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RESEARCH ARTICLE

Effects of the chloro-s-triazine herbicide terbuthylazine on DNA integrity in human and mouse cells

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Abstract

Terbuthylazine belongs to the chloro-s-triazine group of herbicides and acts primarily as a photosynthesis inhibitor. The mechanisms of action related to its exposure, relevant both in animals and humans, are still insufficiently investigated. This comprehensive study focused on the outcomes of terbuthylazine exposure at cell level in vitro, and a mice model in vivo. Experiments in vitro were conducted on whole human peripheral blood, isolated lymphocytes, and HepG2 cells exposed for 4 h to terbuthylazine at 8.00, 0.80, and 0.58 ng/mL, which is comparable with current reference values set by the European Commission in 2011. Terbuthylazine cytotoxicity was evaluated using dual fluorescent staining with ethidium bromide and acridine orange on lymphocytes, and CCK-8 colorimetric assay on HepG2 cells. The levels of DNA damage were measured using alkaline and hOGG1-modified comet assays. The potency of terbuthlyazine regarding induction of oxidative stress in vitro was studied using a battery of standard oxidative stress biomarkers. The in vivo experiment was conducted on Swiss albino mice exposed to terbuthlyazine in the form of an active substance and its formulated commercial product Radazin TZ-50 at a daily dose of 0.0035 mg/kg bw for 14 days. Following exposure, the DNA damage levels in leukocytes, bone marrow, liver, and kidney cells of the treated mice were measured using an alkaline comet assay. In vitro results suggested low terbuthylazine cytotoxicity in non-target cells. The highest tested concentration (8.00 ng/mL) reduced lymphocyte viability by 15%, mostly due to apoptosis, while cytotoxic effects in HepG2 cells at the same concentration were negligible. Acute in vitro exposure of human lymphocytes and HepG2 cells to terbuthylazine resulted in low-level DNA instability, as detected by the alkaline comet assay. Further characterization of the mechanisms behind the DNA damage obtained using the hOGG1-modified comet assay indicated that oxidative DNA damage did not prevail in the overall damage. This was further confirmed by the measured levels of oxidative stress markers, which were mostly comparable to control. Results obtained in mice indicate that both the active substance and formulated commercial product of terbuthylazine produced DNA instability in all of the studied cell types. We found that DNA in liver and kidney cells was more prone to direct toxic effects of the parent compound and its metabolites than DNA in leukocytes and bone marrow cells. The overall findings suggest the formation of reactive terbuthylazine metabolites capable of inducing DNA cross-links, which hinder DNA migration. These effects were most pronounced in liver cells in vivo and HepG2 cells in vitro. To provide a more accurate explanation of the observed effects, additional research is needed. Nevertheless, the present study provides evidence that terbuthylazine at concentrations comparable with current reference values possesses toxicological risk because it caused low-level DNA instability, both at cellular and animal organism level, which should be further established in forthcoming studies.

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Abbreviations

ADI	Acceptable daily intake
AO	Acridine orange
AOEL	Acceptable operator exposure level
ARfD	Acute reference dose
AU	Fluorescence arbitrary unit
EMEM	Eagle's minimum essential medium
EtBr	Ethidium bromide
FBS	Foetal bovine serum
GSH	Glutathione
GSH-Px	Glutathione peroxidase
hOGG1	Human 8-hydroxyguanine DNA-glycosylase 1
LMP	Low melting point
LPO	Lipid peroxidation
NC	Negative control
NMP	Normal melting point
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TB	Terbuthylazine
TBARS	Thiobarbituric acid reactive substances

Introduction

Terbuthylazine, N^2-*tert*-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine, belongs to the chloro-s-triazine group of herbicides and acts primarily as an inhibitor of photosynthesis. It is used mainly in the production of maize but is also applied to potatoes, peas, sugar cane, fruit trees, olives, citrus, coffee, oil palm, cocoa, rubber, as well as in forestry (WHO 2003). It is also an effective algicide, microbicide, and microbistat (USEPA 1995). The use of terbuthylazine has boosted in the last decades as it efficiently substituted atrazine, previously banned in EU countries (Sass and Colangelo 2006). However, recent studies suggest that terbuthylazine, due to its persistence, may present a similar environmental concern as atrazine (Stipičević et al. 2015, 2017).

There are several routes of human exposure to terbuthylazine. The general population is mostly exposed via ingestion of contaminated drinking water and dermal contact. Occupational exposure arises through dermal contact and inhalation at workplaces where terbuthylazine is manufactured or applied, as well as during applications in commercial/industrial settings (USEPA 1995).

In 2011, the European Commission established the following reference values for the active substance terbuthylazine: ADI 0.004 mg/kg bw day, AOEL 0.0032 mg/kg bw day, and ARfD 0.008 mg/kg bw (European Comission 2011; EFSA 2011, 2017). USEPA (2017) and the Committee for Risk Assessment of the European Chemicals Agency (ECHA 2015) concluded that no classification with respect to carcinogenicity is warranted for terbuthylazine. In recent years, the amount of data regarding potential detrimental effects of terbuthylazine has grown and several studies associated the compound with effects on endocrine functions in vitro (Forgacs et al. 2013; Stigaard Kjeldsen et al. 2013; Ghisari et al. 2015) and in vivo (Bossi et al. 2013; Taxvig et al. 2013).

The majority of recent toxicological studies on terbuthylazine and its degradation products and metabolites focused on aquatic organisms (Štepanova et al. 2012; Mikulíkova et al. 2013; Velisek et al. 2015, 2016a, b, 2017; Manera et al. 2016a, b; Stara et al. 2016; Koutnik et al. 2017). Their results indicated that exposure to terbuthlyazine affected the survival, development, and growth of the tested organisms; caused changes in hematological and biochemical parameters; led to various pathological changes in their organs and tissues; and caused oxidative stress. Toxicity studies with terbuthylazine on rodent experimental models are limited. Benković et al. (2012) reported impairments of hematological and some biochemical markers in terbuthylazine-treated male Swiss albino mice. Gebel et al. (1997) studied terbuthylazine toxicity in female and male mice using the bone marrow micronucleus test and did not find genotoxic effects.

Our research group conducted several experiments with terbuthylazine so far, and this report represents an extension of earlier studies, whose results indicated that the compound might impair genome integrity in rat blood cells in vivo (Tariba Lovaković et al. 2017) and human lymphocytes in vitro (Mladinić et al. 2009, 2012). Such findings motivated us to further characterize terbuthlyazine potency towards the infliction of DNA damage using other in vitro and in vivo models and reveal the possible mechanisms behind the observed DNA instability. For the in vitro segment, we selected human peripheral blood lymphocytes and HepG2 cells, considering that both represent well-established models in in vitro toxicology, suitable for the assessment of genotoxicity of direct and indirect mutagens, including pesticides (Villarini et al. 2000; Želježić et al. 2006, 2016; Hreljac et al. 2008; Mladinić et al. 2009; Nikoloff et al. 2014; Bianchi et al. 2015; Valencia-Quintana et al. 2016a, b; Žunec et al. 2016, Kašuba et al. 2017). An additional reason for the selection of HepG2 cells was that these cells retained many phase I and phase II metabolizing enzymes involved in the activation and/or deactivation of DNA-damaging agents (Knasmüller et al. 1998, 2004; Westerink and Schoonen 2007; Guo et al. 2011). To elucidate the toxicity profile of terbuthylazine in vitro, we selected two cell viability assays and a battery of well-established oxidative stress biomarkers (lipid peroxidation, ROS, glutathione,

glutathione peroxidase, superoxide dismutase), along with alkaline and hOGG1-modified comet assays. We expected such a study design would give more insight into the mechanisms behind the DNA instability caused by the tested compound, and point to directions in which future research should go. Since the results of our recent in vivo study (Tariba Lovaković et al. 2017) indicated low-level DNA damage in blood cells of treated rats, to further explore terbuthylazine's genotoxicity, we extended research to another rodent model. So, the second goal of this study was to establish the level of DNA instability caused by terbuthylazine (tested both in the form of active substance and a formulated commercial product) in leukocytes, bone marrow, liver, and kidney cells of Swiss albino mice following 14-day exposure to doses comparable with the currently proposed health-based reference values (European Commission 2011). Taken together, the results of the present study could contribute to understanding terbuthylazine's potential toxicity and the mechanisms of action related to its exposure, relevant both in animals and humans, as this herbicide was designed to specifically affect plant organisms by disturbing photosynthesis.

Materials and methods

Chemicals and reagents

Terbuthylazine (CAS Number 5915-41-3) was purchased as analytical standard purity grade (≤ 100%) PESTANAL® (Sigma-Aldrich Laborchemikalien GmbH, Germany). The formulated commercial terbuthylazine product, Radazin TZ-50 was purchased from Herbos (Croatia). It contained 500 g/L of the active substance. All of the other chemicals used were of analytical grade and bought from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), except otherwise stated.

Experimental design

In vitro study

Selection of tested concentrations and preparation of tested compound Terbuthylazine concentrations tested in vitro (8, 0.8, and 0.58 ng/mL, respectively) were selected based on previous report (Mladinić et al. 2009).

To prepare a stock solution, the tested compound was dissolved in dimethyl sulfoxide (DMSO). Further dilutions were done in RPMI 1640 (×1) growth medium (Gibco®, Life Technologies, Paisley, UK). The final volume of DMSO in the tested samples did not exceed 0.5%.

Treatments in vitro lasted for 4 h in accordance with our previous experiments (Mladinić et al. 2009), and in accordance with OECD guidelines for testing chemicals (OECD 2016).

Experiment on isolated lymphocytes and whole blood

Blood sampling

Venous blood (V = 50 mL) was drawn from a healthy male (non-smoker, 25 years old) without any diagnostic or therapeutic irradiations or exposure to genotoxic agents for 1 year preceding the blood collection. Written informed consent was obtained from the donor, and the research was part of a larger project approved by the Ethics Committee of the Institute for Medical Research and Occupational Health (Zagreb, Croatia). The number of the approval document was Class: 01-18/14-02/6, Reg. No. 100-21/14-5.

Blood was collected in sterile vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) coated with lithium heparin. The sample was immediately divided into two portions, one used in the experiments as whole blood and the other subjected to lymphocyte isolation.

Isolation of lymphocytes and their treatment

Lymphocyte isolation from the whole blood sample was done using the Histopaque®1077 reagent according to the manufacturer's instructions (Sigma-Aldrich 2018). The obtained cell pellet was resuspended with RPMI 1640 medium. Lymphocyte viability was immediately checked by trypan blue staining and exceeded 95%. In the lymphocyte suspension, after isolation, there were 2.35×10^5 cells/mL. Aliquots of the suspension (V = 1 mL each) were incubated for 4 h at 37 °C with the selected terbuthylazine concentrations. An untreated control sample was incubated in parallel. For each experimental group, duplicate samples were prepared. After treatment, the treated and control lymphocytes were used for viable cell counting, genotoxicity estimations by the alkaline and hOGG1-modified comet assays, and determination of oxidative stress parameters.

In vitro treatment of the whole blood

The portion of whole blood was divided into aliquots (V = 1 mL each), which were incubated for 4 h at 37 °C with selected terbuthylazine concentrations. For each experimental group, duplicate samples were prepared, along with an untreated control. After incubation, whole blood was used for measuring the glutathione peroxidase activity, while plasma samples were used to determine the extent of lipid peroxidation, the level of glutathione, and reactive oxygen species. Erythrocytes separated after centrifugation of whole blood (980 g for 10 min) were used for measuring the superoxide dismutase activity.

