Test substance and positive control substance

Terbuthylazine (CAS number 5915-41-3), purity grade 99.0%, was purchased as analytical standard from Pestanal® quality (Fluka, Sigma Aldrich, Germany) and dissolved in ethanol (EtOH) to prepare a stock solution. To prepare treatment solutions, the stock solution was diluted with sterile redistilled water. Ethyl methanesulfonate (EMS) was purchased from Sigma Aldrich, Germany.

Negative controls received water, whereas positive controls received ethyl methanesulfonate (EMS) at 300 mg/kg bw/day the last three days of the experiment. EMS is a monofunctional alkylating agent recommended for *in vivo* comet assay in rodents [38].

2.2 Animals

The study was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health (IMROH), Zagreb, Croatia and the Croatian Ministry of Agriculture (Reg.no. 100-21/14-5, Class 01-18/14-02-2/6 of 11 June 2014). Animal treatments were carried out according to internationally accepted animal welfare guidelines [39]. The study was performed using 25 healthy male adult Wistar rats with an initial body weight from 231 g to 271 g. Free access to standard food (Mucedola, 4RF21, Italy) and tap water was ensured. Animals were kept in clear polycarbonate cages with 40–60% humidity at 22°C and normal 12-hr light/dark cycle. At the start of the study, the animals were weighted and inspected by a licenced veterinarian at IMROH.

2.3 Experimental schedule

Rats were randomly assigned to the five groups composed of five animals as recommended by JaCVAM (Japanese Center for the Validation of Alternative Methods) [40]. Three groups received TERB at doses of 0.004, 0.4 and 2.29 mg/kg bw/day, respectively, for 28 consecutive days by an oral gavage. These doses were selected based on the reference values set by the EFSA [6]. Negative controls received water, whereas positive controls received ethyl methanesulfonate (EMS) at 300 mg/kg bw/day over the last three days of the experiment. All animals were handled in the same manner.

Body weights were regularly monitored (once a week) during the experiment and the doses of TERB were adjusted accordingly. Survival and clinical signs of intoxication were also inspected daily by a licenced veterinarian at IMROH.

The experiment was terminated 24 h after the final gavage. All animals were humanely euthanized by exsanguination under Xylapan/Narketan anaesthesia (Xylapan, Vetoquinol UK Ltd., 12 mg/kg bw *i. p.*/Narketan, Vetoquinol UK Ltd., 80 mg/kg bw) and dissected. Animals were examined for gross pathological changes of the internal organs by a licenced veterinarian at IMROH.

At the end of each treatment, the body weight of rats was determined and compared with the initial body weight. Liver and kidney weight were also measured. Based on the obtained values, relative kidney (ROW_{kidney}) and liver weight (ROW_{liver}) were calculated using the following formula: ([organ weight/body weight at sacrifice day] × 100).

2.4 Slide preparation and the alkaline comet assay

Preparations of single cells were done within 1 h following sacrifice. Livers and kidneys were dissected and rinsed in cold TBS buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.5) [41] until as much blood as possible was removed. A small piece of tissue was put in chilly mincing buffer [75 mM NaCl (Kemika, Zagreb, Croatia) and 24 mM Na_2EDTA, pH 7.5] and minced with a pair of fine scissors to release single cells. The obtained cell suspension was stored on ice for a few seconds to allow large clumps to settle, and the supernatant was used to prepare agarose microgels for the alkaline comet assay. Slides were immersed in chilled lysing solution for at least 3 h in a refrigerator in the dark. Then the slides were rinsed in purified water to remove residual detergent and salts prior to the alkali unwinding step. Slides were randomly placed onto a platform of submarine type electrophoresis unit (Horizon 11.14, Whatman, Florham Park, NJ, USA) in the chilled electrophoresis solution, and left to unwind for 10 min. After 10 min of denaturation, the slides were electrophoresed at 1 V/cm for 10 min, with a constant voltage at approximately 300 mA, at +4°C [41]. The slides were then immersed in neutralisation buffer (0.4 M Tris buffer, pH 7.5) for at least 3 × 5 min. All slides were dehydrated by 70% ethanol and 96% ethanol (10 minutes each), air dried and stored at room temperature protected from humidity.

