



Short communication

DNA damage and methylation induced by glyphosate in human peripheral blood mononuclear cells (*in vitro* study)Marta Kwiatkowska^{a,*}, Edyta Reszka^b, Katarzyna Woźniak^c, Ewa Jabłońska^b, Jaromir Michałowicz^a, Bożena Bukowska^a^a Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland^b Department of Molecular Genetics and Epigenetics, Nofer Institute of Occupational Medicine, Lodz, Poland^c Department of Molecular Genetics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

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ABSTRACT

Glyphosate is a very important herbicide that is widely used in the agriculture, and thus the exposure of humans to this substance and its metabolites has been noted. The purpose of this study was to assess DNA damage (determination of single and double strand-breaks by the comet assay) as well as to evaluate DNA methylation (global DNA methylation and methylation of p16 (CDKN2A) and p53 (TP53) promoter regions) in human peripheral blood mononuclear cells (PBMCs) exposed to glyphosate. PBMCs were incubated with the compound studied at concentrations ranging from 0.1 to 10 mM for 24 h. The study has shown that glyphosate induced DNA lesions, which were effectively repaired. However, PBMCs were unable to repair completely DNA damage induced by glyphosate. We also observed a decrease in global DNA methylation level at 0.25 mM of glyphosate. Glyphosate at 0.25 mM and 0.5 mM increased p53 promoter methylation, while it did not induce statistically significant changes in methylation of p16 promoter. To sum up, we have shown for the first time that glyphosate (at high concentrations from 0.5 to 10 mM) may induce DNA damage in leucocytes such as PBMCs and cause DNA methylation in human cells.

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1. Introduction

Glyphosate (*N*-phosphonomethylglycine) is a total herbicide, which is commonly used as an active ingredient of more than 750 different broad-spectrum herbicide preparations (Mesnage et al., 2015). Glyphosate formulations are made of around 36–48% glyphosate as well as water, salts, and adjuvants such as ethoxylated alkylamines (e.g. POEA). Glyphosate is widely used in genetically modified crops and in the removal of unwanted vegetation from urban areas, which contributes to its occurrence in water (0.1–0.7 mg/dm³), sediments and soils (0.5–5 mg/kg) (Peruzzo et al., 2008) and GM-soy (mean 3.3 mg/kg) (Böhn et al., 2014).

Glyphosate is never used without its adjuvants, which enhance its activity and contribute to stronger toxicity of its preparations (Pieniżek et al., 2004; Dallegre et al., 2007; Koller et al., 2012; Chaufan et al., 2014; Martini et al., 2016).

Several recent studies have shown that glyphosate reveals

adverse health effects to humans including endocrine disrupting activity (Romano et al., 2012; Kwiatkowska et al., 2013, 2016). For example, Thongprakaisang et al. (2013) showed that pure glyphosate at 10⁻¹² to 10⁻⁶ M affected estrogen receptors (ERs) by disruption of their transcriptional activity and expression. Additionally, these authors reported that glyphosate even in environmentally relevant concentrations was capable of exhibiting estrogenic activity.

Recently, Samsel and Seneff (2016) in their review of the literature suggested that glyphosate, acting as a glycine analogue, may be mistakenly incorporated into peptides during protein synthesis. Glycine has unique properties that support peptides by their ability to anchor to the plasma membrane or the cytoskeleton. The authors claimed that glyphosate substitution in place of conserved glycine may lead to development of various disorders such as diabetes, obesity, asthma, chronic obstructive pulmonary disease (COPD), Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease and others.

Although glyphosate has been being used for over 40 years, the assessment of toxic potential of this pesticide still demands

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significant verification. Plenty of data shows mutagenic (in high concentrations), xenoestrogenic and oxidative potential of glyphosate.

The World Health Organization (WHO) and the United States Environmental Protection Agency (US EPA) in 1993–1994 have recognized that glyphosate does not reveal mutagenic and carcinogenic effects. It was classified on E category, which means that there is no evidence of carcinogenic effect of this compound to humans (EPA, 1993). However, after 22 years of extensive research, the WHO in March 2015 decided to change the classification of glyphosate on category 2A (IARC Working Group, 2015), which means that glyphosate is regarded as 'probably carcinogenic to humans'. This category relates to compounds for which limited evidence of carcinogenicity to humans and sufficient evidence of carcinogenicity to experimental animals exist (Guyton et al., 2015; IARC Working Group, 2015). The European Commission decided to extend the approval for the use of glyphosate to the end of 2017.

