ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine
www.aaem.pl

Assessment of genetic damage and mutagenic properties of chlorpyrifos and glyphosate

Krzysztof Sawicki[1,A-D] ✉, **Magdalena Matysiak-Kucharek**[1,C], **Marcin Kruszewski**[1,2,E], **Lucyna Kapka-Skrzypczak**[1,3,E-F]

[1] *Department of Molecular Biology and Translational Research, Institute of Rural Health, Lublin, Poland*
[2] *Centre for Radiobiology and Biological Dosimetry, Institute of Nuclear Chemistry and Technology, Warsaw, Poland*
[3] *World Institute for Family Health, Calisia University, Kalisz, Poland*
A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

Sawicki K, Matysiak-Kucharek M, Kruszewski M, Kapka-Skrzypczak L. Assessment of genetic damage and mutagenic properties of chlorpyrifos and glyphosate. Ann Agric Environ Med. doi:10.26444/aaem/221150

Abstract

Introduction and Objective. Beyond acute and chronic poisoning, scientific attention in recent years has shifted toward the negative effects of pesticides on genetic material. Numerous studies demonstrate that these compounds possess both genotoxic and mutagenic potential. Therefore, the aim of this study is to investigate and compare the potential of chlorpyrifos (CPS) and glyphosate (GLY) to induce DNA damage.

Materials and Method. Human immortalized keratinocytes cell line HaCaT were used as the test organisms in viability (Neutral Red assay) and genotoxicity assays (Comet assay). To test the mutagenicity, *S. typhimurium* strains TA98 and TA100 assay was used in microplate format.

Results. The study demonstrated that CPS exhibits significantly higher toxicity and genotoxicity, compared to GLY. The Ames test showed that CPS possesses mutagenic potential (strain TA98) only after activation with the S9 fraction, remaining inactive against strain TA100. Although GLY did not induce mutations in strain TA98, at the highest concentration (1,000 µM), it caused substitution mutations (strain TA100).

Conclusions. Due to the complexity of pesticide biotransformation mechanisms and their diverse impact on genetic material, relying on a single test system is insufficient. To obtain a reliable safety assessment and capture the full spectrum of potential damage, it is necessary to combine bacterial methods, with cytogenetic studies on mammalian cells. Such a complementary approach eliminates errors resulting from the high cytotoxicity of pesticides toward bacteria, and enables the analysis of DNA mutations in non-bacterial models. Further research into the impact of these compounds on the genetic material of living organisms is therefore necessary, but using a greater number of biological models, as well as other methods to resolve this issue.

Key words

comet assay, chlorpyrifos, glyphosate, *Salmonella typhimurium*, Ames test, HaCaT

INTRODUCTION

The widespread use of pesticides in agriculture has enabled the effective elimination of various pest types, subsequently contributing to increased crop yields. However; alongside the positive aspects of pesticide application, significant concerns exist regarding their impact on the environment and human health. Beyond acute and chronic poisoning, scientific attention in recent years has shifted toward the negative effects of pesticides on genetic material. Numerous studies demonstrate that these compounds possess both genotoxic and mutagenic potential, which may be linked to long-term carcinogenesis [1, 2].

Chlorpyrifos (CPS) (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is a well-established broad-spectrum organophosphate insecticide used to control a wide variety of insects [3]. CPS is primarily known for inhibiting cholinesterase, leading to increased acetylcholine levels at neuronal junctions. Furthermore, many studies have shown that CPS exhibits genotoxic and teratogenic potential [4, 5].

Another representative of organophosphate compounds is Glyphosate (GLY) (N-[phosphonomethyl]glycine), the active ingredient in Roundup® formulations. It is one of the most frequently used herbicides in agriculture and horticulture for weed control [6]. Although GLY is still classified by global regulatory agencies as relatively safe, there are serious contradictions in research findings regarding its genotoxic properties. Literature data remain inconclusive and appear to depend largely on the methods and models employed in the experiments [7].

The terms 'genotoxicity' and 'mutagenicity' have often been used interchangeably, although they are sometimes defined differently [8]. Genotoxicity refers to the ability of substances to damage genomic DNA, resulting in mutagenic effects within cells. Chemically- induced DNA damage includes various modifications, such as small base lesions, bulky DNA adducts, and DNA strand breaks [9]. The genotoxic potential of chemicals can be evaluated using several available bioassays. The Comet assay is a well-established, relatively simple method used in genetic toxicology and environmental biomonitoring, which enables the detection of genetic material damage in individual cells, and allows for the assessment of cellular DNA repair capacity [10].

✉ Address for correspondence: Krzysztof Sawicki, Department of Molecular Biology and Translational Research, Institute of Rural Health, Lublin, Jaczewskiego 2, 20-090 Lublin, Poland
E-mail: sawicki.krzysztof@imw.lublin.pl

Received: 18.02.2026; accepted: 27.04.2026; first published: 07.05.2026

In contrast, mutagenicity refers specifically to gene mutations and chromosomal aberrations [8]. The *Salmonella* mutagenicity test (Ames test) is a rapid and simple bacterial assay based on the detection of histidine-independent revertants in selected *Salmonella* strains following exposure to specific mutagens. These reverse mutations allow the bacterial strains to regain their ability to synthesize histidine. This assay is used worldwide as a preliminary screen to determine the mutagenic potential of both new and existing chemicals [11]. To date, many miniaturized versions of the Ames test have been developed and are widely utilized, offering high sensitivity while using smaller quantities of materials than the standard assay [12].