Slides were stained with ethidium bromide (20 μg/mL; Sigma, St. Louis, MO, USA) and analysed under an epifluorescence microscope (Olympus BX 50, Olympus, Tokyo, Japan), equipped with appropriate filters, under 200x magnification.

Three hundred cells (150 cells from each of two replicate slides, per each animal; five animals per group) were selected and analysed with a Comet Assay IV™ image analysis system (Instem-Perceptive instruments Ltd., Suffolk, Halstead, UK). All the comet measurements were performed on coded/blinded slides by the same person, experienced in scoring. When selecting cells, the areas around air bubbles or at the edges were avoided [42]. Two descriptors of primary DNA damage were selected, TL and a percentage of DNA in tail (T Int, expressed in % DNA).

Nucleoids with >80% DNA in the tail region were excluded from analysis of % tail DNA. They consisted of small or non-existent head and large, diffuse tails, and, according to literature data, they may represent DNA damage resulting from cytotoxicity [43, 44].

2.5 Determination of GPx activity in liver and kidney

Glutathione peroxidase (GPx) activity in liver and kidney supernatant was determined spectrophotometrically according to the European standardised method [45]. Briefly, 50 μl of liver/kidney supernatant was diluted with 500 μl of DL-Dithiothreitol (0.1 mol/L, Sigma, St. Louis, MO, USA). After 5 min of stabilisation, samples were further diluted (10 times) with double strength Drabkin's reagent and kept at 4°C until analysis. Portions of 0.8 mL of reaction mixture containing 0.1 mmol phosphate buffer pH 7.0, 0.01 mmol Na_2EDTA (Merck, Darmstadt, Germany), 1 EU glutathione reductase, 5 μmol of GSH and 0.25 μmol β-NADPH (Sigma, St. Louis, MO, USA) and 100 μl of diluted sample were pipetted into a measurement tube. The reaction was initiated

with 0.1 mL t-butyl hydroperoxide (2.5 μmol, Sigma, St. Louis, MO, USA). The amount of GSH oxidised by t-butyl hydroperoxide was determined by following the decrease in the β-NADPH concentration, and the decrease in absorbance was measured at 340 nm (Cary 50 UV–Vis, Varian Inc. CA, USA). One unit of GPx is the number of micromoles of β -NADPH oxidised per minute. The results were expressed as IU/g protein.

Protein content was measured by Bradford assay [46] using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

2.6 Statistics

Statistical analysis was run using STATISTICA, version 13.2 (Dell Inc., Round Rock, TX, USA) software. Normality of data distribution was tested with Shapiro-Wilk's test. The data obtained for body and organ weight and GPx activity were normally distributed, while the data obtained for DNA damage were not. The results were expressed as means ± standard error, standard deviation, medians and ranges (min–max).

Data on weight of animals on the sacrifice day, liver and kidney weights, relative liver and kidney weights and GPx activity in both tissues were analysed with one-way ANOVA. For pairwise comparison, *post hoc* Tukey's HSD test was used.

Kruskal Wallis ANOVA by Ranks test with multiple comparisons (two tailed) was conducted to examine the differences in two descriptors of the alkaline comet assay (TL and T Int) and different herbicide doses. Statistical significance was set at $p < 0.05$.

3. Results

There was no incidence of mortality recorded in all the examined groups throughout the 28-day exposure period.

3.1 Body and organ mass, and relative organ weight

To assess the toxicity of applied TERB doses, the body mass of rats treated 28 days with 0.004, 0.4 and 2.29 mg/kg of TERB per day was compared with the mass of rats on the day 0 (the day before treatment). The results showed a significant reduction in a body mass of negative control compared to positive control (One-way ANOVA with Tukey's HSD *post hoc* analysis: $F = 5.94$, $df = 4$, $p < 0.001$) and 0.4 mg/kg bw/day group compared to positive control ($F = 5.94$, $df = 4$, $p < 0.05$) on sacrifice day. The weight gain of the rats exposed to TERB at all of the three applied doses was about 20% lower than the weight gain of negative control (**Figure 1**).