Up to now, no study has been conducted in order to assess the impact of glyphosate on DNA damage in human leucocytes, while no research has addressed the evaluation of the effect of this pesticide on DNA methylation in any animal cell type. Therefore, in this work, we have investigated the effect of glyphosate on DNA damage and methylation in human peripheral blood mononuclear cells (PBMCs).

It is widely known that xenobiotics can affect DNA methylation pattern that is closely associated with specific genes modulation. DNA methylation is a type of chemical modification of DNA that involves the addition of methyl group to cytosine to form 5-methylcytosine (5-mC), and it is linked to several processes including tissue-specific silencing of gene expression, genomic imprinting, development of tumors etc. DNA global hypomethylation can affect cancerogenesis via over-expression of oncogenes, while the most important consequence of hypermethylation is down-regulation of tumor suppressor genes, e.g. p16 gene (Łukasik et al., 2009).

2. Materials and methods

2.1. Chemicals

Glyphosate [N-(phosphonomethyl)glycine] (purity 95%) was bought from Sigma-Aldrich, USA. Glyphosate was dissolved in phosphate buffered saline (PBS pH 7.4). Bovine fetal serum (BFS), 4',6'-diamidino-2-phenylindole (DAPI), penicillin-streptomycin, low melting point (LMP) and normal melting point (NMP) agarose were purchased from Sigma-Aldrich (USA). Cells-to-CpG™ Bisulfite Conversion Kit, MethylFlash Methylated DNA Quantification Kit, Methyl Primer Express®, v.1.0 were obtained from Epigentek and Life Technologies.

Lymphocyte separation medium (LSM) (1.077 g/cm³) and RPMI 1640 medium with L-glutamine were bought in Cytogen (Germany). Other chemicals were purchased from Roth (Germany) and POCH (Poland).

2.2. Cells isolation

PBMCs were isolated from leucocyte-buffy coat collected from blood taken in Blood Bank in Łódź, Poland. Blood was collected from 9 healthy volunteers (aged 18–55) with no symptoms of infection disease. For each parameter studied, three leucocytes-buffy coats were taken from three independent blood donors.

The use of human blood (leucocyte buffy-coat) in the investigation of the effect of glyphosate on human PBMCs was approved by Bioethics Committee for Scientific Investigation, University of Łódź (agreement no. KBBN-UŁ/I/3/2013).

PBMCs were diluted with PBS (1:4) and isolated using LSM (1.077 g/cm³) by centrifugation at 600 × g for 30 min at 20 °C. PBMCs were collected, suspended in erythrocyte lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA, pH 7.4) and incubated for 5 min at 20 °C. Then, PBS was added immediately, and the cells were centrifuged at 200 × g for 15 min at 20 °C. The supernatant was decanted, and the cells were washed twice with RPMI with L-glutamine and 10% fetal bovine serum (FBS) at 200 × g for 15 min. The cells were resuspended in RPMI medium with L-glutamine, 10% FBS and penicillin-streptomycin (0.5%) and counted in haemocytometer. The final PBMCs density used in the experiments (after addition of glyphosate) was 1 × 10⁶ cells/ml. After incubation, PBMCs were diluted to a density of 5 × 10⁴ cells/ml for comet assay and condensed to a density of 5 × 10⁶ cells/ml for epigenetic methods. The viability of the cells was over 94%.

2.3. Cells treatment

Glyphosate was dissolved in PBS. The final concentrations of glyphosate used in the comet assay were in the range from 0.25 to 10 mM, while epigenetic changes were assessed after exposure of the cells to glyphosate at 0.25 mM and 0.5 mM. The cells were incubated with glyphosate for 24 h. DNA repair was assessed in time of 120 min.

In the comet assay, the lowest concentration of glyphosate of 0.25 mM was chosen as that, which followed any statistically significant changes in DNA damage. Epigenetic changes were assessed for the concentration of glyphosate of 0.25 mM that did not induce DNA damage, and 0.5 mM, which induced statistically significant DNA damage. Glyphosate concentration of 0.25 mM may be present in the human blood after glyphosate intoxication (Zouaoui et al., 2013).