Despite individual limitations, the Comet assay and the Ames test used in combination can help detect a wide range of chemicals that cause genotoxicity or mutagenicity [1]. Therefore, combining prokaryotic and eukaryotic evaluation systems supports and correlates observations, confirming whether specific xenobiotic compounds significantly affect the genetic material of living organisms [13].

OBJECTIVE

The aim of the study is to investigate the potential of CPS and GLY to induce DNA damage by performing Comet assays on HaCaT cells and the *Salmonella* mutagenicity test to compare their genotoxic and mutagenic potential. The Comet assay was performed using the HaCaT human keratinocyte cell line, which was spontaneously immortalized. This well-characterized cell line has been shown to be a suitable model for toxicology. These cells exhibit good predictive performance and serve as a suitable model for studying various adverse effects of insecticides [14].

MATERIALS AND METHOD

All chemicals, culture media and supplements were purchased from Merck KGaA (Darmstadt, Germany), unless otherwise indicated.

Cell culture. Human immortalized keratinocytes cell line HaCaT (CLS Cell Lines Service GmbH, Eppelheim, Germany) were used as the test organisms in viability (Neutral Red assay) and genotoxicity assays (Comet assay). Keratinocytes were cultured in DMEM medium supplemented with 4,500 mg/l glucose, 1% solution (200 mM L-glutamine, 10,000 units of Penicillin and 10 mg/mL of Streptomycin) and 10% Foetal Bovine Serum. The HaCaT cells were cultured according to the manufacturer's instructions at 37°C and 5% CO_2.

Chemicals tested. Two representatives of organophosphorus compounds were used in the study: the insecticide chlorpyrifos (CPS, O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate; CAS No. 2921-88-2; purity ≥ 98.0%) and the herbicide glyphosate (GLY, N-(Phosphonomethyl)glycine; CAS No. 1071-83-6; purity ≥ 98.0%). Both compounds were dissolved in DMSO, CPS 100 mM (stock solution), GLY 50 mM (stock solution) was divided into aliquots and frozen at -20 °C.

Neutral red. HaCaT cells were seeded in a 96-well plate (TPP, Switzerland) at density 5 x 10^3 per well. After 24 h incubation at 37°C and 5% CO_2, the culture medium was gently removed and dilutions of pesticides in culture medium were added at 100 µl per well (final DMSO concentration in the samples did not exceed 1% (v/v)). The following concentrations of CPS/GLY pesticides were used: (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1,000 µM) for 24 h incubation at 37°C and 5% CO_2. In the next step, the culture medium with pesticide was gently removed and the cells were washed with 150 µl/well PBS with Ca^{2+} and Mg^{2+} ions. Then, 100 µl of NR solution in culture medium (50 µg/ml) was added to the wells and incubated for 3 h at 37°C and 5% CO_2. After incubation, the NR solution was removed, the cells were washed again with 150 µl/well PBS with Ca^{2+} and Mg^{2+} ions and 150 µl/well each of fixative solution (ethyl alcohol 96%; dist. H_2O; acetic acid 99.5% in a ratio of 50:49:1) (Avantor Performance Materials, Poland) was added. In the final step, the 96-well plate was shaken for 15 min and the absorbance of the samples were measured using an Omega FLUOstar Microplate Reader (BMG LABTECH GmbH, Germany) at 540 nm. The analysis was performed in 3 replicates.

Comet assay. HaCaT cells were seeded in a 6-well plate at density 4×10^5 per well. The plates were incubated for 24 h at 37 °C and 5% CO_2 to achieve proper cell attachment. After incubation, the culture medium was gently removed from the wells and the following concentrations of CPS/GLY pesticides were used: (0, 0.1, 1, 10, 100, 1,000 µM) in the culture medium were added instead (final DMSO concentration in the samples did not exceed 1% [v/v]). Cells were incubated with CPS/GLY for 3 or 24 h at 37 °C and 5% CO_2. After pesticide incubation supernatant was gently removed, the cells washed with warm PBS without Ca^{2+} Mg^{2+} and trypsinized, harvested and dry cell pellets were frozen at -80 °C.

On the another day, the dry cell pellet was next re-suspended in 150 µl of PBS without Ca^{2+} Mg^{2+} and mixed 1:1 with 2% type VII agarose in PBS without Ca^{2+} Mg^{2+}. A 100 µl of this suspension were then added to slides coated with 0.5% type IA agarose and incubated for few minutes. Slides were then transferred to a glass slide chamber with lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, Triton X-100 1%, pH 10) and allowed to stand for 1 h at 4 °C in darkness. After lysis, slides were rinsed in PBS without Ca^{2+} Mg^{2+}. Slides were then placed in a chamber in electrophoresis buffer (300mM NaOH, 1mM EDTA, pH 14) and left for 40 min at 4 °C without any light for unwinding. The slides were next subjected to electrophoresis for 30 min (1.35 V/cm; approx. 480 mA) at 4 °C in darkness. The slides were then rinsed 3 times in dist. H_2O, 3 times in Tris HCl buffer (0.4M pH 7.5) and again 3 times in dist. H_2O, and allowed to dry. The slides were then stained with DAPI (0.5 µg/ml) at 100 µl per slide and placed in a humidity chamber overnight. The slides were read off using an Olympus BX51 fluorescence microscope (Olympus, Japan) and Komet 6.0 programme (Andor, USA). One hundred comets were interrogated from each duplicate slide.