The effect of TERB on the organ weights and relative organ weights (ROWs) of the liver and kidney of adult male Wistar rats is shown in **Table 1**.

When compared liver mass, significant reduction in mass of PC compared to NC ($F = 5.89$, $df = 4$, $p < 0.05$), and 0.4 mg/kg bw/day group compared to PC ($F = 5.89$, $df = 4$, $p < 0.05$) was observed. The liver mass of the 0.004 mg/kg bw/day group was significantly lower than NC ($F = 5.89$, $df = 4$, $p < 0.05$) and the 0.4 mg/kg bw/day treated group ($F = 5.89$, $df = 4$, $p < 0.05$). When compared relative liver weights (ROW_{liver}), significant differences between the 0.004 mg/kg bw/day group with regard to PC, 0.4 mg/kg bw/day and 2.29 mg/kg bw/day group ($F = 4.53$, $df = 4$, $p < 0.05$) were observed.

There were no significant differences between the examined groups in kidney mass. A significant difference between PC and the 0.004 mg/kg bw/day group ($F = 4.62$, $df = 4$, $p < 0.001$) was found for relative kidney weight.

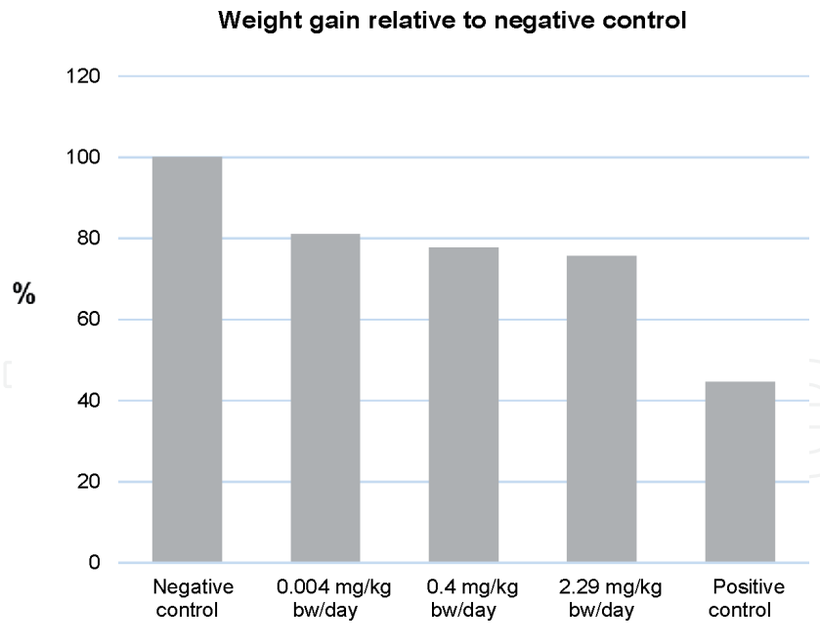

Figure 1.
Weight gain of the rats exposed to terbuthylazine at 28th day of consecutive exposure.