Our previous study showed that glyphosate at the highest concentration of 10 mM after 24 h incubation negligibly (by 2.7%) decreased cell viability (Kwiatkowska et al., 2016).

Additionally, cell viability was assessed after 26 h incubation, the time, which was necessary to incubate PBMCs to analyze DNA damage and repair. The cells were incubated for 24 h with different concentrations of glyphosate, then the cells were centrifuged, the glyphosate was discarded, and the cells were resuspended in RPMI medium and incubated for 2 h. Finally cell viability was determined. We noted that cell viability was 90.0% ± 1.0%, 90.2% ± 2.3%; 90.4% ± 2.2%, 88.5% ± 3.5%, 87.8% ± 2.1% for control, 0.5 mM, 5 mM, 7.5 mM and 10 mM of glyphosate, respectively. Cell viability was determined according to the procedure described by Kwiatkowska et al. (2016).

Each DNA damage experiment included a positive control. Hydrogen peroxide at 20 μM was selected to induce DNA strand-breaks (the cells were incubated with H₂O₂ for 15 min on ice).

2.4. Analysis of DNA strand-breaks

Damage to DNA provoked by glyphosate was assessed by means of the single cell gel electrophoresis (comet assay). In this technique, the cells are immersed in low melting point agarose, placed on microscopic slides, and then lysed. As a result, released DNA is submitted to electrophoresis in alkaline conditions (pH > 13). The comet assay enables identification of single and double strand-breaks (SSBs and DSBs) as well as alkali labile sites (ALSs).

2.4.1. Comet assay - alkaline version

2.4.1.1. Slides preparation and lysis. The comet assay was performed under alkaline conditions according to the procedure of Singh et al. (1988) with some modifications (Klaude et al., 1996) as described previously (Błasiak and Kowalik, 2000). A freshly

prepared cells suspension in 0.75% LMP agarose dissolved in PBS was layered onto microscope slides (Superior, Germany), which were pre-coated with 0.5% NMP agarose. Then, the cells were lysed for 1 h at 4 °C in a buffer containing 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10. After cells lysis, the slides were placed in an electrophoresis unit. DNA was allowed to unwind for 20 min in the solution containing 300 mM NaOH and 1 mM EDTA, pH > 13.

2.4.1.2. Electrophoretic separation and staining. Electrophoretic separation was performed in the solution containing 30 mM NaOH and 1 mM EDTA, pH > 13 at ambient temperature of 4 °C (the temperature of the running buffer did not exceed 12 °C) for 20 min at an electric field strength of 0.73 V/cm (28 mA). Then, the slides were washed in water, drained, stained with 2 µg/ml DAPI and covered with cover slips. In order to prevent additional DNA damage, the procedure described above was conducted under limited light or in the dark.

2.4.1.3. Comets analysis. The comets were observed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Japan) attached to a COHU 4910 video camera (Cohu, Inc., San Diego, CA, USA) equipped with a UV-1 A filter block and connected to a personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Praha, Czech Republic).

Fifty images (comets) were randomly selected from each sample and the mean value of DNA in comet tail was taken as an index of DNA damage (expressed in percent). For one blood donor, three parallel tests with aliquots of the sample of the cells were performed for a total number of 150 comets. A total number of 450 comets (3 blood donors, n = 3) was recorded to calculate mean ± SEM.

2.4.1.4. DNA repair. The control samples and the PBMCs treated with glyphosate at 0.5, 5, 7.5 and 10 mM were washed and resuspended in fresh RPMI 1640 medium with L-glutamine preheated to 37 °C. Aliquots of the suspension were taken immediately (“time zero”) and 120 min later. The samples were placed in an ice bath to stop DNA repair. Next, the preparation of the samples was conducted as described above. DNA repair was assessed by the extent of residual DNA damage detection at each time-point using alkaline version of the comet assay.

2.5. Methylation levels

Genomic DNA from human PBMCs was isolated using QIAamp DNA mini Kit (Qiagen).