Ames test. To test the mutagenic properties of CPS and GLY, an Ames MPF™ 98/100 assay was used, in microplate format developed by Xenometrix AG (Allschwil, Switzerland) according to the OECD 471 guidelines, which used *S. typhimurium* strains TA98 and TA100. Strain TA98 is designed to detect reading frame mutations, while strain TA100 is designed to detect mutations involving base substitutions.

On the first day of the experiment, a bacterial culture was established. Ampoules with TA98 and TA100 strains were thawed. The bacterial pellet was re-suspended and 25 µl each transferred into labelled 50 ml air filter tubes. To the culture was added 10 ml each of GM (growth medium) and 10 µl of ampicillin (50 mg/ml). The bacterial cultures were placed on an orbital shaker (250 rpm) and left for 16 h in an incubator at 37 °C. After the incubation period, the density of the cultures was measured using a spectrophotometer, assuming that the appropriate bacterial culture density for the experiment was within the range of 2.0–3.0 [OD600].

Four 24-well plates were then prepared, to which were added (in 3 replicates):

(a) 6 selected concentrations of pesticide (0.01, 0.1, 1, 10, 100, 1,000 µM) at 10 µl/well.
(b) Positive control at 10 µl/well:
   – 2-nitrofluorene (2-NF) for TA98 strain without metabolic activation of S9;
   – 4-Nitroquinoline-N-Oxide (4-NQO) for strain TA100 without S9 metabolic activation;
   – 2-Aminoanthracene (2-AA) for both strains and under metabolic activation of S9.

All test samples and positive controls were dissolved in DMSO. All analytical results were normalized to control '0' (pure DMSO).

The wells of two 24-well plates were each supplemented with 240 µl of TA98/100 bacterial culture solution in EM (exposure medium). Another two 24-well plates were each supplemented with 240 µl/well of TA98/100 bacterial culture solution in EM supplemented with liver fraction from rat S9 Mix. The 24-well plates prepared in this way were placed on an orbital shaker (250 rpm) and left for 1.5 h in an incubator at 37 °C. After 90 min of incubation, 2.6 ml/well of indicator medium was added to the plates. Then, 50 µl was transferred from each well of the 24-well plate to 48 wells of a 384-well plate. Each 384-well plate corresponded to 4 different conditions of the experiment conducted in 3 replicates. All plates were then placed in a string bag for incubation and left for 48 h in incubator at 37 °C. After the incubation period, positive wells on a 384-well plate were counted in triplicate for each strain and under conditions without/metabolic activation of S9. Positive wells were considered those that were yellow colour, or in which a colony of bacteria was visible at the bottom of the well.

Statistical analysis. The obtained results for viability (NR) and genotoxicity (Comet Assay) were analyzed using MS Office Excel 2013 (Microsoft Corporation, USA), and GrapPad Prism 9.5.1. (GraphPad Software Inc., USA). Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. For variables with lack of normal distribution, Kruskal-Wallis test and *post-hoc* Dunn's Multiple Comparison test was performed. Data are presented as the mean±standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference. Results from the Ames test were analyzed using Xenometrix AG's spreadsheet ver. 3.24u. For statistical analysis, the Cumulative Binomial Distribution test was used to determine the probable difference of 99% of doses with solvent control.

RESULTS

Influence of tested pesticides on the viability of keratinocytes. After 24 hours of exposure to either CPS or GLY, a statistically significant decrease in the viability of HaCaT keratinocytes was observed, compared to controls ($P < 0.05$) (Fig. 1 and 2). The results of the analysis showed that CPS was more toxic to HaCaT cells compared to GLY. At the highest concentration applied (1,000 µM), exposure to CPS reduced the viability of keratinocytes by 66.8 pp (33.25±6.6) (Fig. 1), while exposure to GLY only, reduced the viability by 9.65 pp (90.35±16.76) (Fig. 2).

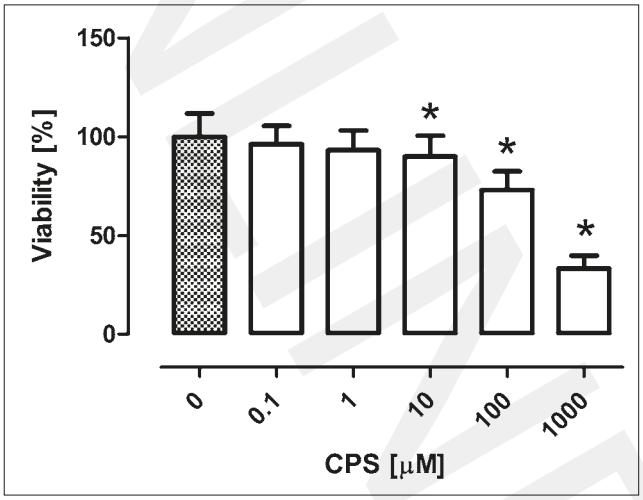

Figure 1. Viability of human keratinocytes HaCaT after 24 hour exposure on CPS. Results are expressed as a percent of the control sample.
* statistically significant difference of means from control (cells not treated with CPS), One-way ANOVA and *post-hoc* Tukey's Multiple Comparison Test ($P < 0.05$); Mean±SD, (n = 18), CPS – chlorpyrifos