Organ	Negative control	Positive control[a]	Terbuthylazine 0.004 mg/kg bw/day	Terbuthylazine 0.4 mg/kg bw/day	Terbuthylazine 2.29 mg/kg bw/day
Animal weight on sacrifice day (g)	351 ± 10.89[PC] 24.35 366 312–368	289.2 ± 6.02 13.46 286 276–310	324 ± 5.61 12.55 318 314–345	333.6 ± 10.92[PC] 24.42 335 307–368	322.4 ± 11.06 24.72 327 286–352
Liver					
Organ weight (g)	10.35 ± 0,32[PC, b] 0.71 10.7 9.25–10.96	8.66 ± 0.37 0.82 8.57 7.58–9.76	8.79 ± 0.20 0.44 8.9 8.11–9.26	10.13 ± 0.51[PC,b] 1.33 10.48 8.96–11.6	9.77 ± 0.40 0.90 9.74 8.66–10.7
ROW_{liver}	2.95 ± 0.01 0.03 2.93 2.92–2.99	2.99 ± 0.09 0.20 2.91 2.75–3.24	2.72 ± 0.07[PC, c] 0.17 2.73 2.49–2.91	3.03 ± 0.06[b] 0.13 3.08 2.87–3.15	3.03 ± 0.05 0.10 3.02 2.92–3.2
Kidney					
Organ weight (g)	1.13 ± 0.04 0.09 1.15 1.01–1.24	1.01 ± 0.04 0.08 1.00 0.93–1.13	0.99 ± 0.03 0.07 0.99 0.93–1.11	1.07 ± 0.04 0.10 1.13 0.95–1.16	1.05 ± 0.03 0.08 1.03 0.96–1.15
ROW_{kidney}	0.32 ± 0.01 0.01 0.32 0.31–0.34	0.35 ± 0.01 0.01 0.35 0.33–0.36	0.31 ± 0.01[PC] 0.01 0.31 0.29–0.32	0.32 ± 0.01 0.01 0.31 0.31–0.34	0.33 ± 0.01 0.02 0.33 0.3–0.36

ROW_{kidney}: relative kidney and ROW_{liver}: relative liver weight.
The data are presented as mean ± SE (n = 5) and were evaluated by one-way ANOVA confirmed by Tukey's test. As an additional information, the data on standard deviation, median and range (min-max) were also obtained.
[a]EMS(ethyl methanesulfonate) 300 mg/kg bw/day for the last three days of application.
[b]Statistically significant compared to 0.004 mg/kg bw/day.
[c]Statistically significant compared to 2.29 mg/kg bw/day.
[PC]Statistically significant compared to positive control.

Table 1.
The effect of terbuthylazine on the organ weights (mean ± SE) and relative organ weights (mean ± SE) (ROWs) of the liver and kidney of adult male Wistar rats.

3.2 Antioxidant response

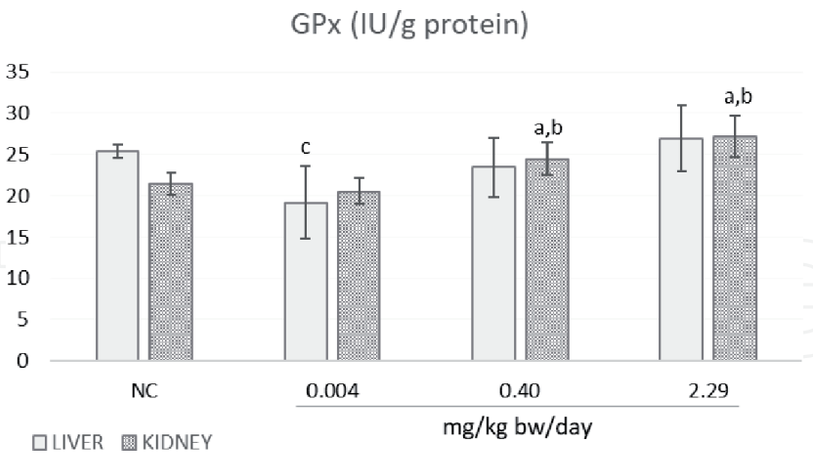

Figure 2.
The effects of the 28-day exposure to terbuthylazine on the activity of glutathione peroxidase (GPx) in the liver and kidney tissue of Wistar rats. Values are expressed as mean ± standard deviation of five rats per group (one way ANOVA followed by post hoc Tukey's HSD test for between groups comparisons, $p < 0.05$; a – significantly different from negative control (NC), b – significantly different from group treated with 0.004 mg/kg bw/day of terbuthylazine, c - significantly different from the group treated with 2.29 mg/kg bw/day of terbuthylazine).