Experiment on HepG2 cells HepG2 cells [Hep G2 [HEPG2] (ATCC® HB8065™)] were bought from the American Type Culture Collection (Rockville, MD, USA) and were seeded in cell culture flasks at a density of 5×10^4 cells/mL and cultured until reaching exponential growth phase in Eagle's Minimum Essential Medium (EMEM; Gibco®, Life Technologies, Paisley, UK), enriched with 10% (*v*/*v*) foetal bovine serum (FBS) (Gibco®, Life Technologies, Paisley, UK) and 1%

(*v*/*v*) penicillin/streptomycin solution. Cultures were maintained in a moisturized atmosphere with 5% CO_2 at 37 °C and 95% relative humidity. Culture medium was replaced every 72 or 96 h during sub-culturing.

HepG2 cells were exposed for 4 h to different concentrations of terbuthylazine in EMEM without FBS. Untreated control cells were cultivated in parallel. After treatment, the medium was discarded. The cells were detached from the culture flask bottom using 0.025% trypsine solution (*w*/*v*) and 0.53 mM EDTA in PBS (pH 7.4). The enzymatic reaction was stopped by adding FBS. The cells were then rinsed in EMEM three times, and used for cell viability count, genotoxicity evaluations, and the determination of oxidative stress parameters.

Fluorescent viability assay with ethidium bromide and acridine orange (EtBr/AO) staining Lymphocyte viability was studied using dual fluorescent staining with AO and EtBr (Duke and Cohen 1992). Prior to the staining, both dyes dissolved in PBS were mixed at the final concentrations of 100 μg/mL (1:1; *v*/*v*). A total of 5 μL of the mixture of dyes was added to the tested cell suspension (V = 10 μL), and the preparations were immediately analyzed under an epifluorescence microscope (Olympus BX51, Japan; ×400 magnification). Triplicate tests with aliquots of each sample were accomplished and a total of 300 lymphocytes per sample per experiment were scored. Quantifications were made by determining the percentage of viable, apoptotic, and necrotic lymphocytes. Viable cells had a bright green-stained nucleus due to intercalation of AO in their DNA. Dead cells fluoresced dark orange/red due to the binding of EtBr in their DNA. Apoptotic cells in their early phase showed fragmented nuclei apoptosis, and orange staining of cytoplasm in late apoptosis.

CCK-8 colorimetric assay HepG2 cells were checked for viability using colorimetric CCK-8 assay, which utilized the highly water-soluble tetrazolium salt WST-8 [(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. Prior to the treatment, the cells were seeded in 96-well tissue culture plates (1×10^5 cells/100 μL culture medium per well) in EMEM and incubated overnight at 37 °C. Then, the cells (in quintuplicate wells per sample) were exposed for 4 h to selected terbuthylazine concentrations. Control cells were cultured in parallel. Following treatment, 10 μL of WST-8 solution was put into each well. After 4 h of incubation at 37 °C, the optical density at 450 nm was determined using a multilabel plate reader (Victor3™, Perkin Elmer, MA, USA). Data are expressed as a percentage of absorbance compared with the appropriate negative control.

Alkaline comet assay The assay was performed following the protocol by Singh et al. (1988), with minor modifications. Agarose microgels were prepared on fully frosted microscopic slides coated with 1 and 0.6% normal melting point (NMP) agarose. Isolated lymphocytes and HepG2 cells (V = 10 μL per slide) were embedded in a 0.5% low melting point (LMP) agarose. Two microgels per sample were prepared for each treated sample and controls. All of the slides were coded. The gels were immersed overnight at 4 °C in a freshly prepared lysis solution (pH 10.0; 100 mM Na_2EDTA, 2.5 M NaCl, 1% Na lauroylsarcosinate, 10 mM Tris-HCl, 10% dimethyl sulfoxide, and 1% Triton X-100). Denaturation and electrophoresis (1 V/cm, 300 mA) were performed at 4 °C, protected from light in alkaline buffer (pH 13.0; 300 mM NaOH, 1 mM Na_2EDTA), and lasted for 20 min each. Then, the microgels were neutralized using 0.4 M Tris-HCl buffer (pH 7.5). They were stained with EtBr (20 μg/mL). One well-trained scorer performed all of the comet measurements on coded/blinded slides. The level of DNA damage in individual cells was assessed with an image analysis system (Comet Assay IV™, Instem-Perceptive Instruments Ltd., UK) using an epifluorescence microscope (Leitz, Germany) equipped with appropriate filters, under ×250 magnification. A total of 100 randomly selected comets per each tested concentration were measured on replicate slides in two independent evaluations. As indicators of DNA damage, tail length (presented in micrometers), tail intensity (i.e., DNA% in tail), and total area (represents the overall surface area of the comet) were selected.

hOGG1-modified comet assay The assay was performed as proposed by Smith et al. (2006). Quadruplicate slides were prepared per each sample, as described above. Microgels were lysed overnight in a freshly prepared chilled lysis solution (pH 10.0). Then, they were immersed in three changes of the 1X FLARE™ reaction buffer (Trevigen, MD, USA). One of the two matching microgels from each replica was treated with the hOGG1 enzyme diluted in a reaction buffer. At the same time, another parallel slide from each replica was kept in a reaction buffer without the enzyme. Incubations lasted for 30 min in a humidified chamber at 37 °C, in line with the manufacturer's instructions (Trevigen 2017). Denaturation was performed at 4 °C in an electrophoretic buffer (1 mM Na_2EDTA, 0.3 M NaOH, pH 13) for 20 min. Electrophoresis was run for a further 20 min at 0.7 V/cm and 300 mA. Afterwards, the gels were neutralized with a 0.4 M Tris-HCl buffer (pH 7.5). After staining with ethidium bromide, the coded/blinded slides were evaluated by one well-trained scorer, following the same procedure as for the alkaline comet assay. The level of oxidative damage was judged based on the difference in the mean values of tail length and tail intensity measured on microgels treated with the hOGG1 enzyme and microgels treated with the reaction buffer (Smith et al. 2006).

Determination of the parameters of oxidative stress Before measurements, lymphocytes were resuspended in RPMI 1640 (V = 1 mL), while HepG2 cells were resuspended in PBS (V = 2 mL). Cells were sonicated at 60% amplitude for a total of 1.5 min (in cycles of 30 s ON and 15 s OFF) at room temperature. Following 10 min of centrifugation at 2460 g at 4 °C, the supernatant was frozen at − 20 °C until analysis within 24 h (Mateos et al. 2004).

Lipid peroxidation The level of thiobarbituric reactive substances (TBARS), as a marker of lipid peroxidation, was measured in plasma samples using a modified method by Drury et al. (1997). Five microliters of 0.2% (*w*/*v*) butylated hydroxytoluene (BHT) and 750 μL 1% (*v*/*v*) phosphoric acid were added to 50-μL plasma samples. After mixing, 250 μL 0.6% (*w*/*w*) thiobarbituric acid (TBA) and 445 μL H_2O were added, and the reaction mixture was incubated at 90 °C for 30 min. After cooling, absorbance was measured spectrophotometrically at 532 nm (Cecil 9000 spectrophotometer, Cecil Instruments Limited, Cambridge, UK). The TBARS concentration was calculated using the standard curves of increasing 1,1,3,3-tetramethoxypropane (1.5–30.35 μM) concentrations, and expressed as $\mu mol/g_{protein}$.

For lymphocytes and HepG2 cells, we used a TBARS assay modified by Ohkawa et al. (1979) and Pareek et al. (2013). In short, the sample (V = 0.2 mL) was mixed with the same volume of TBA-TCA (trichloroacetic acid) reagent and heated at 90 °C for 30 min. After cooling and centrifugation, absorbances were measured spectrophotometrically at 530 nm. TBARS concentration in unknown samples was calculated using a standard curve constructed with 1,1,3,3-tetrametoxypropane (0.3–6.0 μM) and expressed as $\mu mol/g_{protein}$.

ROS detection The amount of ROS in blood plasma, lymphocytes, and HepG2 cells was estimated using 2′,7′-dichlorofluorescin diacetate (DCFDA) reagent (Griffiths et al. 2011). Briefly, 10% blood plasma samples were prepared by dilution with ice-cold PBS (pH 7.4). Portions of 0.1 mL of 10% diluted blood plasma were pipetted into the wells of a 96-well plate following the addition of 20 μL of 0.12 mM DCFDA dye to PBS. The final dilution of blood plasma in wells was 8.3%. Lymphocytes were pipetted into the wells undiluted, and the final dilution in the wells was 83%. HepG2 cells samples were handled in the same way as for the viability assay. After the treatment, they were rinsed and 120 μL of 0.12 mM DCFDA dye in PBS was put into each well. Control for dye autofluorescence was prepared without adding the dye. Each experiment included negative controls. All samples were incubated for 30 min at 37 °C and measured using a multilabel plate reader (Victor3™, Perkin Elmer, MA, USA) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Data were displayed in florescence arbitrary units (AU).

Quantification of glutathione (GSH) GSH concentration was measured using a monochlorobimane dye, which reacts specifically with GSH to generate a fluorescent product (Kamencic et al. 2000). Blood plasma, lymphocyte suspensions, and HepG2 cells were prepared identically as for ROS detection and incubated for 20 min at 37 °C. The amount of GSH in blood plasma and lymphocyte samples was analyzed using a multilabel plate reader (Victor3™, Perkin Elmer, MA, USA) at an excitation wavelength of 355 nm and emission wavelengths of 460 nm. Each experiment included negative controls. Data were displayed in fluorescence arbitrary units (AU).

Glutathione peroxidase (GSH-Px) activity The GSH-Px activity was estimated by the European standardized method (Belsten and Wright 1995a). The amount of GSH oxidized by t-butyl hydroperoxide was measured by observing the decrease in the β-NADPH concentration. The decrease in absorbance at 340 nm was measured spectrophotometrically (Cary 50 UV-Vis, Varian Inc. CA, USA). One unit of GSH-Px is defined as the number of micromoles of β-NADPH oxidized per minute. The activity of GSH-Px in whole blood was expressed per gram of hemoglobin (U/g Hb), while the results obtained in lymphocytes and HepG2 cells were expressed per gram of total protein ($U/g_{protein}$).

Superoxide dismutase activity (SOD) We applied the European standardized method (Belsten and Wright 1995b) using the Ransod kit (Randox, Crumlin, UK) in line with the manufacturer's instructions. The SOD activity was measured spectrophotometrically (Cary 50 UV-Vis, Varian Inc. CA, USA) according to the degree of inhibition of this reaction at 505 nm. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) under the conditions of the assay. The SOD activity in erythrocytes was expressed per gram of hemoglobin (U/g_{Hb}), while the results obtained in lymphocytes and HepG2 cells were expressed per gram of total protein ($U/g_{protein}$). Hemoglobin in whole blood and erythrocytes was spectrophotometrically measured at 540 nm by a standard cyanmethaemoglobin method using the haemiglobincyanide standard. To validate the analytical method, three levels of Hb were used.

Protein quantification Protein content was measured by the Bradford assay (1976) using bovine serum albumin as the standard.