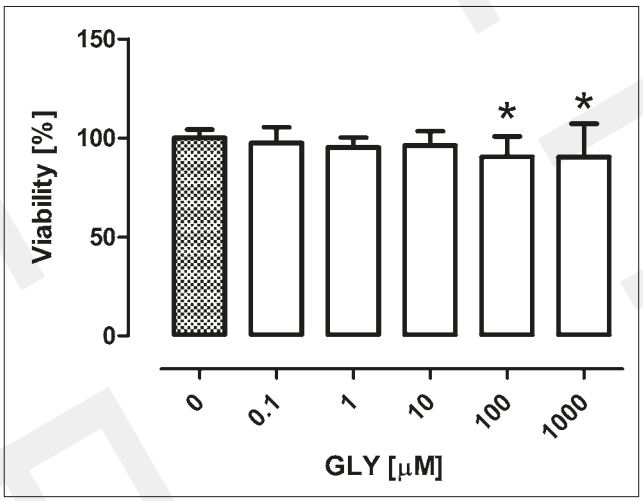

Figure 2. Viability of human keratinocytes HaCaT after 24 hour exposure on GLY. Results are expressed as a percent of the control sample.
* statistically significant difference of means from control (cells not treated with GLY), One-way ANOVA and *post-hoc* Tukey's Multiple Comparison Test ($P < 0.05$); Mean±SD, (n = 18); GLY – glyphosate

Genotoxicity analysis. Comet assay analysis showed that exposure of HaCaT cells to CPS induces significant DNA damage, compared to control sample ($P < 0.05$) (Fig. 3). The highest, more than 2-fold DNA damage, was observed after a 3-hour exposure with 100 µM CPS, whereas, a reduction in the level of DNA damage was observed after 24-hour exposure to

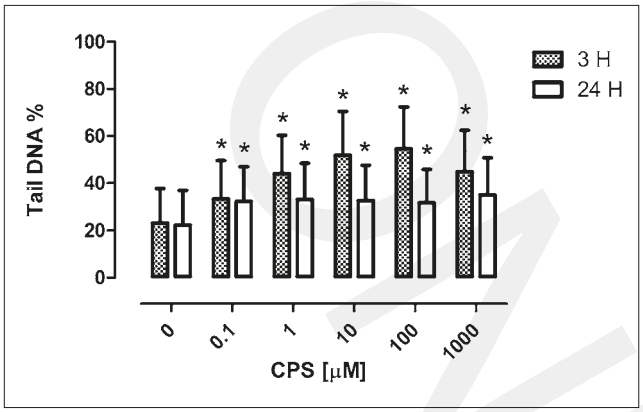

Figure 3. Genotoxic effect of CPS after 3 or 24 hour exposure of human keratinocytes HaCaT.
* statistically significant difference of means from control (cells not treated with CPS), Kruskal-Wallis test and post-hoc Dunn's Multiple Comparison Test ($P < 0.05$). Mean±SD, (n = 200); CPS – chlorpyrifos

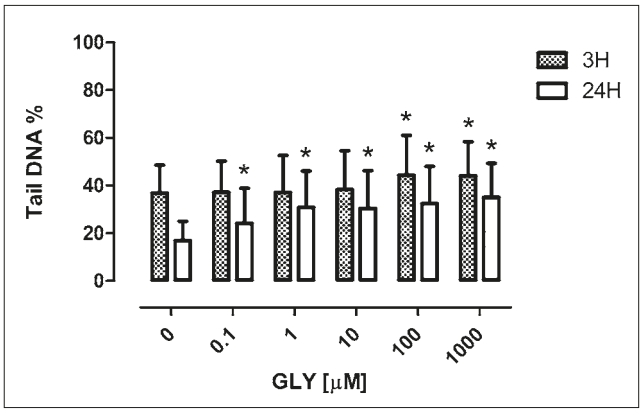

Figure 4. Genotoxic effect of GLY after 3 or 24 hour exposure of human keratinocytes HaCaT.
*denotes statistically significant difference of means from control (cells not treated with GLY), Kruskal-Wallis test and *post-hoc* Dunn's Multiple Comparison Test ($P < 0.05$). Mean±SD, (n = 200); GLY – glyphosate

Table 1. Mutagenicity analysis of chlorpyrifos using *S. typhimurium* assay with TA98 and TA100 strain with or without metabolic activation (Ames test)