2.5.1. Methylation of p16 (CDKN2A) and p53 (TP53) promoter regions

Chemical modification of 500 ng of genomic DNA was performed with Cells-to-CpG™ Bisulfite Conversion Kit (Life Technologies). For methylation analysis, quantitative methylation-specific real-time PCR assay (qMSP) was conducted in three independent experiments (three blood donors) with FastStart SYBR Green Master (Roche). All samples were amplified in triplicate. To determine the methylation status of particular gene expressed as methylation index (MI) in percentage, the Ct values of the methylated gene of interest were compared with the Ct values of the unmethylated gene of interest. The DNA sequences around the transcription sites (from -1000 to +300 bp) of both genes, which usually contain a promoter region, were obtained from the DBTSS (Database of Transcriptional Start Sites, <http://dbtss.hgc.jp>). Methylated and unmethylated primers were designed by means of Methyl Primer Express®, v.1.0 (Life Technologies). For CDKN2A promoter region covering 9 CpG islands primers as follows MF were

applied: 5' TGTTAACGTTGGTTTGGC 3', MR: 5' AAAAACC CGGA-TATCTTTCC 3', UF: 5' TTTTGTAAATGTTGGTTTGGT 3', UR: 5' AAAAACCACAATATCTTTCCAAA 3' and for TP53 with 14 CpG islands MF: 5' TTGGGAGCGTGTITTTTAC 3', MR: 5' CAACGATTTCCCGAACTA 3', UF: 5' GGGTTGGGAGTGTITTTTAT 3', UR: 5' CAA-CATTTTCCCAAATAAAA 3'.

2.5.2. Global DNA methylation

Global DNA methylation was determined by means of DNA quantification using 5-mC monoclonal antibodies in ELISA-like reaction with MethylFlash Methylated DNA Quantification Kit (Epigentek). Methylation levels were calculated relatively to the methylated control DNA and expressed as a percentage of methylated DNA. DNA (100 ng) isolated from whole blood PBMCs was used for analyses. Each sample was analyzed in duplicate and the determination was repeated whenever there was a failure in detection. The calculation of 5-mC amount was done with the use of standard curve created by using defined dilutions of methylated genomic DNA. Methylation levels were calculated relatively to the methylated control DNA (included in the kit) and expressed as a percentage of total methylated DNA.

2.6. Statistical analysis

The mean value was obtained for three independent experiments (three blood donors), whereas for each individual (one

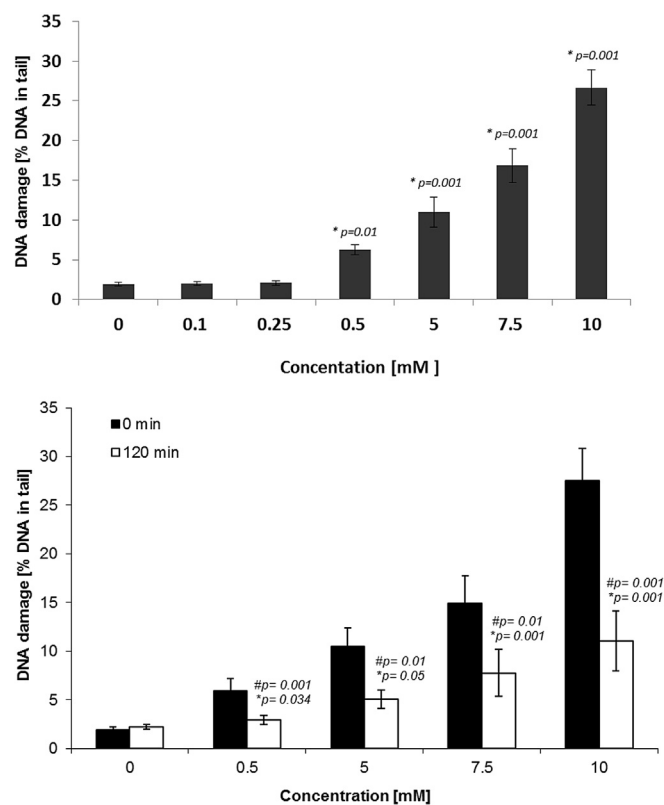


Fig. 1. A. DNA damage in human PBMCs incubated with glyphosate in the concentrations ranging from 0.1 to 10 mM for 24 h at 37 °C. Error bars denote SEM; *p value as compared with control. B. Time course of the repair of damaged DNA in human PBMCs after 24 h incubation with glyphosate in the concentrations ranging from 0.5 to 10 mM. The repair was assessed as a decrease in the extent of DNA damage measured after 120 min of post-incubation (the percentage of the DNA in comet tail) using the alkaline version of the comet assay. The results are mean of three independent experiments (donors). Error bars denote SEM, #p value as compared with the extent of DNA damage in time of 0 min, *p value as compared with control after 120 min.

donor), an experimental point was a mean value of at least 2 (methylation analysis) or 3 replications (DNA damage analysis). Statistical analysis was conducted using the Mann-Whitney test (samples with distributions departing from normality) and the Student's *t*-test (samples with the normal distribution) and one-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey test). The differences were considered to be statistically significant when *P* value was less than 0.05. Data analysis was performed using STATISTICA software (StatSoft, Inc, Tulsa, USA).