In vivo study

Animals Male Swiss albino mice (2–3 months old, weighing 20–25 g) were obtained from the breeding colony at the Department of Biology, Faculty of Science, University of Zagreb, Zagreb

(Croatia). Animals were maintained under standard conditions, 12-h light/dark cycle, 22 °C with ad libitum access to standard GLP certified food (Mucedola, 4RF21, Italy) and water. Protocols for the care, treatment, and euthanasia of the mice were approved by the Institutional Animal Care and Use Committee. The experiment was carried out in compliance with international standards and national legislation to protect animal welfare. The experiment was conducted in line with the EU Directive 2010/63/EU for animal experiments.

Selection of the tested dose and preparation of the compounds The dose of terbuthylazine and its formulated commercial product Radazin TZ-50 used in in vivo testing (0.0035 mg/kg bw day) was selected according to current health-based reference values (European Commission 2011). Solutions used for the treatments were prepared by dissolving the tested compounds in sterile 0.9% sodium chloride solution (Croatian Institute for Transfusion Medicine, Zagreb, Croatia).

Experimental schedule Mice were randomly assigned to the following three groups: group 1: mice administered terbuthylazine at a dose of 0.0035 mg/kg bw day; group 2: mice administered a formulated commercial product of terbuthylazine (Radazin TZ-50) daily standardized at the same dose of the active substance; group 3: mice administered 0.9% sodium chloride solution daily. Each group comprised five animals. Mice were treated for 14 days via an intraperitoneal (*i.p.*) injection of tested solutions (V = 0.5 mL). At the end of each treatment, the body weight of mice was measured and compared with the initial body weight. The experiment was terminated 24 h after the last treatment. All of the animals were anesthetized with adequate intraperitoneal administration of a combination of Narketan® (Vétoquinol SA, BP 189 Lure Cedex, France; active substance ketamine; 0.8 mL/kg) and Xylapan® (Vetoquinol Biowet Sp., Gorzow, R. Poland; active substance xylazine; 0.6 mL/kg). Peripheral blood samples were taken from the tail vein into heparinised vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Animals were sacrificed with cervical dislocation and then the other samples were taken. To obtain bone marrow cells, the femurs were removed and then the cells were extracted with foetal calf serum, precipitated, and used for the preparation of microgels. Livers and kidneys were dissected out and rinsed in cold saline. Small pieces of freshly resected liver and kidney were then put into a chilled 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 cellular suspensions and blood samples were also immediately used for preparation of agarose microgels for the alkaline comet assay.

Alkaline comet assay The protocol used for the alkaline comet assay was the same as described for the in vitro experiment. For each mouse, duplicate microgels for each tissue were prepared. Slides were analyzed using Comet Assay IV™ software (Instem-Perceptive Instruments Ltd., Suffolk, UK) under an epifluorescence microscope (Leitz, Germany) equipped with appropriate filters, at ×250 magnification. A total of 100 randomly selected comets per mice were measured in two independent evaluations. Tail length, tail intensity, and total comet area were chosen as indicators of DNA damage.

Statistics Statistical calculations were done using the Dell™ Statistica™ 13.2 software (StatSoft, Tulsa, USA). Comparisons between the values observed for cell viability, apoptosis, and necrosis were performed by Pearson's χ^2 test for two-by-two contingency tables. The data acquired by both comet assays were first evaluated using descriptive statistics. The comparisons between samples in the alkaline comet assay were completed using one-way analysis of variance on $\log_{10}$ transformed data. Afterwards, the *post-hoc* Tukey HSD test was applied for the calculations regarding pair-wise comparisons. For the hOGG1 modified comet assay, statistical significance between the mean tail length and tail intensity values measured with buffer and the means for the matching hOGG1 enzyme-treated slide was tested using the Mann-Whitney U test. For the biochemical assays data analysis, we applied descriptive statistics and for evaluations between independent samples, a *t* test. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Assessment of terbuthylazine toxicity in vitro

Cell viability

Exposure to terbuthylazine lowered lymphocyte viability in a concentration-dependent manner (Fig. 1), which was statistically significant compared to the control ($P < 0.05$). Apoptosis prevailed over necrosis in all of the samples.

Cytotoxicity of terbuthylazine in vitro has thus far not been extensively studied, and its potential to induce apoptosis has also not been documented in the available literature. However, activation of apoptosis, as programmed cell death, can be regarded as a deliberate self-elimination pathway of potentially mutant cells that fixed unrepairable DNA damage following the treatment. Pesticides in general trigger apoptosis both via the caspase-dependent and the mitochondrial pathway (Franco et al. 2009). Since the EtBr/AO fluorescent viability assay allows only for the morphological detection of apoptotic lymphocytes, we unfortunately cannot propose the precise mechanisms behind apoptosis, and this remains to be investigated in forthcoming studies by means of molecular methods.

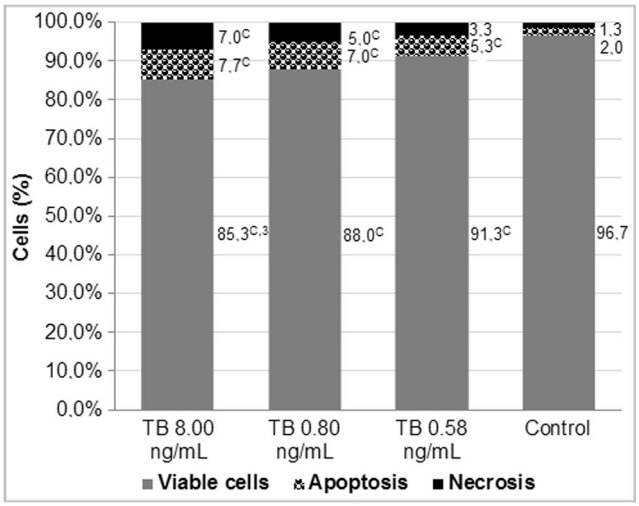

Fig. 1 Viability of terbuthylazine (TB)-treated and control lymphocytes determined using a quantitative fluorescent assay for the simultaneous identification of apoptotic and necrotic cells Mean values of three independent evaluations are shown (3 × 100 cells per sample per each experimental point were analyzed). The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance ($P < 0.05$): C—control; 3—sample treated with 0.58 ng/mL of terbuthylazine

In contrast to lymphocytes, exposure to terbuthylazine did not significantly impair HepG2 cell viability at the same tested concentrations (Fig. 2).

The obtained results indicate that terbuthylazine at the tested concentrations posed a relatively weak cytotoxic threat to non-target cells. However, low cytotoxicity does not necessarily indicate low genotoxic effects. This observation was a strong argument for the evaluation of terbuthylazine's genotoxic potency using the comet assay, considering the fact that one of the most important criteria for such testing involves cell viability greater than 70–75% (Henderson et al. 1998; Tice et al. 2000).

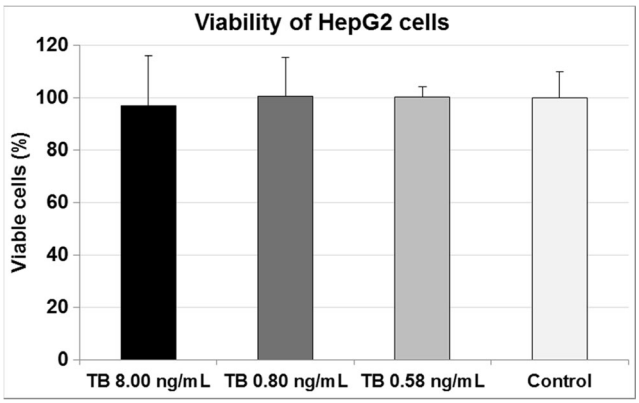

Fig. 2 Viability of HepG2 cells treated with terbuthylazine (TB) in vitro for 4 h estimated by CCK-8 assay. Mean values ± SD of five independent evaluations are shown

The alkaline comet assay

To assess the genotoxic profile of terbuthylazine, we first applied an alkaline version of the comet assay, which identified the broadest spectrum of DNA damage (Tice et al. 2000; Liao et al. 2009; Azqueta and Collins 2013; Langie et al. 2015). There are limited literature sources on similar in vitro and in vivo comet assay studies with terbuthylazine and related herbicides. Mladinić et al. (2012) found that terbuthylazine was capable of producing primary DNA damage in 14-day extended lymphocyte culture. Other available data are rather ambiguous. Ribas et al. (1995) reported genotoxicity of atrazine, alachlor, maleic hydrazide, paraquat, and trifluralin on human lymphocytes. Villarini et al. (2000) observed increased DNA damage in terbuthryn-treated human leukocytes at concentrations of 100 and 150 μg/mL. Želježić et al. (2006) reported that atrazine was not genotoxic to human lymphocytes in vitro at concentrations 0.047, 0.47, and 4.7 μg/mL.

The results obtained in the present study after 4 h in vitro treatments with terbuthylazine indicated the DNA-damaging potential of the tested compound in human lymphocytes and HepG2 cells. However, there were significant differences in the levels of primary DNA damage between them. Although endogenous levels of DNA damage in both cell types were low, judging by the measured comet parameters, lymphocyte DNA was generally more susceptible to breakage following treatment than DNA in HepG2 cells (Fig. 3). Exposure of lymphocytes to the two higher terbuthylazine concentrations caused a significantly increased DNA migration compared to the control cells. In HepG2 cells, we found significantly higher DNA migration compared to controls only at 0.80 ng/mL of terbuthylazine. The observed changes in damage levels between HepG2 cells and lymphocytes could be related both to the toxic properties of the tested compound and the inherent differences between these cells. Among them, the quiescence state of lymphocytes should be pointed out. It is characterized by lower metabolic and many other activities (Yusuf and Fruman 2003). DNA damage in lymphocytes probably could not be efficiently repaired due to saturation of DNA processes. Previous findings (Green et al. 1994) suggest that dormant lymphocytes are more vulnerable to mutagenesis since their deoxynucleotide pools are low and the efficiency of some repair processes decreased when compared with mitogen stimulated cells. Taken together, these factors could contribute to the higher level of DNA lesions in lymphocytes, which have been detected using the alkaline comet assay in our study. The results obtained on the HepG2 cell model also suggest that these cells possibly had more efficient mechanisms for repairing single-strand breaks than lymphocytes. Furthermore, one also has to take into account the possibility that the parent compound underwent some degree of metabolic conversion in these cells, which also may account for decreased DNA migration, as observed here. Results of previous