Test substance	Concentration [μM]	Mean positive wells ± SD	Baseline	Fold increase over baseline	Binomial B-value
TA98 S9 (-)					
CPS	0	1.67 ± 1.15	2.82		
	0.1	0.67 ± 1.15		0.24	0.12
	1	1.33 ± 1.15		0.47	0.43
	10	0.33 ± 0.58		0.12	0.03
	100	0.67 ± 0.58		0.24	0.12
	1000	1.33 ± 0.58		0.47	0.43
2-NF	2.0 [μg/ml]	**48.00 ± 0.00**		**17.01**	**1.00**
TA98 S9 (+)					
CPS	0	0.33 ± 0.58	1.00		
	0.1	**2.67 ± 1.15**		**2.67**	**1.00**
	1	1.33 ± 1.15		1.33	0.99
	10	**2.00 ± 0.00**		**2.00**	**0.99**
	100	1.67 ± 1.15		1.67	0.99
	1000	1.00 ± 1.00		1.00	0.98
2-AA	0.55 [μg/ml]	**48.00 ± 0.00**		**48.00**	**1.00**
TA100 S9 (-)					
CPS	0	2.67 ± 1.15	3.82		
	0.1	4.00 ± 1.00		1.05	0.94
	1	4.00 ± 3.00		1.05	0.94
	10	4.67 ± 1.53		1.22	0.98
	100	3.00 ± 2.65		0.79	0.72
	1000	2.67 ± 1.15		0.70	0.59
4-NQO	0.1 [μg/ml]	**48.00 ± 0.00**		**12.56**	**1.00**
TA100 S9 (+)					
CPS	0	4.33 ± 1.15	5.49		
	0.1	4.67 ± 1.53		0.85	0.68
	1	6.00 ± 0.00		1.09	0.93
	10	6.00 ± 0.00		1.09	0.93
	100	3.00 ± 2.65		0.55	0.15
	1000	5.33 ± 1.15		0.97	0.84
2-AA	1.25 [μg/ml]	**48.00 ± 0.00**		**8.75**	**1.00**

All test samples and positive controls were dissolved in DMSO. All analytical results were normalized to control "0" (pure DMSO). The binomial value B indicates the probability that spontaneous mutation events alone will result in, at most, n = the total number of positive wells at a given sample concentration. A binomial value of B ≥ 0.99 indicates that the chance ≤ 1% that this result is due to spontaneous mutation. Data points with an increase in multiplicity ≥ 2 and a binomial B-value ≥ 0.99 are marked, as mutagenic concentration. 2-AA, 2-aminoanthracene, 2-NF, 2-nitrofluorene, 4-NQO, 4- nitroquinoline-N-oxide, CPS, chlorpyrifos, GLY, glyphosate

Table 2. Mutagenicity analysis of glyphosate using *S. typhimurium* assay with TA98 and TA100 strain with or without metabolic activation (Ames test).

Test substance	Concentration [μM]	Mean positive wells ± SD	Baseline	Fold increase over baseline	Binomial B-value
TA98 S9 (-)					
GFS	0	0.67 ± 0.58	1.24		
	0.1	0.67 ± 1.15		0.54	0.67
	1	2.00 ± 2.65		1.61	**0.99**
	10	1.33 ± 0.58		1.07	0.94
	100	1.67 ± 1.15		1.34	0.98
	1000	0.33 ± 0.58		0.27	0.40
2-NF	2.0 [μg/ml]	47.33 ± 0.58		**38.05**	**1.00**
TA98 S9 (+)					
GFS	0	0.67 ± 1.15	1.82		
	0.1	0.67 ± 1.15		0.37	0.67
	1	1.00 ± 1.00		0.55	0.85
	10	0.67 ± 1.15		0.37	0.67
	100	1.00 ± 0.00		0.55	0.85
	1000	0.67 ± 0.58		0.37	0.67
2-AA	0.55 [μg/ml]	47.67 ± 0.58		**26.17**	**1.00**
TA100 S9 (-)					
GFS	0	3.00 ± 0.00	3.00		
	0.1	5.33 ± 1.53		1.78	**0.99**
	1	5.00 ± 1.73		1.67	0.98
	10	3.33 ± 1.53		1.11	0.70
	100	3.67 ± 2.08		1.22	0.80
	1000	6.00 ± 2.00		**2.00**	**0.99**
4-NQO	0.1 [μg/ml]	48.00 ± 0.00		16.00	1.00
TA100 S9 (+)					
GFS	0	3.67 ± 2.89	6.55		
	0.1	8.33 ± 3.21		1.27	**1.00**
	1	7.00 ± 2.00		1.07	**0.99**
	10	6.00 ± 2.65		0.92	0.98
	100	9.67 ± 1.53		1.48	**1.00**
	1000	9.67 ± 1.15		1.48	**1.00**
2-AA	1.25 [μg/ml]	47.67 ± 0.58		**7.27**	**1.00**

All test samples and positive controls were dissolved in DMSO, and all analytical results normalized to control '0' (pure DMSO). The binomial value B indicates the probability that spontaneous mutation events alone will result in, at most, n = the total number of positive wells at a given sample concentration. A binomial value of B ≥ 0.99 indicates that the chance ≤ 1% that this result is due to spontaneous mutation. Data indicates that with an increase in multiplicity ≥ 2 and a binomial B-value ≥ 0.99 are marked, as mutagenic concentration. 2-AA, 2-aminoanthracene, 2-NF, 2-nitrofluorene, 4-NQO, 4- nitroquinoline-N-oxide, CPS, chlorpyrifos, GLY, glyphosate

CPS, compared to 3-hour exposure at all of the concentrations used. In contrast, after 3-hour exposure of keratinocytes to GLY, a slight but statistically significant increase in the level of DNA damage was observed only at the 2 highest concentrations (100, 1,000 μM) ($P < 0.05$) (Fig. 4). Similar to CPS, a reduction in the level of DNA damage was shown after 24-hour exposure to GLY, compared to 3-hour exposure of keratinocytes. The analysis demonstrated that CPS has a higher genotoxic potential against HaCaT cells than GLY.

Mutagenic analysis

Ames test in microplate format demonstrated that CPS in tested doses for TA98 strain (detecting reading frame mutations) is probably mutagenic only in the presence of microsomal S9 fraction from rat liver. In contrast, no mutagenic properties were observed for CPS tested on TA100 strain (detecting substitution mutations) (Tab. 1). Whereas no mutagenic properties were detected for GLS regarding reading frame mutations (TA98 strain). On the other hand, it was observed that the highest concentration of GLY (1,000 μM) caused a mutation involving base substitution (TA100 strain) (Tab. 2).