2.7. Results

2.7.1. Analysis of DNA strand-breaks and DNA repair

Fig. 1A shows percentage of DNA in the comets tail derived from human PBMCs exposed for 24 h to glyphosate. Glyphosate increased DNA damage (single and double strand-breaks and alkali-labile sites formation) from the concentration of 0.5 mM. It was also noted that glyphosate at 10 mM caused a significant increase in the parameter studied, which exceeded 13 times the control value (control -1.93 ± 0.27 versus 10 mM -26.68 ± 2.25).

It was noticed that PBMCs after 120 min incubation significantly repaired DNA lesions induced by glyphosate (5.61% (0 min) vs 2.93% (120 min) at 0.5 mM, 10.51% (0 min) vs 5.07% (120 min) at 5 mM, 14.91% (0 min) vs 7.76% (120 min) at 7.5 mM and 27.49% (0 min) vs 11.05% (120 min) at 10 mM of glyphosate (Fig. 1B).

Selected comets originating from PBMCs exposed to glyphosate are shown in Fig. 2.

2.7.2. DNA methylation status

Statistically significant changes were observed in 5-mC percentage and p53 promoter methylation in PBMCs treated with glyphosate. As compared to control cells, percentage of global DNA methylation level was significantly decreased by glyphosate at both

concentrations, with significant decrease at 0.25 mM ($p = 0.017$) and with border line significance at 0.5 mM ($p = 0.084$) (Fig. 3). On the contrary, p53 promoter methylation was significantly increased as compared to control cells at both concentrations of glyphosate used i.e. $p = 0.013$ and $p = 0.011$ for 0.25 mM and 0.5 mM, respectively (Fig. 4). For the p16 gene promoter, methylation increased after treatment of PBMCs with glyphosate, however this change was not statistically significant ($p = 0.101$) (Fig. 5).

2.8. Discussion

Glyphosate is an active ingredient of the most widely used herbicide, and it is believed to be less toxic than other herbicides.

It has been proven that genotoxic effects that are provoked by various xenobiotics may contribute to cancer development (Harris, 2013; Gao et al., 2016). Similarly, changes in DNA methylation

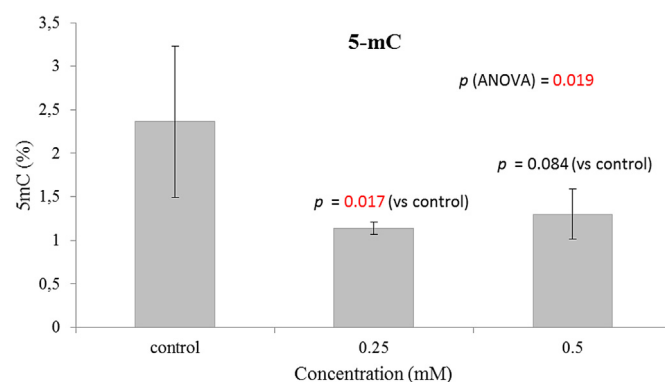


Fig. 3. 5-methylcytosine (%) in human PBMCs incubated with glyphosate in the concentrations of 0.25 mM and 0.5 mM for 24 h. Data presented as mean \pm SD.