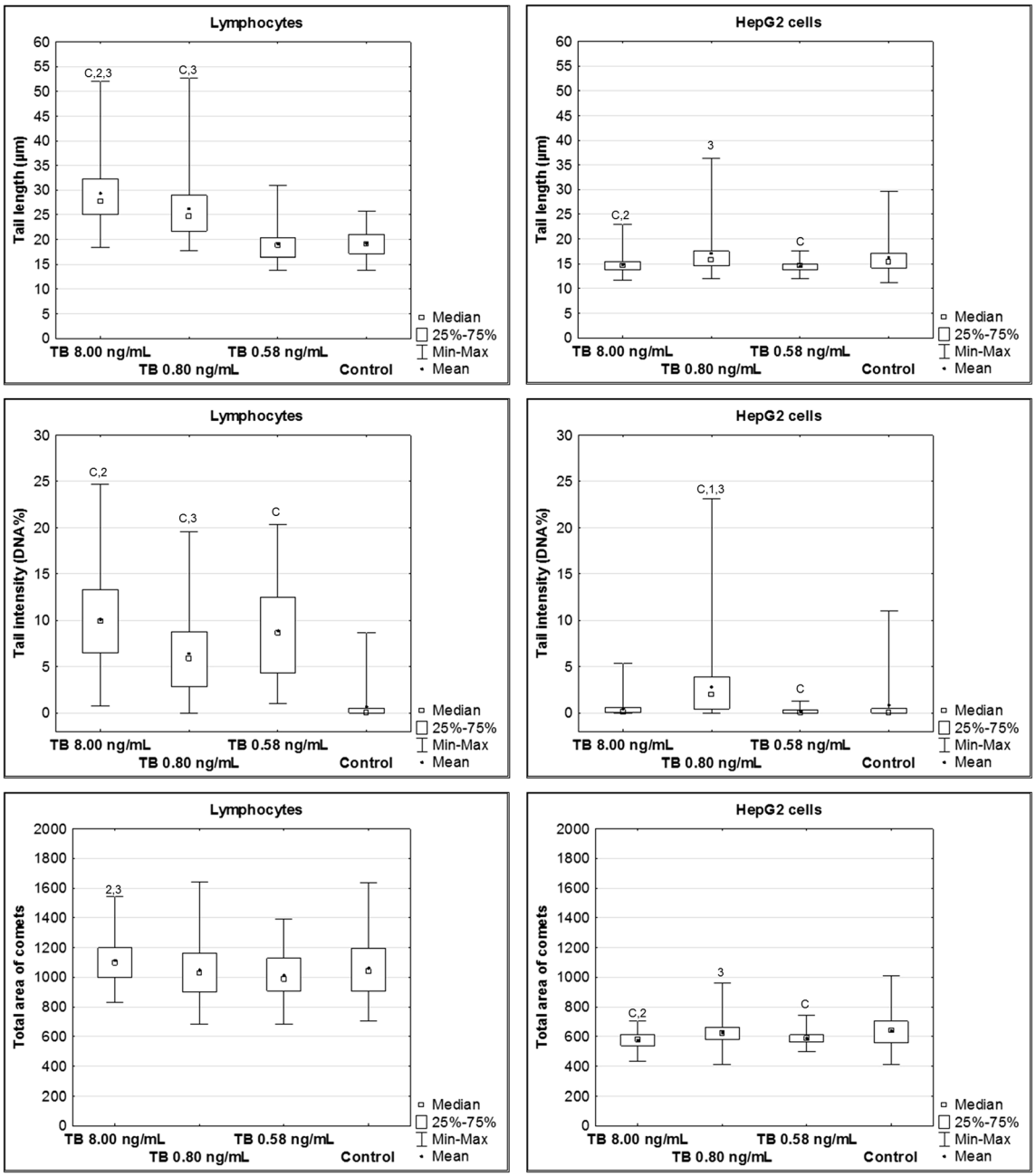

Fig. 3 Primary DNA damage in peripheral blood lymphocytes and HepG2 cells treated in vitro with terbuthylazine (TB) for 4 h as determined by the alkaline comet assay. The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance: C—control; 1—sample treated with 8.00 ng/mL; 2—sample treated with 0.80 ng/mL; 3—sample treated with 0.58 ng/mL

studies that tested toxicity of herbicides on the same cell type suggest that they represent a good model system to assess genotoxic effects, due to their xenobiotic-metabolizing activities (Powell et al. 2011; Hartnett et al. 2013; Nikoloff et al. 2014). Of course, the outcomes obtained on such a cell system are certainly not the same as expected in liver in vivo, due to the lack of a full phase I and phase II enzyme activity. Nevertheless, the HepG2 cell model gets an insight into DNA damage caused by treatments compared to lymphocytes that do not possess endogenous metabolic activity at all.

As the results of the alkaline comet assay revealed significantly higher DNA migration in lymphocytes, using the hOGG1-modified comet assay, we tried to establish the contribution of oxidative damage to the total DNA damage measured. As known, human 8-hydroxyguanine DNA-glycosylase 1 (hOGG1) is the chief enzyme responsible for the repair of 8-oxoguanine, which is considered a marker of cellular oxidative stress important for carcinogenesis (Kasai 1997). hOGG1-modification of the comet assay is particularly efficient for the recognition of 8-oxoguanine and

formamidopyrimidine products of guanine, since the hOGG1 enzyme can repair these oxidized bases only when paired with cytosine (Smith et al. 2006).

The results of the hOGG1-modified comet assay (Fig. 4) suggest that after treatment with terbuthylazine a small amount of modified guanine products was formed in the lymphocyte DNA. Despite a small rise in the rate of hOGG1-sensitive sites detected in the DNA in all of the terbuthylazine-treated samples, no clear concentration-dependent DNA oxidation was noted. Statistically significant responses for parameter tail length were observed at 0.80 and 0.58 ng/mL (Fig. 4a), and for parameter tail intensity at 8.00 and 0.58 ng/mL (Fig. 4b).

It has to be noted that the difference between the hOGG1-treated and buffer-treated samples obtained for tail intensity at 8.00 ng/mL was only 1.2%, which points to a quite low damage level (Fig. 4). For comparison, when lymphocytes of the same donor were treated with 1 mM hydrogen peroxide for 10 min (positive control), the difference between the hOGG1-treated and buffer-treated microgels for tail intensity was 12.6 ± 7.6%. Such results suggest that terbuthylazine has a rather low potential towards the infliction of oxidative damage in lymphocyte DNA. This was further supported by the fact that when we compared the tail length or tail intensity values measured using two versions of the comet assay, greater statistical differences among the treated and control cells were obtained for the alkaline than for the hOGG1 modified comet assay at all of the tested concentrations. This implies that under our experimental conditions, the amount of oxidative damage, measured by the hOGG1 modified comet assay, was lower than the amount of strand breakage, which can be efficiently measured by the alkaline comet assay. Therefore, based on the findings obtained, we cannot unambiguously propose that terbuthylazine exerts a clear capacity towards oxidative DNA damage, and additional investigation in this regard has to be performed.

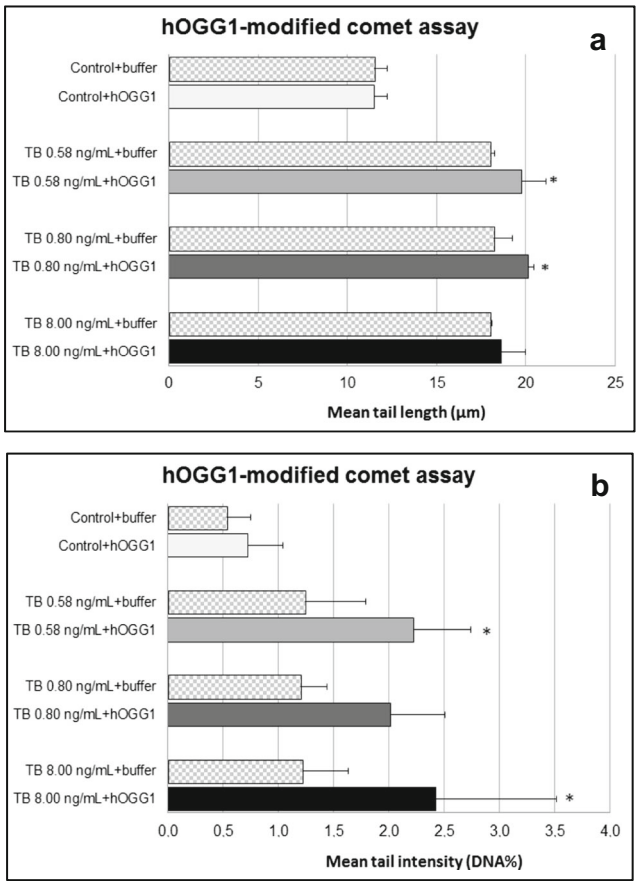

Fig. 4 Primary DNA damage in peripheral blood lymphocytes treated with terbuthylazine in vitro for 4 h as determined by hOGG1-modified comet assay. A single asterisk indicates a statistically significant difference ($P < 0.05$) from the corresponding sample treated with buffer

Parameters of oxidative stress The findings of the hOGG1-modified comet assay motivated us to further characterize the potency of terbuthlyazine regarding induction of oxidative stress in vitro. The obtained results are presented in Table 1.

As reported in Table 1, the concentrations of terbuthylazine tested in the present study mostly did not provoke significant changes in oxidative stress markers. We observed significantly increased lipid peroxidation only in plasma at the highest tested terbuthylazine concentration. ROS levels in plasma were significantly increased compared to controls at all of the tested terbuthylazine concentrations, as well as in lymphocytes treated at 0.80 and 0.58 ng/mL of terbuthylazine. Non-elevated ROS levels in HepG2 cells at the same tested concentrations might reflect the better efficiency of antioxidant defense in this cell type. GSH concentrations after treatment did not significantly deviate from respective controls in any of the biological matrices.

Antioxidant enzyme changes due to exposure to terbuthylazine have not been documented sufficiently well in the existing literature. This study evaluated the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD). Both enzymes scavenge free radicals to prevent oxidative damage and overcome increased production of ROS (Matés et al. 1999). Our results indicate decreased activities of GSH-Px in whole blood and SOD in erythrocytes after terbuthylazine treatment. In contrast, their activities increased both in HepG2 cells and lymphocytes, possibly as a response to disturbed oxidative/antioxidative equilibrium due to treatment. The differences in the enzyme activities observed between lymphocytes and HepG2 cells apparently originated from metabolic variations between these distinct cell types, which resulted in different antioxidant capacities.