DISCUSSION

The presented study attempted to compare the genotoxic and mutagenic potential of two representatives of organophosphorus compounds – CPS and GLY. Studies utilizing the neutral red uptake assay demonstrated that both tested compounds, CPS and GLY, inhibit the viability of HaCaT cells in a dose-dependent manner. However, it should be emphasized that CPS exhibits significantly higher toxicity, compared to GLY.

The obtained results are consistent with other published studies in which a significant, dose-dependent decrease in

cell viability was observed after 24-hour exposure to CPS in models such as: mouse 3T3-L1 preadipocytes, human hepatocellular carcinoma HepG2, and mouse neuronal HT22 cells [15, 16, 17]. It is postulated that CPS toxicity stems primarily from the induction of oxidative stress and mitochondrial dysfunction [17].

Conversely, in the case of exposure to GLY, the situation appears more complex and may depend on both the cell type used and the duration of exposure. In a study conducted on several cell lines (human colorectal adenocarcinoma CaCo-2, HepG2, human epidermoid carcinoma A431, HaCaT, human melanoma SK-MEL-5, and mouse macrophages RAW 264.7), cells were exposed to various concentrations of GLY (0–1 mM) for 24 and 48 hours. It was observed that GLY did not affect the viability of CaCo-2 cells, and had only a minimal impact on the other lines (HepG2, A431, HaCaT, and RAW 264.7). On the other hand, the SK-MEL-5 line was the only cell type among those tested in which viability significantly decreased after exposure to 1 mM GLY at both 24 and 48 hours, with reductions of 23.3% and 18.7%, respectively [18].

Subsequently, the genotoxic potential of CPS and GLY was investigated using the immortalized human keratinocyte model. The comet assay revealed that CPS possesses a stronger genotoxic potential toward HaCaT cells than GLY. Exposure to CPS induced significant DNA damage ($P < 0.05$), reaching over a two-fold increase after 3 hours at 100 μM. In the case of GLY, a small but statistically significant increase in damage was recorded only at the highest concentrations (100 and 1,000 μM). In both instances, extending the exposure time to 24 hours resulted in a reduction in the levels of DNA damage, compared to the 3-hour results, suggesting the activation of genetic material repair processes.

Confirmation of the genotoxic potential of the studied compounds can be found in research conducted on erythrocytes isolated from the blood of the broad-snouted caiman (*Caiman latirostris*), exposed to CPS and GLY, among others. Comet assay results showed that both compounds cause a statistically significant increase in DNA damage. The authors postulate that an excess of reactive oxygen species (ROS) and the impairment of antioxidant enzymes may be responsible for the genetic damage [19].

The genotoxic potential of CPS was also confirmed in studies on lymphocytes isolated from male and female rats [3, 4], in which it was found that CPS increases the activity of superoxide dismutase, glutathione peroxidase, and catalase. Thus, the most likely mechanism for DNA damage and chromosomal breaks induced by CPS appears to be ROS production.

Similarly, for GLY, research confirms significant genotoxic potential across a wide spectrum of cell models – from human erythrocytes and kidney cells (HEK293) to mouse lines and *in vivo* rat studies. A key conclusion is that this genotoxicity is not the result of a single mechanism but a cascade of molecular processes. It is proposed that the primary factor determining GLY genotoxicity is the induction of oxidative stress [6, 7]. GLY-based formulations stimulate excessive ROS production, leading to oxidative damage to lipids, proteins, and nucleic acids.

Particularly significant is the observation by Milić et al. (2018) regarding a molecular substitution mechanism in which glyphosate replaces glycine. This process leads to the accumulation of unrepaired 8-oxoG adducts and the formation of double-strand breaks, posing a serious threat to genomic stability [20]. Furthermore, analysis of results obtained from HEK293 cells sheds new light on the persistence of induced changes. The demonstration that at a concentration of 3500 μg/L, cells lose the ability to effectively repair DNA strand breaks even after exposure ceases, suggests that glyphosate may permanently impair cellular repair systems. This is of concern because damage was observed at concentrations considered environmentally relevant (70 μg/L) [21].

In the final stage of the study, the mutagenic potential of both compounds was analyzed. The Ames test showed that CPS possesses mutagenic potential (frameshift mutations, strain TA98) only after activation with the S9 fraction, remaining inactive against strain TA100. While GLY did not induce mutations in strain TA98, at the highest concentration (1000 μM), it caused substitution mutations (strain TA100). A key observation was the selective mutagenicity of CPS toward TA98, occurring exclusively in the presence of the S9 fraction. This suggests that CPS itself does not possess direct mutagenic properties regarding frameshift mutations, acquiring this ability only through metabolic transformation. According to the toxicological mechanism of thioester-type organophosphorus compounds (P=S), their full biological activity is revealed after conversion to the oxon form (P=O). As shown in this study, it is this transformation, occurring in the presence of the S9 microsomal fraction, that may be responsible for inducing mutations in the TA98 strain.