	Kidney cells	
	Tail length ± SE SD Median Min.-max.	Tail intensity ± SE SD Median Min.-max.
Positive control EMS (300 mg/kg bw/day) for the last three days of treatment	$29.97 \pm 0.15^{NC,a,b,c}$ 5.33 30 12.92–54.17	$14.51 \pm 0.21^{NC,a,b,c}$ 7.28 13.77 0.01–39.29
Negative control	$18.33 \pm 0.11^{PC,a,c}$ 4.07 17.08 10–44.17	$1.33 \pm 0.08^{PC,a,b,c}$ 3.13 0.07 0–48.13
0.004 mg/kg bw/day	$19.33 \pm 0.16^{PC,c}$ 6.03 17.5 10.83–65.83	$2.15 \pm 0.11^{PC,NC,c}$ 4.31 0.13 0–39.74
0.4 mg/kg bw/day	$19.74 \pm 0.16^{PC,NC,c}$ 6.06 18.33 9.58–55.42	$2.17 \pm 0.11^{PC,NC,c}$ 4.32 0.17 0–38.71
2.29 mg/kg bw/day	$20.78 \pm 0.18^{PC,NC,a,b}$ 6.80 19.58 10.83–67.92	$2.42 \pm 0.11^{PC,NC,a}$ 4.17 0.22 0–26.63

The data were analysed using non-parametric Kruskal Wallis ANOVA by Ranks test with multiple comparisons (two tailed) for comparison between different treatment groups. Statistical significance was set at $p < 0.05$.
[PC]Significant compared to positive control.
[NC]Significant compared to negative control.
[a]Significant compared to 0.004 mg/kg bw/day.
[b]Significant compared to 0.4 mg/kg bw/day.
[c]Significant compared to 2.29 mg/kg bw/day.

Table 2.
The comet assay parameters determined in kidney cells of rats exposed to terbuthylazine and corresponding controls.

In the current study, GPx activity in liver and kidney tissue homogenate was determined in order to assess the liver and kidney function and TERB-induced injury. The effect of 28 consecutive days of oral exposure to TERB on the activity of GPx in liver and kidney is presented in **Figure 2**.

A significant increase of GPx activity was observed in livers of animals treated with TERB ($F = 6.839$, $p < 0.05$, One way ANOVA followed by *post hoc* Tukey's HSD test for between groups comparisons). However, there was no significant difference between negative controls and treated animals. A comparison between groups showed a significantly higher activity of liver GPx in rats treated with 2.29 mg/kg bw/day of TERB compared to those treated with 0.004 mg/kg bw/day.

GPx activity significantly increased in kidney of animals treated with TERB ($F = 18.128$, $p < 0.001$, One way ANOVA followed by *post hoc* Tukey's HSD test for between groups comparisons). A comparison between groups showed a significantly higher activity of kidney GPx in rats treated with 0.4 and 2.29 mg/kg bw/day of TERB compared to negative control and rats treated with 0.004 mg/kg bw/day.

	Liver- non-parenchymal cells		**Liver-parenchymal cells**	
	Tail length ± SE SD Median Min.-max.	**Tail intensity ± SE SD Median Min.-max.**	**Tail length ± SE SD Median Min.-max.**	**Tail intensity ± SE SD Median Min.-max.**
Positive control EMS (300 mg/kg bw/day) for the last three days of treatment	34.91 ± 0.15^{NC,a,b,c} 5.83 34.58 10–61.25	16.21 ± 0.18^{NC,a,b,c} 6.97 15.72 0–37.60	38.50 ± 0.15^{NC,a,b,c} 5.86 38.33 14.58–77.5	15.20 ± 0.15^{NC,a,b,c} 5.92 15.07 0–38.48
Negative control	18.40 ± 0.13^{PC,a,b,c} 4.40 17.08 9.58–44.17	1.19 ± 0.08^{PC,a,b,c} 2.65 0.05 0–33.37	22.54 ± 0.14^{PC,a,b,c} 4.84 21.67 12.5–63.75	1.53 ± 0.09^{PC,a,c} 3.24 0.07 0–32.26
0.004 mg/kg bw/day	20.21 ± 0.13^{PC,NC,b,c} 5.06 20 7.08–47.08	2.49 ± 0.11^{PC,NC,b} 4.22 0.34 0–28.35	23.79 ± 0.14^{PC,NC,b,c} 5.32 23.33 11.67–52.5	2.80 ± 0.12^{PC,NC,b} 4.78 0.33 0–32.47
0.4 mg/kg bw/day	21.79 ± 0.16^{PC,NC,a} 5.52 20.83 11.25–59.58	1.47 ± 0.09^{PC,NC,a,c} 2.97 0.14 0–26.11	25.39 ± 0.13^{PC,NC,a} 4.79 25.42 10.83–54.17	1.55 ± 0.08^{PC,a,c} 2.99 0.09 0–18.49
2.29 mg/kg bw/day	22.53 ± 0.19^{PC,NC,a} 7.20 21.67 11.67–62.5	2.31 ± 0.11^{PC,NC,b} 4.07 0.41 0–32.39	25.75 ± 0.16^{PC,NC,a} 6.17 25.42 11.67–65.42	2.51 ± 0.11^{PC,NC,b} 4.29 0.35 0–34.10

