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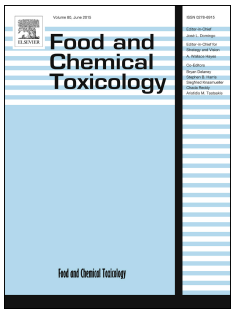

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The mechanism of DNA damage induced by Roundup 360 PLUS, glyphosate and AMPA in human peripheral blood mononuclear cells - genotoxic risk assessement

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Abstract

Glyphosate is the most heavily applied among pesticides in the world, and thus human exposure to this substance continues to increase. WHO changed classification of glyphosate to probably cancerogenic to humans, thus there is urgent need to assess in detail genotoxic mechanism of its action. We have assessed the effect of glyphosate, its formulation (Roundup 360 PLUS) and its main metabolite (aminomethylphosphonic acid, AMPA) in the concentration range from 1 to 1000 µM on DNA damage in human peripheral blood mononuclear cells (PBMCs) incubated. The cells were incubated for 24 h. The compounds studied and formulation induced DNA single and double strand-breaks and caused purines and pyrimidines oxidation. None of compounds examined was capable of creating adducts with DNA, while those substances increased ROS (including $^{\bullet}OH$) level in PBMCs. Roundup 360 PLUS caused damage to DNA even at 5 µM, while glyphosate and particularly AMPA induced DNA lesions from the concentration of 250 µM and 500 µM, respectively. DNA damage induced by glyphosate and its derivatives increased in order: AMPA, glyphosate, Roundup 360 PLUS. We may conclude that observed changes were not associated with direct interaction of xenobiotics studied with DNA, but the most probably they occurred through ROS–mediated effects.

Keywords: glyphosate, Roundup 360 PLUS, PBMCs, DNA bases, DNA adducts, reactive oxygen species

1. Introduction

Damage to DNA leads to disturbances in numerous cellular processes, which may result in cancer development. DNA single strand-breaks (SSBs) and double strand-breaks (DSB), oxidative DNA bases modifications and DNA adducts formation are the most

common types of DNA lesions (Christmann and Kaina 2013). The assessement of genotoxic potential of xenobiotics influencing human organism is crucial for evaluation of human safety.

Glyphosate (N-(phosphonomethyl)glycine)) (Scheme 1) is a chemical that is produced in massive amounts as it is one of the most commonly used pesticide worldwide, and thus environmental and occupational exposure to this substance has been noted for decades. Glyphosate has been extensively used in the past 40 years under the assumption that side effects induced by this pesticide are minimal. However, in recent years, concerns have increased worldwide about the potential wide ranging direct and indirect health effects caused by glyphosate to animals and human (Van Bruggen et al.2018).

Benbrook (2016) described that in 2014, farmers sprayed enough glyphosate to apply ~1.0 kg/ha (0.8 pound/acre) on every hectare of U.S.-cultivated cropland and nearly 0.53 kg/ha (0.47 pounds/acre) on all cropland worldwide. Glyphosate-based herbicides, consisting of glyphosate and formulates, are the most frequently applied pesticides worldwide. The declared active ingredient glyphosate inhibits the activity of 5-enolpyruvyl shikimate 3-phosphate synthase, the enzyme of the shikimate pathway, which governs the synthesis of aromatic amino acids in higher plants, algae, bacteria, and fungi. Moreover, this substance is capable of chelating of macro- and micronutrients, essential for many plant physiological processes and pathogen resistance (Mertens et al. 2018).

Glyphosate has been detected in blood of humans in a mean concentration of 73.6 ± 28.2 µg/L (0.435 ± 0.166 µM) who were not directly exposed to elevated doses of this herbicide (Aris Leblanc, 2011). Moreover, some cases of acute poisoning with glyphosate have been reported (Roberts et al. 2010). Zouaoui et al. (2013) showed that in the case of glyphosate intoxication, its content in blood was in the range from 0.6 to 150 mg/L (3.54 ± 887.21 µM).

These cases were associated with consumption of high amounts of glyphosate-preparation for suicide-related purposes. Acute poisoning with glyphosate is characterized by disturbances in function of kidneys, liver, alimentary tract as well as cardiovascular and respiratory systems. Glyphosate has also been shown to enter human body as a result of spraying with inhaled air or/and direct dermal contact. In these cases, the most common symptoms include eyes and skin irritation, stomach ache and vomiting (You et al. 2015). On the other hand, side-effects associated with everyday usage of glyphosate formulation of the spraying solutions have been seldom observed. According to the most recent data, only one case of peripheral neuropathy following exposure to large amounts of glyphosate-based formulation has been noted (Kawagashira et al. 2017).

Although the effects of acute poisoning with glyphosate and its metabolite AMPA in mammals are rare, there are animal data raising the possibility of adverse health effects associated with chronic, ultra-low doses related to accumulation of these compounds in the environment (Van Bruggen et al. 2018).

Alarming and controversial are the reports showing probable carcinogenic potential of glyphosate and its derivatives. 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". The European Commission decided to extend the approval for the use of glyphosate to the end of 2022. On the other hand, European Food Safety Authority (EFSA) concluded "that there is very limited evidence for an association between glyphosate-based formulations and non-Hodgkin lymphoma (NHL), overall inconclusive for a causal or clear associative relationship between glyphosate and cancer in human studies" (EFSA, 2015). That is why, the investigations are still being conducted to estimate potential carcinogenic effect of glyphosate.