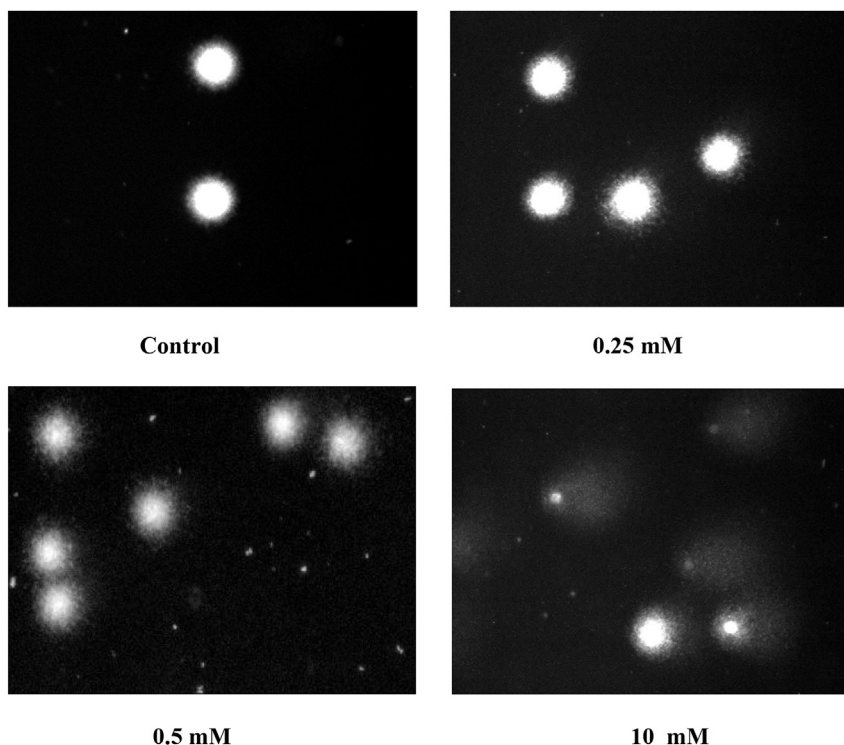


Fig. 2. Typical fluorescence microscopic images of the DAPI-stained DNA of human PBMCs incubated with glyphosate (A –control, B –0.25 mM, C –0.5 mM, D –10 mM) for 24 h at 37 °C.

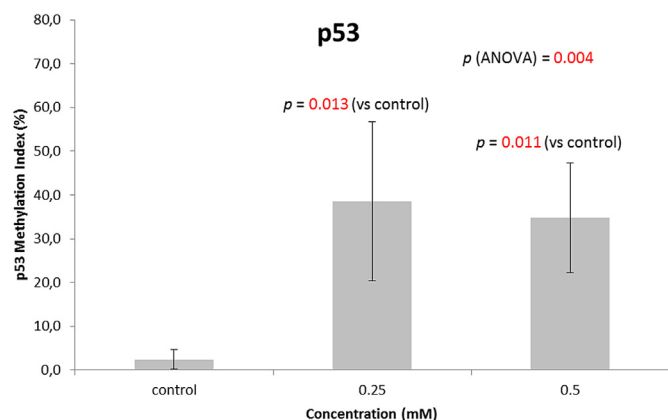


Fig. 4. P53 methylation index (%) in human PBMCs incubated with glyphosate in the concentrations of 0.25 mM and 0.5 mM for 24 h. Data presented as mean \pm SD.

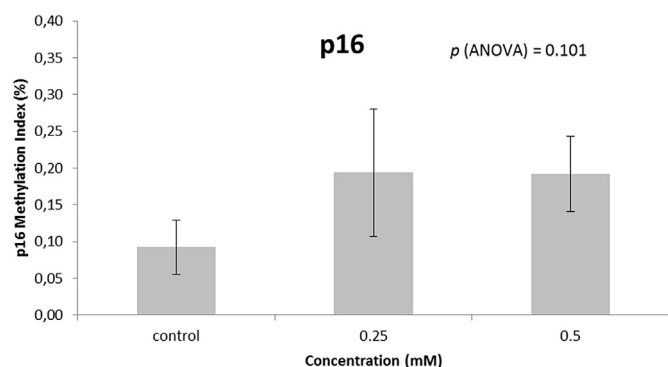


Fig. 5. P16 methylation index (%) in human PBMCs incubated with glyphosate in the concentrations of 0.25 mM and 0.5 mM for 24 h. Data presented as mean \pm SD.

pattern may lead to genetic instability and ultimately to cancer (Toyota and Yamamoto, 2011).