The data were analysed using non-parametric Kruskal Wallis ANOVA by Ranks test with multiple comparisons (two tailed) for comparison between different treatment groups. Statistical significance was set at $p < 0.05$. [PC] *Significant compared to positive control.*
[NC] *Significant compared to negative control.*
[a] *Significant compared to 0.004 mg/kg bw/day.*
[b] *Significant compared to 0.4 mg/kg bw/day.*
[c] *Significant compared to 2.29 mg/kg bw/day.*

Table 3.
The comet assay parameters determined in parenchymal and non-parenchymal liver cells of rats exposed to terbuthylazine and corresponding controls.

3.3 The alkaline comet assay

Tables 2 and **3** report results regarding the comet assay parameters determined in kidney, and parenchymal and non-parenchymal liver cells of rats exposed to TERB and corresponding controls. Background levels of primary DNA damage in all tissues were low. Treatment with TERB at all three applied doses caused increased DNA instability in both tissues compared to negative control. The DNA of liver cells was more prone to breakage after TERB treatment compared to DNA in kidney cells. In liver cells, there was no dose-related increase of DNA damage, in contrast to kidney cells, where such a damage pattern was observed, both in terms of TL and T Int (**Table 2**). Inter-group differences in the levels of DNA damage measured in all cell types and their statistical significance are reported in **Tables 2** and **3**.

4. Discussion

This study aimed to assess the DNA damage and impairment of GPx activity in kidney and liver cells in a consecutive 28-day oral exposure of adult male Wistar rats to low doses of TERB. An EFSA document [6] pointed out that in mammals exposed through the gastrointestinal tract, animal skin, or inhalation routes, TERB acute toxicity can be low to moderate. Besides, it also causes slight eye and skin irritation, as well as sensitisation. Short-term exposure may affect body weight and food consumption in rats, mice, dogs, and rabbits. Long-term exposure may further affect organ weights in rats and mice, as well as haematological parameters in rats.

The results regarding body weight and organ weight changes observed after treatments with TERB in this study suggest that this herbicide at the tested doses and applied experimental schedule was able to produce acute toxicity, which led to changes in the overall fitness of the exposed *vs*. control rats. In toxicological studies, body weight and relative organ weight are widely accepted as a parameter associated with treatment-related effects. In official documents of regulatory agencies [6, 47] a significant decrease in body weight as the main effect of acute and long-term oral TERB exposure in experimental animal models was indicated. Such changes were usually connected with decreased food consumption. Since rats in this experiment had free access to food and water, treatment-related distress possibly reduced their appetite. Furthermore, the weight loss in exposed rats could also be related to its detrimental effect of treatment on intestinal absorption. Furthermore, the effects on liver weight reduction could be related both to functional liver deficiencies, but also to the impairment of different essential processes at cell level. It is possible that treatment produces loss of hepatocytes, due to cytotoxicity and apoptotic potential of the tested herbicide.