Since most of the previous studies that focused on terbuthylazine-related oxidative stress were conducted on in vivo models, there are no similar reports to validate and compare our results. Previous reports on lipid peroxidation are inconclusive. Plhalova et al. (2012) found significantly increased TBARS levels in zebrafish only after 28 days exposure

Table 1 Effects of 4-h treatment with terbuthylazine on the extent of lipid peroxidation, level of intracellular reactive oxygen species (ROS), concentration of glutathione (GSH), and on the activity of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in blood samples, lymphocytes, and HepG2 cells

	Blood samples				
Parameter/concentration	TBARS (μmol/$g_{protein}$)	ROS (AU)	GSH (AU)	GSH-Px (U/g Hb)	SOD (U/g Hb)
TB 8.00 ng/mL	0.337 ± 0.033*,[a]	1352.5 ± 56.3*	3090.5 ± 375.9	181.4 ± 1.6*	2792.9 ± 4.9*
TB 0.80 ng/mL	0.225 ± 0.006	1434.3 ± 31.9*	3150.0 ± 87.0	181.3 ± 11.8	2823.9 ± 73.1
TB 0.58 ng/mL	0.220 ± 0.003	1451.0 ± 40.4*	3393.0 ± 157.5	161.3 ± 17.6	2841.2 ± 7.6
Control	0.244 ± 0.003	1651.8 ± 108.9	3448.5 ± 353.4	191.7 ± 2.9	2870.5 ± 28.4
	Lymphocytes				
Parameter/concentration	TBARS (μmol/$g_{protein}$)	ROS (AU)	GSH (AU)	GSH-Px (U/$g_{protein}$)	SOD (U/$g_{protein}$)
TB 8.00 ng/mL	0.628 ± 0.12	3689.0 ± 201.0	3380.3 ± 100.6	1359.6 ± 6.3	168.4 ± 9.3*
TB 0.80 ng/mL	0.781 ± 0.00	3954.0 ± 24.1*	3366.7 ± 240.7	1338.7 ± 1.6	140.8 ± 21.3
TB 0.58 ng/mL	0.416 ± 0.00	4044.0 ± 127.5*	3373.3 ± 102.0	1219.1 ± 33.1	141.7 ± 10.2
Control	0.783 ± 0.02	3269.3 ± 139.2	3281.7 ± 86.0	1296.7 ± 46.4	139.6 ± 1.8
	HepG2 cells				
Parameter concentration	TBARS (μmol/$g_{protein}$)	ROS (AU)	GSH (AU)	GSH-Px (U/$g_{protein}$)	SOD (U/$g_{protein}$)
TB 8.00 ng/mL	108.6 ± 7.7	1839.5 ± 75.7	40,277.0 ± 3847.4	16,065 ± 1739*	5338.4 ± 264.0
TB 0.80 ng/mL	90.1 ± 0.3	1822.5 ± 115.5	38,656.0 ± 617.2	14,716 ± 2347*	4586.9 ± 90.4
TB 0.58 ng/mL	162.6 ± 19.5	1892.0 ± 85.3	40,311.8 ± 2576.6	29,429 ± 5785*	4989.5 ± 110.1
Control	86.3 ± 7.8	1873.0 ± 51.8	39,040.8 ± 1462.0	8025 ± 1175	4633.8 ± 323.6

Values are presented as mean ± SD of four independent measurements

TBARS thiobarbituric reactive substances, *AU* fluorescence arbitrary units

*Significantly different vs. control

[a] significantly different vs. all other treated samples ($P < 0.05$)

to 1000 μg/L of terbuthylazine. Stara et al. (2016) found significantly increased lipid peroxidation in the hepatopancreas of red swamp crayfish (*Procambarus clarkii*) after 14 days of exposure to terbuthylazine-desethyl at 2.9 and 580 μg/L, along with increased GSH levels in the hepatopancreas at 580 μg/L. Several studies reported changes in SOD activity following exposure to terbuthylazine or its degradation products. Velisek et al. (2014) found decreased SOD activity in common carp larvae exposed for 35 days to terbuthylazine-2-hydroxy at doses ranging from 0.0029 to 3.5 mg/L. In another study, Velisek et al. (2016a) found changes in the SOD activity of common carp embryos and larvae exposed for 7 days to the real environmental concentration (1.8 μg/L). The authors suggested that a decline in SOD activity may be due to increased ROS generation induced by treatments. Tariba Lovaković et al. (2017) found significantly decreased plasma SOD activity in rats given terbuthylazine for 28 days at 0.004 and 0.4 mg/kg bw day, and significantly decreased erythrocyte SOD activity at 2.29 mg/kg bw day compared to negative control. They also found significantly increased GSH-Px activity in whole blood at all three of the administered doses.

Based on our findings and those from the abovementioned studies, it can be concluded that oxidative stress-related phenomena become important mostly after prolonged exposure to terbuthylazine at doses significantly higher than studied here.

When the results of oxidative stress markers and both modifications of the comet assay are considered in their entirety, it seems that several mechanisms might account for the low level of DNA instability observed after terbuthylazine exposure. A part of DNA damage measured with the comet assay was undoubtedly caused by indirect mechanisms, and mediated through free radicals that contributed to the production of single-strand breaks, oxidized bases, and other DNA lesions, which were readily identified by the employed comet versions. Oxidative DNA damage definitely did not prevail. Nevertheless, our results suggest that terbuthylazine was capable of inducing a certain amount of primary DNA damage by a direct mechanism not yet described in the existing literature. Since levels of DNA damage were higher in lymphocytes, which do not possess intrinsic metabolic activation, most of the observed effects in this cell type could be attributed to the parent compound. However, in HepG2 cells, the effects of some metabolites can also be expected. Lang et al. (1996, 1997) studied the in vitro metabolism of terbuthylazine using liver microsomes from humans, pigs, and rats and found that all of the species formed the same classes of metabolites, but their ratios were species-specific. The major metabolites of terbuthylazine, relevant as environmental contaminants, are desethylterbuthylazine, desisopropyatrazine, hydroxyterbuthylazine, desethylhydroxyatrazine, and

desethylhydroxyterbuthylazine (Papadopoulos et al. 2007). Their toxicity profiles have not yet been investigated well. However, there are some indications that terbuthylazine toxicity and at least one of its metabolites strongly depend on the presence of chlorine in their molecular structure (EFSA 2016). To be precise, desethylterbuthylazine, which contains chlorine, was toxic to algae, while three other terbuthylazine metabolites that do not contain chlorine showed low toxicity to algae (EFSA 2016). Whether chlorine also plays an important role in the DNA-damaging potency of terbuthylazine remains to be discovered. However, this assumption does make sense, as something similar was observed for the insecticide cypermethrin, which contains two chlorine atoms in the molecule (Saxena et al. 2005; Kocaman and Topaktas 2009). Saxena et al. (2005) suggest that cypermethrin interacts with DNA directly and that its binding to DNA possibly occurs due to the strong polarization associated with chlorine atoms. Such polarization may destabilize DNA structure and cause the unwinding of the DNA helix resulting in damage. The authors suggested that the proposed mechanism is useful in predicting the genotoxic potential of the related compounds. However, it remains to be elucidated whether the same is valid for terbuthylazine.

Our results obtained on HepG2 cells suggest another possible mechanism of terbuthylazine action at DNA level. We found that terbuthylazine applied at the two lower concentrations caused a significant reduction of mean tail length in HepG2 cells compared to control. The decreased extent of DNA migration might have been due to impaired DNA denaturation, which often arises as a consequence of inter- or intrastrand crosslinks. The existence of this specific type of DNA damage could be specifically detected using a modified protocol for the comet assay (Spanswick et al. 2010; Wu and Jones 2012). However, some comet assay studies (Buschfort et al. 1997; Thomale et al. 1998; Benítez-Bribiesca et al. 2001; Buschfort-Papewalis et al. 2002; Jurica et al. 2017) suggested that cross-links could also be indirectly detected by evaluation of the third comet assay parameter—total area, which represents the overall surface area of the comet. A detailed analysis we performed in that manner suggests that, at all of the tested concentrations, the mean total area of terbuthylazine-treated HepG2 cells was reduced compared to control, which was most prominent at the highest tested concentration (Fig. 3). The same terbuthylazine effect was not shown in lymphocytes, which suggests that it could be associated with the metabolism of the tested compound.

We have to stress that the use of in vitro cell models along with the comet assay has some limitations, and the obtained results therefore have to be taken with certain precautions. First of all, the results obtained in vitro cannot be directly extrapolated to real in vivo situations and they are not directly applicable to human risk assessments. This is especially the case when the observed DNA damage results from a process that was specific to the used in vitro system or particular experimental conditions not likely to be reached in vivo. In our case, this potential limitation was minimalized by the simultaneous use of two metabolically distinct cell types for all evaluations. Nevertheless, to document whether the same effects of terbuthylazine at DNA level could be detected in vivo, we extended our research to a mouse model and evaluated the effects of the compound in leukocytes, bone marrow, hepatocytes, and kidney cells of the exposed animals.

Assessment of terbuthylazine genotoxicity in vivo

We evaluated the DNA-damaging potency of both the active substance and formulated product of terbuthylazine (Radazin TZ-50), considering that unwanted human exposure commonly arises as a consequence of the use of various commercial pesticide products.

After 14 days of treatment, no deaths and no signs of systemic toxicity were observed. Gross necropsy did not reveal any treatment-related findings. Body weight gain in the terbuthylazine-treated group of mice at the end of experiment was 7.9%, and 7.6% in mice exposed to Radazin TZ-50. Both values did not significantly differ from the control mice, whose body weight gain was 8.0%. Such a finding is in line with our recent study on rats (Tariba Lovaković et al. 2017), where significant terbuthylazine effects on body weights were observed only after 28 days of treatment.

Results regarding tail lengths and tail intensities measured using the alkaline comet assay in leukocytes, hepatocytes, bone marrow, and kidney cells of mice exposed to the tested compounds for 14 days at a daily dose of 0.0035 mg/kg are shown in Fig. 5. We found that exposure to both of the tested compounds resulted in significantly increased mean tail lengths and tail intensities in leukocytes, bone marrow cells, and liver cells compared to the values measured in respective control groups. In kidney cells, only exposure to the formulated product Radazin TZ-50 caused a significant increase of mean tail lengths and tail intensities compared to the values measured in control mice.

The differences in DNA susceptibility we observed between different cell types, apparently originated from the intrinsic metabolic differences between them, as well as differences in their inherent mechanisms, which counteract damage (antioxidative defense, DNA repair enzymes, etc.). Leukocytes and bone marrow, for instance, represent a heterogeneous group of nucleated cells that might slightly differ in their sensitivity towards genotoxins. However, their DNA damage levels could also be indirectly affected by the presence of enzymes like SOD in erythrocytes, as well as SOD, GSH-Px, and catalase in plasma. Liver cells could, on the other hand, display a different pattern of DNA damage compared to other cell types owing to the metabolism of the tested compound.

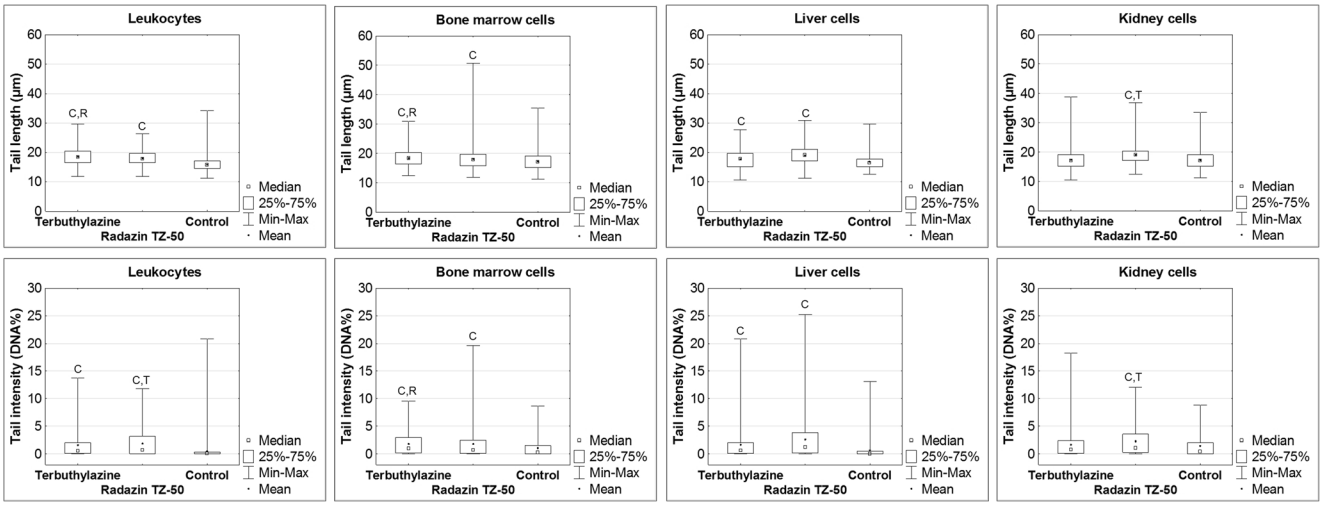

Fig. 5 Results regarding the values of tail lengths and tail intensities measured using the alkaline comet assay in leukocytes, bone marrow, liver, and kidney cells of Swiss albino mice treated for 14 days with terbuthylazine, applied as active substance and its commercial formulation Radazin TZ-50 at a daily dose of 0.0035 mg/kg bw. The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance ($P < 0.05$): C—control mice; R—mice treated with Radazin TZ-50; T—mice treated with terbuthylazine

In leukocytes and bone marrow of mice given terbuthylazine as an active substance, we measured significantly higher mean tail length than in those given a formulated product (Fig. 5). Quite the opposite was observed in hepatocytes and kidney cells, where both mean tail lengths and mean tail intensities were significantly higher in mice given a formulated product (Fig. 5).