These results differ from earlier literature reports. In studies by Gollapudi et al. (1995) and Yaduvanshi et al. (2012), no mutagenic activity of CPS toward TA98 was found, regardless of the S9 fraction's presence [2, 22]. This discrepancy may result from the doses used and the toxicity of the compound. In a study by Yaduvanshi et al. (2012), doses of 5,000 and 10,000 μg/plate were strongly cytotoxic, which could have masked the mutagenic effect. A lack of CPS mutagenicity was also shown by Isidori et al. (2009); however, these authors operated in a much lower concentration range (0.713–5.70 μM) than those applied in the present study [23]. This suggests the existence of a concentration threshold above which CPS (after activation) begins to interact with bacterial DNA. The lack of CPS activity toward strain TA100 in the presented study is consistent with most literature findings [22, 23], except for reports by Egorova et al. (2020), who recorded a positive result only for this strain under non-activation conditions (-S9) [12]. Such discrepancies highlight the influence of experimental conditions on the sensitivity of the Ames test.

For GLY, the induction of substitution mutations (TA100) was recorded only at the highest tested concentration (1,000 μM). This result partially aligns with observations by Rank et al. (1993), who showed weak mutagenicity of the Roundup formulation toward TA100 at doses near toxic levels [24]. It should be emphasized that pure glyphosate is over 100 times less toxic compared to the commercially available formulation, due to the presence of surfactants (including polyethoxylated tallow amine – POEA). Therefore, pure glyphosate has yielded negative results in many tests, including in a study by Ilyushina et al. (2019) [25]. The difference between the presented results and Ilyushina's data may stem from reaching a critical concentration (1 mM), which under specific test conditions can lead to DNA damage, despite GLY being widely recognized as a non-clastogenic substance in mammalian tests (e.g., micronucleus test).

CONCLUSIONS

This study compared the genotoxicity and mutagenicity of CPS and GLY. Research on human keratinocytes has shown that both compounds CPS and GLY reduce cell viability, with CPS being significantly more toxic than GLY. The toxicity mechanism for both may be primarily linked to the disruption of antioxidant mechanisms and the induction of oxidative stress. Nevertheless, the toxicity of both compounds depends on the choice of research model and the dose applied. Genotoxicity analysis confirmed that CPS damages HaCaT cell DNA more severely than GLY, although both compounds exhibit genotoxic potential, likely due to ROS overproduction. The Ames test demonstrated that CPS is mutagenic (strain TA98) only after metabolic activation (S9 fraction), which is linked to its conversion into the oxon form. Conversely, GLY showed weak mutagenic effects (strain TA100) only at the highest concentrations. Discrepancies with some literature can be explained by differences in concentrations and experimental conditions, highlighting the role of cytotoxicity in masking mutagenic effects.

Due to the complexity of pesticide biotransformation mechanisms and their diverse impact on genetic material, relying on a single test system is insufficient. To obtain a reliable safety assessment and capture the full spectrum of potential damage (from gene mutations to chromosomal and genomic damage), it is necessary to combine bacterial methods, such as the Ames test, with cytogenetic studies on mammalian cells (e.g., comet assay, micronucleus test). Such a complementary approach eliminates errors resulting from the high cytotoxicity of pesticides toward bacteria and enables the analysis of DNA mutations in non-bacterial models.

Further research into the impact of these compounds on the genetic material of living organisms is therefore necessary, but using a greater number of biological models, as well as other methods to resolve this issue.

Funding: This research was funded by the Institute of Rural Health in Lublin from statutory funds allocated for the financing of scientific study No. 23030.