The environmental contaminants such as herbicides modulate antioxidant defensive systems causing oxidative stress, an abnormal phenomenon, which occurs in our cells or tissues when production of oxygen radicals exceeds their antioxidant capacity. Antioxidant enzymes catalyse the decomposition of ROS. GPx enzymes are the most important hydrogen peroxide (H_2O_2) removing enzymes in mammalian cells [48]. The liver is the major organ attacked by ROS [49]. Parenchymal cells or hepatocytes are primary cells subjected to oxidative stress-induced injury in the liver. To maintain the redox homeostasis in the liver, a sophisticated antioxidant system in mammals has been developed. Moreover, systemic oxidative stress arising during liver disease can also cause damage to the kidney [50]. Literature data suggests that systemic oxidative stress is considered to play a critical role in the pathophysiology of several kidney diseases [51, 52]. All parts of the kidney are affected by oxidative stress. Both directly and indirectly,

vascular reactivity and renal hemodynamics, as well as glomerular filtration and tubular reabsorption and secretion in all nephron segments are included [53]. Disturbances in the antioxidant system could play a role in pathogenesis of chronic liver disease [54, 55]. GPx is one of the key enzymes in protecting the liver from the products of free radical reaction.

To the best of our knowledge, very little is known about the effects of TERB on oxidative stress parameters and antioxidant defence as well as primary DNA damage in mammals [15]. Studies on the influence of TERB exposure on parameters of oxidative stress are very rare. A few were performed in aquatic organisms such as the common carp (*Cyprinus carpio*) and red swamp crayfish (*Procambarus clarkii*). The results indicate that parameters of oxidative stress are altered under exposure to main degradation products of TERB at environmentally relevant concentrations, while TERB itself does not generally affect the oxidant/antioxidant balance in such conditions (reviewed in [15]).

In the current study, a slight disturbance in oxidant/antioxidant status was reflected in changes of the activities of GPx, mainly in the kidney. A significant increase in GPx activity was observed at 0.4 and 2.29 mg/kg bw/day compared to negative controls and animals exposed to 0.004 mg/kg bw/day of TERB. In the liver, no significant difference was observed between the negative control group and treated animals. However, significantly higher activity of liver GPx was observed in rats treated with 2.29 mg/kg bw/day of TERB compared to those treated with 0.004 mg/kg bw/day indicating that this effect could possess a certain toxicological risk at only slightly higher concentrations. The results suggest that repeated daily exposure to low doses of TERB stimulates the defending antioxidant mechanisms in order to alleviate the toxic effects of the produced reactive species.

It has previously been reported that triazine pesticides have a direct effect on kidney structure and function in freshwater fish [56–58]. The caudal kidney of common carps exposed to TERB showed alteration of tubular system of caudal kidney and the authors suggested that it was possible to describe TERB as a primary nephrotoxic substance. However, no similar study exists on rats with which we could compare our results.

In our earlier studies [15, 26], it was shown that TERB disturbs the oxidant/antioxidant balance in erythrocytes and plasma at the applied concentrations. Plasma SOD activity decreased at 0.004 and 0.4 mg/kg bw/day, as well as plasma CAT activity at 2.9 mg/kg bw/day. The observed increase of SOD activity in erythrocytes was most prominent at the highest applied concentration. An increase of whole blood GPx activity was observed at 0.4 mg/kg bw/day. Total antioxidant capacity expressed as plasma antioxidant power (FRAP) significantly increased at 0.004 and 0.4 mg/kg bw/day. In these experimental conditions, TERB did not induce lipid peroxidation.

The literature on primary DNA damage caused by TERB and related triazine herbicides in various cell types of *in vivo* exposed rodents is relatively rare. To the best of our knowledge, only a few studies used the alkaline comet assay as a method of choice in the evaluation of primary DNA damage in leukocytes of exposed mice or rats. Only a few studies have focused on *in vivo* [24] and *in vitro* experimental models [25, 59]. Using bone marrow micronucleus assay, Gebel et al. [24] did not find a genotoxic effect for TERB either in female or in male mice. In an environmental study in fish erythrocytes, Polard et al. [60] highlighted TERB as a potential water contaminant. Tennant et al. [61] tested the genotoxic effects of atrazine, simazine and cyanazine in mice following acute exposure at different doses, up to the maximum tolerated doses. They found relatively low genotoxicity in leukocytes. Singh et al. [62] studied the genotoxic effects of atrazine in male rats at the

high dose of 300 mg/kg bw in 7, 14 and 21 days study. They reported a significant increase in comet TL in atrazine-exposed animals compared to controls, in blood and liver cells. In a study of prometryn on mice exposed to three doses for 28 days, Đikić et al. [63] found dose- and exposure-related DNA damage in the leukocytes.