Such a finding is not surprising if one takes into account the possibility that other ingredients of a formulated product might possess DNA-damaging effects as well (Cox and Surgan 2006). Besides the active substance, formulated pesticide products contain various inert ingredients. Among them are different adjuvants, added to the product aimed at improving its effectiveness, storage, handling, safety, or application properties. These may be solvents that dissolve the active ingredient, carriers added to a pesticide product to aid in the delivery of the active ingredient, and adjuvants that help make the pesticide stick to or spread out on the application surface.

The results of many previous toxicity studies with pesticides suggest that the use of formulated products is often associated with genotoxicity higher than observed after exposure to the active substance (Bolognesi et al. 1997; Soloneski et al. 2002; Grisolia et al. 2004; Ündeğer and Başaran 2005; Želježić et al. 2006; Molinari et al. 2009; Cavas 2011; Guilherme et al. 2012; Valencia-Quintana et al. 2016a, b).

In this study, the tested compounds were administered via intraperitoneal route. This type of administration leads to the absorption of the tested compound through the portal circulation, which must pass through the liver before reaching systemic circulation and other organs (Turner et al. 2011). In the liver, parent terbuthlyazine is subjected to phase I metabolism, mediated via P450 (Lang et al. 1997), and results in the formation of several metabolites, with a different half-life in the body. The major routes of the metabolism are hydrolysis of the chlorine moiety and mono- or didealkylation. Hydroxylation of one or both of the dealkylated amine groups may also occur (US EPA 1995). Abass et al. (2012) also reported that an important reaction in terbuthylazine metabolism is N-deethylation, mediated at least in part by CYP1A1, CYP1A2, CYP2C19, and CYP3A4 enzymes. Overall terbuthylazine metabolism and clearance are rapid (50% is excreted by 16–17 h). It is completely metabolized and did not accumulate in tissues. In male rats, terbuthylazine is excreted equally in urine and feces, which contain up to 25 and 15 identified metabolites, respectively (US EPA 1995).

The treatment schedule we applied caused a constant delivery of new amounts of the tested compounds. Therefore, during 14 days, all cells were continuously exposed both to the parent compound and its metabolites, all of which possess certain potential to damage DNA, and the damage level we measured after treatment is a sum of their direct effects on DNA, indirect effects mediated via free radicals, but also of the outcomes of DNA repair, which might also introduce a certain amount of additional damage. It also has to be stressed that after 14 days, the equilibrium between DNA damage infliction and repair might occur, which also contributed to the values measured by the comet assay.

Taken together, our results indicate a low level of DNA instability caused by terbuthylazine on a mouse in vivo model, in spite of the relatively high tested dose, which ranged between current ADI and AOEL values proposed for the compound by European Union regulative (European Commission 2011; EFSA 2011). The results obtained in this comet assay study on mice are also comparable to results obtained in our former study (Tariba Lovaković et al. 2017), where the

alkaline comet assay was performed on blood cells of rats exposed for 28 days to terbuthylazine at doses of 0.004, 0.4, and 2.29 mg/kg bw day.

Unfortunately, we cannot verify and discuss our findings within the context of other sources, since no other rodent studies evaluated terbuthylazine genotoxicity thus far. A few studies evaluated the DNA-damaging effects of terbuthylazine-related triazine herbicides. Tennant et al. (2001) conducted an alkaline comet assay study on mice treated for 24 h with atrazine, simazine, and cyanazine, and their findings suggested small and marginal increases in DNA damage in leukocytes. Želježić and Garaj-Vrhovac (2004) found that pesticide formulations containing atrazine induced a statistically significant increase of comet tail length in blood, kidney, liver, bone marrow, and spleen of exposed mice. Mahmoud (2008) also reported that atrazine caused DNA damage in liver and kidney cells of mice measurable by the comet assay.

However, none of these studies suggested potential mechanisms responsible for the observed DNA instability. Although there is a general lack of studies that specifically point to interactions of terbuthylazine with DNA, some data are available for related compounds. Riahi et al. (2010) investigated how the structure and dynamics of DNA-binding was influenced by the herbicide terbuthryn. Using molecular modeling, they discovered that the complex between terbuthryn and DNA was completely capable of participating in the creation of a stable intercalation site. Other studies investigated mechanisms of atrazine interactions with DNA double-helix (Prance et al. 2010; Stobiecka et al. 2010) and concluded that atrazine causes the unwinding of double stranded DNA (or negative supercoiling), making it more vulnerable to attacks by herbicide adjuvants or other toxicants.

Based on our results, it is likely that terbuthlyazine is to a certain extent capable of producing cross-links in vivo, as we recognized based on the results obtained in vitro. This hypothesis is further corroborated by the results obtained for the third comet assay parameter, i.e., total area of the comets, which was evaluated here after in vivo exposure, as well (Fig. 6).

From the results shown in Fig. 6, it can be concluded that DNA in liver cells (possibly due to intense metabolism of the tested compound), and DNA in kidney cells (due to renal excretion of the metabolites) were more prone to direct toxic effects of the parent compound and its metabolites than leukocytes and bone marrow cells. In these particular cell types of exposed mice, we found a significant lowering of the parameter total comet area with respect to the control mice, similarly as observed in terbuthlyazine-treated HepG2 cells in vitro. Taken together, the same trend for the reduction of the total area of comets observed both in vitro in metabolically competent HepG2 cells and in vivo in hepatocytes and kidney cells of mice, which are exposed to different metabolites of

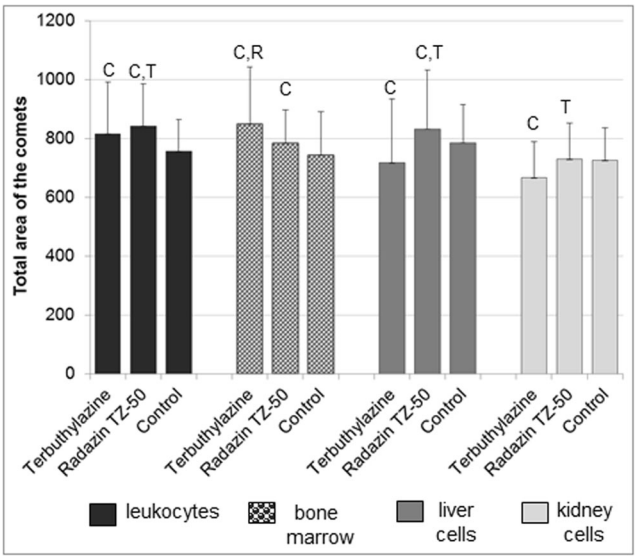

Fig. 6 Results regarding total area of comets measured using the alkaline comet assay in leukocytes, bone marrow, liver, and kidney cells of Swiss albino mice treated for 14 days with terbuthylazine and Radazin TZ-50 at a daily dose of 0.0035 mg/kg bw. The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance ($P < 0.05$): C—control mice; T—mice treated with terbuthylazine; R—mice treated with Radazin TZ-50

terbuthlyazine suggest the possibility for specific interactions at DNA level, which remain to be clarified in future studies after employing more sensitive methods.

Conclusions and future perspectives

The most important information this study added to existing knowledge is that acute in vitro exposure of human lymphocytes and HepG2 cells to terbuthylazine, and subacute 14-day exposure of mice to active substance terbuthylazine and its commercial formulation Radazin TZ-50 led to low-level DNA instability. Terbuthylazine genotoxicity was not primarily mediated by oxidative stress phenomena. The obtained results also suggest that terbuthylazine metabolism possibly results in the formation of reactive metabolites capable of inducing DNA cross-links, which hinder DNA migration, and this effects was most pronounced in liver cells in vivo and HepG2 cells in vitro. Our results also confirmed that simultaneous evaluation of several comet assay parameters is strongly advised to prevent erroneous conclusions regarding overall primary DNA damage measured by the comet assay.

Since the comet assay pointed only to primary DNA damage caused by the treatment, before drawing general conclusions on terbuthylazine genotoxicity, further studies are needed. They should focus on other cytogenetic tests that would allow more insight into its possible aneugenic effects, epigenetic mechanisms of DNA damage, and potential cell-cycle disturbances.

Terbuthylazine was previously stipulated as a persistent contaminant and was detected in hair samples of exposed agriculture workers (Mercadante et al. 2012a, b), which was evidently a result of their cumulative exposure. Since the highest terbuthylazine concentration tested in vitro, which was 2.5 times higher than the current occupational exposure limit set by European Commission (2011) and EFSA (2017), showed significant responses with majority of the assays used, we assume this finding should be interesting from the point of occupational hygiene and worthy of additional investigation. As many aspects of terbuthylazine toxicity remain unanswered, further research should focus on the effects of this herbicide in other in vitro and in vivo models by employing the same and other sensitive biomarkers of effect, along with different exposure scenarios.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Abass K, Turpeinen M, Rautio A, Hakkola J, Pelkonen O (2012) Metabolism of pesticides by human cytochrome P450 enzymes in vitro—a survey, Insecticides - advances in integrated pest management. In: Perveen F (ed). http://www.intechopen.com/books/insecticides-advances-in-integrated-pest-management/metabolism-ofpesticides-by-human-cytochrome-p450-enzymes-in-vitro-a-survey. Accessed 15 Feb 2018

Azqueta A, Collins AR (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch Toxicol 87(6):949–968

Belsten JL, Wright AJ (1995a) European Community–FLAIR common assay for whole-blood glutathione peroxidase (GSH-Px); results of an inter-laboratory trial. Eur J Clin Nutr 49(12):921–927

Belsten JL, Wright AJ (1995b) European community–FLAIR common assay for erythrocyte superoxide dismutase (SOD); results of an inter-laboratory trial. Eur J Clin Nutr 49(12):928–931

Benítez-Bribiesca L, Sánchez P, Toledo J, Peñarroja R, Flores M, Sosa J (2001) Differential staining of DNA strand breaks in dried comet assay slides. J Histochem Cytochem 49(7):921–922

Benković V, Đikić D, Grgorinić T, Mladinić M, Želježić D (2012) Haematology and blood chemistry changes in mice treated with terbuthylazine and its formulation Radazin TZ-50. Bull Environ Contam Toxicol 89(5):955–959