REFERENCES

1. Franco-Bernardes MF, Rocha OP, Pereira LC, et al. The herbicides trifluralin and tebuthiuron have no genotoxic or mutagenic potential as evidenced by genetic tests. Environ Sci Pollut Res Int. 2017 Oct;24(30):24029–24037. doi:10.1007/s11356-017-9966-5
2. Yaduvanshi SK, Srivastava N, Marotta F, et al. Evaluation of micronuclei induction capacity and mutagenicity of organochlorine and organophosphate pesticides. Drug Metab Lett. 2012 Sep 1;6(3):187–97. doi:10.2174/1872312811206030006
3. Essa SS, El-Saied EM, El-Tawil OS, et al. Nanoparticles of zinc oxide defeat chlorpyrifos-induced immunotoxic effects and histopathological alterations. Vet World. 2019;12(3):440–448. doi:10.14202/vetworld.2019.440-448
4. Sandhu MA, Saeed AA, Khilji MS, et al. Genotoxicity evaluation of chlorpyrifos: a gender related approach in regular toxicity testing. J Toxicol Sci. 2013;38(2):237–44. doi:10.2131/jts.38.237
5. Lu YC, Chiang CY, Chen SP, et al. Chlorpyrifos-induced suppression of the antioxidative defense system leads to cytotoxicity and genotoxicity in macrophages. Environ Toxicol Pharmacol. 2024 Jun;108:104468. doi:10.1016/j.etap.2024.104468
6. Ayanda OI, Tolulope A, Oniye SJ. Mutagenicity and genotoxicity in juvenile African catfish, Clarias gariepinus exposed to formulations of glyphosate and paraquat. Sci Prog. 2021 Apr-Jun;104(2):368504211021751. doi:10.1177/00368504211021751
7. Alvarez-Moya C, Reynoso-Silva M. Assessment of Genetic Damage Induced via Glyphosate and Three Commercial Formulations with Adjuvants in Human Blood Cells. Int J Mol Sci. 2023 Feb 25;24(5):4560. doi:10.3390/ijms24054560
8. Hayashi M. Opinion: regulatory genotoxicity: past, present and future. Genes Environ. 2022 Apr 22;44(1):13. doi:10.1186/s41021-022-00242-5
9. Sassa A, Fukuda T, Ukai A, et al. Follow-up genotoxicity assessment of Ames-positive/equivocal chemicals using the improved thymidine kinase gene mutation assay in DNA repair-deficient human TK6 cells. Mutagenesis. 2021 Oct 6;36(5):331–338. doi:10.1093/mutage/geab025
10. Kašuba V, Micek V, Milić M, et al. The effect of low doses of chlorpyrifos on blood and bone marrow cells in Wistar rats. Arh Hig Rada Toksikol. 2022 Sep 30;73(3):223–232. doi:10.2478/aiht-2022-73-3665
11. Madia F, Kirkland D, Morita T, et al. EURL ECVAM Genotoxicity and Carcinogenicity Database of Substances Eliciting Negative Results in the Ames Test: Construction of the Database. Mutat Res Genet Toxicol Environ Mutagen. 2020 Jun-Jul;854–855:503199. doi:10.1016/j.mrgentox.2020.503199. Erratum in: Mutat Res Genet Toxicol Environ Mutagen. 2020 Oct – Dec;858–860:503274. doi:10.1016/j.mrgentox.2020.503274
12. Egorova OV, Ilyushina NA, Rakitskii VN. Mutagenicity evaluation of pesticide analogs using standard and 6-well miniaturized bacterial reverse mutation tests. Toxicol In Vitro. 2020 Dec;69:105006. doi:10.1016/j.tiv.2020.105006
13. Zeyad MT, Kumar M, Malik A. Mutagenicity, genotoxicity and oxidative stress induced by pesticide industry wastewater using bacterial and plant bioassays. Biotechnol Rep (Amst). 2019 Oct 24;24:e00389. doi:10.1016/j.btre.2019.e00389
14. Cortés-Eslava J, Gómez-Arroyo S, Arenas-Huertero F, et al. The role of plant metabolism in the mutagenic and cytotoxic effects of four organophosphorus insecticides in Salmonella typhimurium and in human cell lines. Chemosphere. 2013 Aug;92(9):1117–25. doi:10.1016/j.chemosphere.2013.01.058
15. Naime AA, Lopes MW, Colle D, et al. Glutathione in Chlorpyrifos-and Chlorpyrifos-Oxon-Induced Toxicity: a Comparative Study Focused on Non-cholinergic Toxicity in HT22 Cells. Neurotox Res. 2020 Oct;38(3):603–610. doi:10.1007/s12640-020-00254-5
16. Czajka M, Sawicki K, Matysiak-Kucharek M, et al. Exposure to Chlorpyrifos Alters Proliferation, Differentiation and Fatty Acid Uptake in 3T3-L1 Cells. Int J Mol Sci. 2023 Nov 7;24(22):16038. doi:10.3390/ijms242216038
17. Montanarí C, Franco-Campos F, Taroncher M, et al. Chlorpyrifos induces cytotoxicity via oxidative stress and mitochondrial dysfunction in HepG2 cells. Food Chem Toxicol. 2024 Oct;192:114933. doi:10.1016/j.fct.2024.114933
18. Silva AM, Martins-Gomes C, Silva TL, et al. In Vitro Assessment of Pesticides Toxicity and Data Correlation with Pesticides Physicochemical Properties for Prediction of Toxicity in Gastrointestinal and Skin Contact Exposure. Toxics. 2022 Jul 8;10(7):378. doi:10.3390/toxics10070378
19. López González EC, Odetti LM, Latorre MA, et al. A comprehensive approach using multiple biomarkers to detect damage induced by pesticides in broad-snouted caiman (Caiman latirostris) under ex-situ conditions. Heliyon. 2021 Dec 24;8(1):e08667. doi:10.1016/j.heliyon.2021.e08667
20. Milić M, Žunec S, Micek V, et al. Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate. Arh Hig Rada Toksikol. 2018 Jun 1;69(2):154–168. doi:10.2478/aiht-2018-69-3114
21. Roma D, Cecchini ME, Tonini MP, et al. Toxicity assessment and DNA repair kinetics in HEK293 cells exposed to environmentally relevant concentrations of Glyphosate (Roundup® Control Max). Toxicol Res (Camb). 2023 Sep 30;12(5):970–978. doi:10.1093/toxres/tfad089
22. Gollapudi BB, Mendrala AL, Linscombe VA. Evaluation of the genetic toxicity of the organophosphate insecticide chlorpyrifos. Mutat Res. 1995 Mar;342(1–2):25–36. doi:10.1016/0165-1218(95)90087-x
23. Isidori M, Caterino E, Criscuolo E, et al. Antimutagenic and antigenotoxic effects of vegetable matrices on the activity of pesticides. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2009 Jul;26(7):1049–62. doi:10.1080/02652030902896194
24. Rank J, Jensen AG, Skov B, et al. Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test. Mutat Res. 1993 Jun;300(1):29–36. doi:10.1016/0165-1218(93)90136-2
25. Ilyushina N, Egorova O, Rakitskii V. Limitations of pesticide genotoxicity testing using the bacterial in vitro method. Toxicol In Vitro. 2019 Jun;57:110–116. doi:10.1016/j.tiv.2019.02.018