As for DNA damage, our results of alkaline comet assay showed that the T Int in kidney cells was significantly different at all three doses as compared to controls, while TL was significantly different in 0.4 and 2.29 mg/kg bw/day groups compared to controls. In non-parenchymal liver cells, significant DNA damage (TL and T Int) was observed in all applied doses of TERB. Significant differences in TL were recorded in parenchymal liver cells at all applied TERB doses, while T Int was significantly different from controls at doses of 0.004 and 2.29 mg/kg bw/day.

The absence of a clear dose-response as seen from the T Int descriptor of the alkaline comet assay results (T Int in dose 0.004 mg/kg bw/day is greater than in 0.4 mg/kg bw/day dose, and in the highest applied dose) reported in **Table 2** may be explained by the fact that at higher TERB doses, actual DNA damage is possibly greater, and these highly damaged cells are lost from scoring. In this study, we excluded nucleoids whose TL exceeded 80, and head intensity was under 60. Thus, it is possible that the remaining cells that we measured by the image analysis system had less DNA damage. Such results could be the consequence of the presence of apoptotic cells. These cells have highly fragmented nucleoids which can be "washed" out from agarose gel during the comet assay processing. In such conditions, less damaged nucleoids will be measured, and the obtained values would be lower than real damage. The influence of apoptosis on DNA damage was established in Choucroun et al. and Roser et al. comet assay studies [64, 65].

The obtained results confirmed exposure-related genotoxicity in both types of liver cells. We could assume that a possible outcome of 28-day repeated exposure to TERB is a decrease in overall hepatic function.

Since there are no related comet assay studies on rats administered TERB in similar low doses, it is not possible to draw a parallel between our findings and other literature sources and propose the mechanisms behind the observed primary DNA damage.

In our earlier studies [26, 66], a slight induction of DNA damage in TL in rats leucocytes using alkaline comet assay, as well as reticulocyte frequency in MN *in vivo* assay, was observed. Two descriptors of the comet assay, TL and T Int were both lower at the two higher concentrations. Such results lead us to conclude that one of the possible mechanisms of action of TERB on DNA molecule could be intercalation, which could result in slower migration of DNA during electrophoresis. These effects could be explained by the ability of adaptation to the repeated doses of TERB. Kaware [67] suggested that liver tissue morphological changes during exposure to a toxicant could be adaptive mechanisms that allow animals to rapidly get rid of toxic compounds from the liver, through rapid metabolism and excretion, as sustained insults may lead to possible irreversible damages.

In a study on mice exposed intraperitoneally to TERB for 14 days at a daily dose of 0.0035 mg/kg, Želježić et al. [68] found a significant increase in mean TL and T Int in leucocytes, bone marrow cells, and liver cells compared to the control group. They found a significant increase of mean TL and T Int compared to controls in kidney cells of animals exposed to the formulated product Radazin TZ-50. Their results suggested that TERB metabolism possibly results in the formation of reactive metabolites capable of inducing DNA cross-links, which hinder DNA migration, and these effects were most pronounced in liver cells *in vivo*. The authors pointed to the fact that the differences in DNA damage between different cell types originated from the intrinsic metabolic differences between them.

5. Conclusions

From a toxicological point of view, this study shows that repeated *in vivo* exposure to low doses (0.004, 0.4 and 2.29 mg/kg bw/day) of TERB led to low-level DNA instability in kidney and non-parenchymal and parenchymal liver cells of adult male Wistar rats. An influence of applied low doses on GPx activity in kidneys was detected. Such results could point to the possible role of GPx as a key enzyme in kidney protection from hazardous products of free radical reactions. They can also reflect the response to increased oxidative stress.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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