Bianchi J, Cabral-de-Mello DC, Marin-Morales MA (2015) Toxicogenetic effects of low concentrations of the pesticides imidacloprid and sulfentrazone individually and in combination in in vitro tests with HepG2 cells and *Salmonella typhimurium*. Ecotoxicol Environ Saf 120:174–183

Bolognesi C, Bonatti S, Degan P, Gallerani E, Peluso M, Rabboni R, Roggieri P, Abbondandolo A (1997) Genotoxic activity of glyphosate and its technical formulation Roundup. J Agric Food Chem 45(5):1957–1962

Bossi R, Vinggaard AM, Taxvig C, Boberg J, Bonefeld-Jørgensen EC (2013) Levels of pesticides and their metabolites in Wistar rat amniotic fluids and maternal urine upon gestational exposure. Int J Environ Res Public Health 10(6):2271–2281

Bradford MM (1976) A rapid and sensitive assay for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Buschfort C, Muller M, Seeber S, Rajewsky MF, Thomale J (1997) DNA excision repair profiles of normal and leukemic human lymphocytes: functional analysis at the single-cell level. Cancer Res 57(4):651–658

Buschfort-Papewalis C, Moritz T, Liedert B, Thomale J (2002) Down-regulation of DNA repair in human CD34(+) progenitor cells corresponds to increased drug sensitivity and apoptotic response. Blood 100(3):845–853

Cavas T (2011) In vivo genotoxicity evaluation of atrazine and atrazine-based herbicide on fish *Carassius auratus* using the micronucleus test and the comet assay. Food Chem Toxicol 49(6):1431–1435

Cox C, Surgan M (2006) Unidentified inert ingredients in pesticides: implications for human and environmental health. Environ Health Perspect 114(12):1803–1806

Drury JA, Nycyk JA, Cooke RWI (1997) Comparison of urinary and plasma malondialdehyde in preterm infants. Clin Chim Acta 263(2):177–185

Duke RC, Cohen JJ (1992) Morphological and biochemical assays of apoptosis. In: Coligan JE, Kruisbeal AM, Margulies DH, Shevach EM, Strober W (eds) Current protocols in immunology. Wiley, New York, pp 3.17.1–3.17.16

European Chemicals Agency (2015) Committee for Risk Assessment RAC Opinion proposing harmonised classification and labelling at EU level of Terbuthylazine (ISO); N-tert-butyl-6-chloro-N'-ethyl-1, 3,5-triazine-2,4-diamine. https://echa.europa.eu/documents/10162/7ae57f3d-2f2b-469d-ab34-5b97e48f493a. Accessed 12 Feb 2018

European Comission (2011) Review report for the active substance terbuthylazine finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 17 June 2011 in view of the approval of terbuthylazine as active substances in accordance with Regulation (EC) No 1107/2009. http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.ViewReview&id=467. Accessed 12 Feb 2018

European Food Safety Authority (EFSA) (2011) Conclusion on the peer review of the pesticide risk assessment of the active substance terbuthylazine. http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2011.1969/epdf. Accessed 12 Feb 2018

European Food Safety Authority (2016) Outcome of the consultation with Member States, the applicant and EFSA on the pesticide risk assessment for terbuthylazine in light of confirmatory data. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2016.EN-919. Accessed 12 Feb 2018

European Food Safety Authority (EFSA) (2017) Peer review of the pesticide risk assessment for the active substance terbuthylazine in light of confirmatory data submitted. http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2017.4868/epdf. Accessed 12 Feb 2018

Forgacs AL, D'Souza ML, Huhtaniemi IT, Rahman NA, Zacharewski TR (2013) Triazine herbicides and their chlorometabolites alter steroidogenesis in BLTK1 murine Leydig cells. Toxicol Sci 134(1): 155–167

Franco R, Sánchez-Olea R, Reyes-Reyes EM, Panayiotidis MI (2009) Environmental toxicity, oxidative stress and apoptosis: ménage à trois. Mutat Res 674(1):3–22

Gebel T, Kevekordes S, Pav K, Edenharder R, Dunkelberg H (1997) In vivo genotoxicity of selected herbicides in the mouse bone-marrow micronucleus test. Arch Toxicol 71(3):193–197

Ghisari M, Long M, Tabbo A, Bonefeld-Jørgensen EC (2015) Effects of currently used pesticides and their mixtures on the function of

thyroid hormone and aryl hydrocarbon receptor in cell culture. Toxicol Appl Pharmacol 284(3):292–303

Green MH, Waugh AP, Lowe JE, Harcourt SA, Cole J, Arlett CF (1994) Effect of deoxyribonucleosides on the hypersensitivity of human peripheral blood lymphocytes to UV-B and UV-C irradiation. Mutat Res 315(1):25–32

Griffiths HR, Dunston CR, Bennett SJ, Grant MM, Phillips DC, Kitas GD (2011) Free radicals and redox signalling in T-cells during chronic inflammation and ageing. Biochem Soc Trans 39(5):1273–1278

Grisolia CK, Bilich MR, Formigli LM (2004) A comparative toxicologic and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. Ecotoxicol Environ Saf 59(1):123–126

Guilherme S, Santos MA, Barroso C, Gaivão I, Pacheco M (2012) Differential genotoxicity of Roundup formulation and its constituents in blood cells of fish (*Anguilla anguilla*): considerations on chemical interactions and DNA damaging mechanisms. Ecotoxicology 21(5):1381–1390

Guo L, Dial S, Shi L, Branham W, Liu J, Fang J-L, Green B, Deng H, Kaput J, Ning B (2011) Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. Drug Metab Dispos 39(3):528–538

Hartnett S, Musah S, Dhanwada KR (2013) Cellular effects of metolachlor exposure on human liver (HepG2) cells. Chemosphere 90(3):1258–1266

Henderson L, Wolfreys A, Fedyk J, Bourner C, Windebank S (1998) The ability of the Comet assay to discriminate between genotoxins and cytotoxins. Mutagenesis. 13(1):89–94

Hreljac I, Zajc I, Lah T, Filipic M (2008) Effects of model organophosphorous pesticides on DNA damage and proliferation of HepG2 cells. Environ Mol Mutagen 49(5):360–367

Jurica K, Brčić Karačonji I, Benković V, Kopjar N (2017) In vitro assessment of the cytotoxic, DNA damaging, and cytogenetic effects of hydroquinone in human peripheral blood lymphocytes. Arh Hig Rada Toksikol 68(4):322–335

Kamencic H, Lyon A, Paterson PG, Juurlink BH (2000) Monochlorobimane fluorometric method to measure tissue glutathione. Anal Biochem 286(1):35–37

Kasai H (1997) Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. Mutat Res 387(3):147–163

Kašuba V, Milić M, Rozgaj R, Kopjar N, Mladinić M, Žunec S, Lucić Vrdoljak A, Pavičić I, Marjanović Čermak AM, Pizent A, Lovaković BT, Želježić D (2017) Effects of low doses of glyphosate on DNA damage, cell proliferation and oxidative stress in the HepG2 cell line. Environ Sci Pollut Res Int 24(23):19267–19281

Knasmüller S, Parzefall W, Sanyal R, Ecker S, Schwab C, Uhl M, Mersch-Sundermann V, Williamson G, Hietsch G, Langer T, Darroudi F, Natarajan AT (1998) Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutat Res 402(1–2):185–202

Knasmüller S, Mersch-Sundermann V, Kevekordes S, Darroudi F, Huber WW, Hoelzl C, Bichler J, Majer BJ (2004) Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. Toxicology 198(1–3): 315–328

Kocaman AY, Topaktas M (2009) The in vitro genotoxic effects of a commercial formulation of α-cypermethrin in human peripheral blood lymphocytes. Environ Mol Mutagen 50(1):27–36

Koutnik D, Stara A, Zuskova E, Kouba A, Velisek J (2017) The chronic effects of terbuthylazine-2-hydroxy on early life stages of marbled crayfish (*Procambarus fallax* f. virginalis). Pestic Biochem Physiol 136:29–33

Lang D, Criegee D, Grothusen A, Saalfrank RW, Böcker RH (1996) In vitro metabolism of atrazine, terbuthylazine, ametryne, and terbutryne in rats, pigs, and humans. Drug Metab Dispos 24(8): 859–865

Lang DH, Rettie AE, Böcker RH (1997) Identification of enzymes involved in the metabolism of atrazine, terbuthylazine, ametryne, and terbutryne in human liver microsomes. Chem Res Toxicol 10(9): 1037–1044

Langie SAS, Azqueta A, Collins AR (2015) The comet assay: past, present, and future. Front Genet 6:266. https://doi.org/10.3389/fgene.2015.00266

Liao W, McNutt MA, Zhu WG (2009) The comet assay: a sensitive method for detecting DNA damage in individual cells. Methods 48(1):46–53

Mahmoud IAEMD (2008) Studies of atrazine effects on DNA in mice by chromosomal aberration test and agarose gel electrophoresis assay. J Egypt Soc Toxicol 39:25–38

Manera M, Giari L, Depasquale JA, Dezfuli BS (2016a) European sea bass gill pathology after exposure to cadmium and terbuthylazine: expert versus fractal analysis. J Microsc 261(3):291–299

Manera M, Sayyaf Dezfuli B, DePasquale JA, Giari L (2016b) Multivariate approach to gill pathology in European sea bass after experimental exposure to cadmium and terbuthylazine. Ecotoxicol Environ Saf 129:282–290

Mateos R, Goya L, Bravo L (2004) Determination of malondialdehyde by liquid chromatography as the 2,4-dinitrophenylhydrazone derivative: a marker for oxidative stress in cell cultures of human hepatoma HepG2. J Chromatogr B 805(1):33–39

Matés JM, Pérez-Gómez C, Núñez de Castro I (1999) Antioxidant enzymes and human disease. Clin Biochem 32(8):595–603

Mercadante R, Polledri E, Fustinoni S (2012a) Determination of terbuthylazine and desethylterbuthylazine in human urine and hair samples by electrospray ionization-liquid chromatography/triple quadrupole mass spectrometry. Anal Bioanal Chem 404(3):875–886

Mercadante R, Polledri E, Giavini E, Menegola E, Bertazzi PA, Fustinoni S (2012b) Terbuthylazine in hair as a biomarker of exposure. Toxicol Lett 210(2):169–173

Mikulikova I, Modra H, Blahova J, Kruziková K, Marsalek P, Bedanova I, Svobodova Z (2013) Recovery ability of common carp (Cyprinus carpio) after a short-term exposure to terbuthylazine. Pol J Vet Sci 16(1):17–23

Mladinić M, Perković P, Želježić D (2009) Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay. Toxicol Lett 189(2):130–137

Mladinić M, Želježić D, Shaposhnikov SA, Collins AR (2012) The use of FISH-comet to detect c-Myc and TP 53 damage in extended-term lymphocyte cultures treated with terbuthylazine and carbofuran. Toxicol Lett 211(1):62–69

Molinari G, Soloneski S, Reigosa MA, Larramendy ML (2009) In vitro genotoxic and cytotoxic effects of ivermectin and its formulation ivomec® on Chinese hamster ovary (CHOK1) cells. J Hazard Mater 165(1–3):1074–1082

Nikoloff N, Larramendy ML, Soloneski S (2014) Assessment of DNA damage, cytotoxicity, and apoptosis in human hepatoma (HepG2) cells after flurochloridone herbicide exposure. Food Chem Toxicol 65:233–241