The results of our study provided preliminary information on the effect of glyphosate on DNA integrity and DNA methylation. Based on the obtained results, it can be concluded that glyphosate affect the above mentioned parameters in human PBMCs in high concentrations of 0.25 mM and 0.5 mM. Glyphosate was determined in blood (mean $73.6 \pm 28.2 \mu\text{g/L}$ – $0.435 \mu\text{M}$) (Aris and Leblanc, 2011) and urine (0.1 – $3.3 \mu\text{g/kg bw/day}$) (Niemann et al., 2015) of humans who were indirectly exposed to significant amounts of this substance. As a result of glyphosate intoxication, its content in blood ranged from 0.6 to 150 mg/L (3.54 – $887.21 \mu\text{M}$), whereas during moderate poisoning with this pesticide, its concentrations were in the range from 690 mg/L (4.1 mM) to 7480 mg/L (44.2 mM) (Zouaoui et al., 2013). Our results showed that glyphosate induced DNA damage and DNA methylation in PBMCs in the concentrations, which may occur in blood of humans intoxicated with this pesticide.

Alvarez-Moya et al. (2014) using comet assay showed that glyphosate at 7 mM (*in vitro*) and 0.7 mM (*in vivo*) caused DNA damage in blood cells of Nile Tilapia (*Oreochromis niloticus*). Genotoxicity of glyphosate was also reported by Manas et al. (2009) who examined the effect of this compound on Hep-2 cell line. A statistically significant increase in DNA damage (tail moment) was observed after incubation of the above cell type with glyphosate at 3 mM . In our studies we also observed that glyphosate induced DNA damage in PBMCs in the concentrations range from 0.5 to 10 mM (Fig. 1A). Moreover, we noticed that PBMCs significantly

repaired glyphosate-induced DNA damage, but they were unable to repair completely DNA strand-breaks after 120 min post-incubation (2.23% (control) vs 2.93% (0.5 mM), 5.07% (5 mM), 7.76% (7.5 mM) and 11.05% (10 mM), respectively (Fig. 2B). Unrepaired DNA damage can lead to mutations that may cause genetic instability, and tumor growth (Harris, 2013; Gao et al., 2016).

Recent toxicological studies (Kier and Kirkland, 2013) and human genotoxicity studies (Kier, 2015) have suggested that glyphosate and glyphosate based formulations do not constitute a significant genotoxic hazard to humans environmentally exposed.

Epigenetic changes occur in all human cancers and are known to be associated with genetic alterations, which drive cancer phenotype. These changes involve DNA methylation, histone modifications, chromatin remodeling and other alterations in chromatin. Epigenetic changes may lead to mutations, and, conversely, mutations are frequently observed in genes that modify the epigenome (Baylin and Jones, 2016). Interestingly, hypermethylation of promoter regions of tumor suppression genes in human malignant tissues is often associated with decreased 5-mC level of global genomic DNA; therefore making distinctive cancer features (Kulis and Esteller, 2010).

Our study revealed that glyphosate significantly decreased global DNA methylation in PBMCs at concentration of 0.25 mM . Surprisingly, the effect of glyphosate at concentration of 0.5 mM on global DNA methylation was not statistically significant, though the mean value from three independent experiments was still decreased as compared to control, and a decrease was also observed in each experiment separately. The border line significance for this effect ($p = 0.084$) may suggest that the number of observations (blood donors) was too small. Along with the decreased global DNA methylation, we have observed significantly increased methylation of p53 promoter at two glyphosate concentrations i.e. 0.25 mM and 0.5 mM . Altered p53 promoter hypermethylation is an epigenetic pattern frequently observed in human cancers. Thus, the results of this study suggest that glyphosate at high concentration (0.25 mM) may cause down-regulation of p53 gene expression and activate protooncogenes or retrotransposable sequences, which may induce genomic alterations by insertion and/or homologous recombination. Nardemir et al., (2015) investigated glyphosate for its genotoxic effects on genome of wheat (*Triticum aestivum* L). They observed that glyphosate caused DNA damage and hypermethylation. Moreover, recent findings from genome-wide *in vitro* studies indicated that organophosphorous pesticides such as fonofos, parathion, terbufos (Zhang et al., 2012a) and diazinon (Zhang et al., 2012b) induced similar methylation changes in promoter regions of numerous genes in hematopoietic K562 human cell line. Interestingly, diazinon applied in increasing doses did not induce changes in DNA methylation level (Zhang et al., 2012b).

This study, for the first time showed that glyphosate may induce DNA damage in human leucocytes and cause epigenetic alterations in animal cells. In order to confirm our findings, it is necessary to conduct additional analyses by the use of other cells types and performing *in vivo* studies.

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Transparency document

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