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95(2):351–358

Organisation for Economic Co-operation and Development (OECD) (2016) OECD guideline for the testing of chemicals. Test Guideline 487. http://www.oecd.org/env/test-no-487-in-vitro-mammalian-cell-micronucleus-test-9789264224438-en.htm. Accessed 9 Feb 2018

Papadopoulos N, Gikas E, Zalidis G, Tsarbopoulos A (2007) Simultaneous determination of terbuthylazine and its major hydroxy and dealkylated metabolites in wetland water samples using solid-

phase extraction and high-performance liquid chromatography with diode-array detection. J Agric Food Chem 55(18):7270–7277

Pareek A, Godavarthi A, Issarani R, Prakash Nagori B (2013) Antioxidant and hepatoprotective activity of *Fagonia schweinfurthii* (Hadidi) Hadidi extract in carbon tetrachloride induced hepatotoxicity in HepG2 cell line and rats. J Ethnopharmacol 150(3):973–981

Plhalova L, Stefanova S, Blahová J, Praskova E, Hostovsky M, Skoric M, Zelnickova L, Svobodova Z, Bedanova I (2012) The effects of subchronic exposure to terbuthylazine on zebrafish. Neuroendocrinol Lett 33(Suppl 3):113–119

Powell ER, Faldladdin N, Rand AD, Pelzer D, Schrunk EM, Dhanwada KR (2011) Atrazine exposure leads to altered growth of HepG2 cells. Toxicol in Vitro 25(3):644–651

Prance A, Coopersmith K, Stobiecka M, Hepel M (2010) Biosensors for the detection of DNA damage by toxicants. ECS Trans:33(8):3–33(8)15

Riahi S, Mashhadi A, Eynollahi S, Ganjali MR, Norouzi P (2010) Effect of herbicide terbutryn on the DNA base pairs: design of new herbicide with the minimum toxicity. Int J Electrochem Sci 5:955–966

Ribas G, Frenzilli G, Barale R, Marcos R (1995) Herbicide-induced DNA damage in human lymphocytes evaluated by the single-cell gel electrophoresis (SCGE) assay. Mutat Res 344(1–2):41–54

Sass JB, Colangelo A (2006) European Union bans atrazine, while the United States negotiates continued use. Int J Occup Environ Health 12(3):260–267

Saxena PN, Chauhan LK, Gupta SK (2005) Cytogenetic effects of commercial formulation of cypermethrin in root meristem cells of *Allium sativum*: spectroscopic basis of chromosome damage. Toxicology 216(2–3):244–252

Sigma-Aldrich Inc (2018) Histopaque®-1077 (Procedure No. 1077). http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/1/10771pis.pdf. Accessed 9 Feb 2018

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of damage in individual cells. Exp Cell Res 175(1):184–191

Smith CC, O'Donovan MR, Martin EA (2006) hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. Mutagenesis 21(3):185–190

Soloneski S, González M, Piaggio E, Reigosa MA, Larramendy MA (2002) Effect of dithiocarbamate pesticide zineb and its commercial formulation, azzurro III. Genotoxic evaluation on Chinese hamster ovary (CHO) cells. Mutat Res 514(1–2):201–212

Spanswick VJ, Hartley JM, Hartley JA (2010) Measurement of DNA interstrand crosslinking in individual cells using the single cell gel electrophoresis (comet) assay. In: Fox K (ed) Drug-DNA interaction protocols. Methods in molecular biology (methods and protocols), vol 613. Humana Press, pp 267–282

Stara A, Zuskova E, Kouba A, Velisek J (2016) Effects of terbuthylazine-desethyl, a terbuthylazine degradation product, on red swamp crayfish (*Procambarus clarkii*). Sci Total Environ 566-567:733–740

Štepanova S, Plhalova L, Doleželova P, Prokeš M, Maršalek M, Škorič M, Svobodova Z (2012) The effects of subchronic exposure to terbuthylazine on early developmental stages of common carp. Sci World J Article ID 615920, 7 pages. https://doi.org/10.1100/2012/615920

Stigaard Kjeldsen L, Ghisari M, Bonefeld-Jørgensen EC (2013) Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. Toxicol Appl Pharmacol 272(2):453–464

Stipičević S, Galzina N, Udiković-Kolić N, Jurina T, Mendaš G, Dvoršćak M, Petrić I, Barić K, Drevenkar V (2015) Distribution of terbuthylazine and atrazine residues in crop-cultivated soil: the effect of herbicide application rate on herbicide persistence. Geoderma 259-260:300–309

Stipičević S, Mendaš G, Dvoršćak M, Fingler S, Galzina N, Barić K (2017) Dissipation dynamics of terbuthylazine in soil during the maize growing season. Arh Hig Rada Toksikol 68(4):336–342

Stobiecka M, Coopersmith K, Cutler S, Hepel M (2010) Novel DNA-hybridization biosensors for studies of DNA underwinding caused by herbicides and pesticides. ECS Trans 28(34):1–12

Tariba Lovaković B, Pizent A, Kašuba V, Kopjar N, Micek V, Mendaš G, Dvoršćak M, Mikolić A, Milić M, Žunec S, Lucić Vrdoljak A, Želježić D (2017) Effects of sub-chronic exposure to terbuthylazine on DNA damage, oxidative stress and parent compound/metabolite levels in adult male rats. Food Chem Toxicol 108(Pt A):93–103

Taxvig C, Hadrup N, Boberg J, Axelstad M, Bossi R, Bonefeld-Jørgensen EC, Vinggaard AM (2013) In vitro—in vivo correlations for endocrine activity of a mixture of currently used pesticides. Toxicol Appl Pharmacol 272(3):757–766

Tennant AH, Peng B, Kligerman AD (2001) Genotoxicity studies of three triazine herbicides: in vivo studies using the alkaline single cell gel (SCG) assay. Mutat Res 493(1–2):1–10

Thomale J, Müller MR, Buschfort C, Seeber S, Rajewsky MF (1998) Alterations in DNA repair: implications for leukemia cell biology. In: Hiddemann W et al (eds) Acute Leukemias VII. Haematology and blood transfusion/Hämatologie und Bluttransfusion. Springer, Berlin, pp 3–12

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35(3):206–221

Trevigen (2017) hOGG1 FLARE™ Assay Kit. https://trevigen.com/docs/protocol/protocol_4130-100-FK.pdf. Accessed 9 Feb 2018

Turner PV, Brabb T, Pekow C, Vasbinder M (2011) Administration of substances to laboratory animals: routes of administration and factors to consider. J Am Assoc Lab Anim Sci 50(5):600–613

Ündeğer Ü, Başaran N (2005) Effects of pesticides on human peripheral lymphocytes in vitro: induction of DNA damage. Arch Toxicol 79(3):169–176

United States Environmental Protection Agency (USEPA) (1995) Reregistration Eligibility Decision (RED): Terbuthylazine 738-R-95-005. http://archive.epa.gov/pesticides/reregistration/web/pdf/2645.pdf. Accessed 9 Feb 2018

USEPA Office of Pesticide Programs, Chemicals Evaluated for Carcinogenic Potential (2017) Annual Cancer Report 2017. http://npic.orst.edu/chemicals_evaluated.pdf. Accessed 12 Feb 2018

Valencia-Quintana R, Gómez-Arroyo S, Sánchez-Alarcón J, Milić M, Olivares JL, Waliszewski SM, Cortés-Eslava J, Villalobos-Pietrini R, Calderón-Segura ME (2016a) Assessment of genotoxicity of Lannate-90® and its plant and animal metabolites in human lymphocyte cultures. Arh Hig Rada Toksikol 67(2):116–125

Valencia-Quintana R, Gómez-Arroyo S, Sánchez-Alarcón J, Milić M, Olivares JL, Waliszewski SM, Cortés-Eslava J, Villalobos-Pietrini R, Calderón-Segura ME (2016b) Genotoxic effects of the carbamate insecticide Pirimor-50® in *Vicia faba* root tip meristems and human lymphocyte culture after direct application and treatment with its metabolic extracts. Arh Hig Rada Toksikol 67(4):266–276

Velisek J, Stara A, Koutnik D, Machova J (2014) Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Biomed Res Int 621304. https://doi.org/10.1155/2014/621304

Velisek J, Stara A, Koutnik D, Zuskova E (2015) Effects of terbuthylazine on early life stages of common carp. Neuro Endocrinol Lett 36(Suppl 1):120–125

Velisek J, Koutnik D, Zuskova E, Stara A (2016a) Effects of the terbuthylazine metabolite terbuthylazine desethyl on common carp embryos and larvae. Sci Total Environ 539:214–220

Velisek J, Stara A, Zuskova E (2016b) Effect of single and combination of three triazine metabolites at environmental concentrations on early

life stages of common carp (*Cyprinus carpio* L.). Environ Sci Pollut Res Int 23(23):24289–24297

Velisek J, Stara A, Zuskova E, Kouba A (2017) Effects of three triazine metabolites and their mixture at environmentally relevant concentrations on early life stages of marbled crayfish (*Procambarus fallax* f. virginalis). Chemosphere 175:440–445

Villarini M, Scassellati-Sforzolini G, Moretti M, Pasquini R (2000) In vitro genotoxicity of terbutryn evaluated by the alkaline single-cell microgel-electrophoresis "comet" assay. Cell Biol Toxicol 16(5):285–292

Westerink WM, Schoonen WG (2007) Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. Toxicol in Vitro 21(8):1592–1602

World Health Organization (WHO) (2003) Terbuthylazine in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality. Geneva, World Health Organization WHO/SDE/WSH/03.04/63. http://www.who.int/water_sanitation_health/dwq/chemicals/terbuthylazine.pdf. Accessed 12 Feb 2018

Wu JH, Jones NJ (2012) Assessment of DNA interstrand crosslinks using the modified alkaline comet assay. In: Parry J, Parry E (eds) Genetic toxicology. Methods in Molecular Biology (Methods and Protocols), vol 817. Springer, New York, pp 165–181

Yusuf I, Fruman DA (2003) Regulation of quiescence in lymphocytes. Trends Immunol 24(7):380–386

Želježić D, Garaj-Vrhovac V (2004) Genotoxicity evaluation of pesticide formulations containing alachlor and atrazine in multiple mouse tissues (blood, kidney, liver, bone marrow, spleen) by comet assay. Neoplasma 51(3):198–203

Želježić D, Garaj-Vrhovac V, Perković P (2006) Evaluation of DNA damage induced by atrazine and atrazine-based herbicide in human lymphocytes in vitro using a comet and DNA diffusion assay. Toxicol in Vitro 20(6):923–935

Želježić D, Mladinić M, Žunec S, Lucić Vrdoljak A, Kašuba V, Tariba B, Živković T, Marjanović AM, Pavičić I, Milić M, Rozgaj R, Kopjar N (2016) Cytotoxic, genotoxic and biochemical markers of insecticide toxicity evaluated in human peripheral blood lymphocytes and an HepG2 cell line. Food Chem Toxicol 96:90–106

Žunec S, Kašuba V, Pavičić I, Marjanović AM, Tariba B, Milić M, Kopjar N, Pizent A, Lucić Vrdoljak A, Rozgaj R, Želježić D (2016) Assessment of oxidative stress responses and the cytotoxic and genotoxic potential of the herbicide tembotrione in HepG2 cells. Food Chem Toxicol 94:64–74