Recently, epidemiological survey performed by Andreotti et al. (2017) covered 54,251 applicators of pesticides including 44,932 (82.8%) who used glyphosate. In this large, prospective cohort study, no association was apparent between glyphosate usage and any solid tumors or lymphoid malignancies overall, including NHL and its subtypes. They suggested that there was some evidence of increased risk of acute myeloid leukemia (AML) among the highest exposed group that requires confirmation.

Many times, the efforts have been undertaken in order to assess genotoxicity of glyphosate-based herbicide preparations (isopropylamino salt) *in vitro* including human lymphocytes (Alvarez-Moya et al. 2014) or animal (*Chaetophractus villosus*) lymphocytes (Roundup Full II®) (Luaces et al.. 2017). The studies have also been conducted on epithelial cells of human cheek - TR146) (Keller et al. 2012), human placental JEG3 cells (Richard et al. 2005) or fish cells (Guilherme et al. 2012; Marques et al. 2014).

The studies of the effect of pure glyphosate on human PBMCs (Kwiatkowska et al., 2017), human hepatocytes (HepG2) (Kašuba et al. 2017) and crab (*Eriocheir sinensi*) hematocytes (Hong et al. 2017) as well as the effect of AMPA on fish (*Anguilla Anguilla*) (Guilherme et al. 2014) have also been realized.

However, it must be underlined that no research study has assessed the effect of both glyphosate, its formulation and its main metabolite on the same biological model using the methods that in-depth elucidate mechanism of genotoxic action of these substances. That is why, in this study, we have assessed genotoxic potential of glyphosate, commonly used glyphosate-preparation – Roundup 360 PLUS and main glyphosate metabolite – AMPA. We have employed the comet assay in order to assess SSBs and DSBs formation and DNA purines and pyrimidines lesions as well as conformational test using plasmid to evaluate DNA adducts formation. Moreover, we have used flow cytometry to determine cell viability and ROS (including hydroxyl radical ($^{\bullet}$OH)) formation in human PBMCs

2. Methods

2.1. Chemicals

N-(phosphonomethyl)glycine (glyphosate) (purity 95%) was bought from Sigma Aldrich, USA, while aminomethylphosphonic acid (AMPA) (purity 95%) was provided by the Institute of Industrial Organic Chemistry, Warsaw, Poland.

Roundup 360 PLUS (N-(phosphonomethyl)glycine in form of potassium salt) was bought commercially. Fetal bovine serum (FBS), penicylin-streptomycin, low-melting point (LMP) and normal melting point (NMP) agarose and plasmid pUC19 were bought from Sigma-Aldrich (USA).

RPMI 1640 medium with L-glutamine and lymphocyte separation medium (LSM) (1.077 g/cm3) were purchased in Cytogen (Germany), while other chemicals were bought from Roth (Germany) and POCh (Poland) and were of analytical grade.

2.2. Cells isolation

PBMCs were isolated from leucocyte-buffy coat obtained from blood purchased in Blood Bank in Lodz, Poland. Blood was obtained from healthy, non-smoking volunteers (aged 18-55), who showed no signs of infection disease symptoms at the time the blood samples were collected. The investigations was approved by the Bioethics Committee of the University of Lodz No. 1/KBBN-UŁ/II/2017.

Leucocyte-buffy coat was diluted with PBS (1:4). The cells were isolated using LSM (1.077 g/cm^3) by centrifugation at 600*g* for 30 min at 20 °C. Then, the cells were collected, suspended in erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.4) and incubated for 5 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 penicylin-streptomycin (0.5%) and counted in Bürker chamber. The final PBMCs density after addition of the compounds studied was 1×10^6 cells/ml. After incubation, PBMCs were diluted to a density of 5×10^4 cells/ml for comet assay.

The viability of the cells was over 90%. The Trypan Blue dye exclusion test was used to determine the number of viable cells present in a cell suspension. For each parameter studied, three leucocytes buffy coats were taken from two or three independent blood donors depending on the method.

2.3. Cells treatment

Roundup 360 PLUS, glyphosate and AMPA were dissolved in phosphate buffered saline (pH 7.4). The concentrations of Roundup 360 PLUS (expressed as glyphosate contained in preparation) were from 0.001 to 10 µM (0.0017- 1.7 mg/L) glyphosate from 0.5 to 500 µM (0.085-170 mg/L) and AMPA from 0.5 to 1000 µM (0.055-111 mg/L).

Roundup concentrations were expressed in µM of glyphosate contained in a preparation. The preparation contains 360 g of glyphosate (active substance) in 1 litre, which corresponds (including glyphosate and excluding potassium ion and other substances present in a preparation) to 2.12 mol of glyphosate per 1 litre.

Based on the results of PBMCs viability obtained after the exposure of the cells to Roundup, glyphosate or AMPA, we have selected different concentrations of these compounds for genotoxic tests. Roundup from 50 µM caused significant decrease in cell viability, which excluded the possibility of conducting genotoxic studies at higher concentrations of this compound. Moreover, the concentrations of the compounds studied

have been chosen on the basis of initial experiments conducted by means of alkaline version of the comet assay. Our intention was also to study (select) the lowest concentrations of the compounds examined, which affect DNA damage in PBMCs.

Because Roundup induced DNA damage at 5 µM and 10 µM, we have used these concentrations in further experiments. Glyphosate and AMPA have been shown to be less toxic as they caused DNA damage at the concentrations of 250 µM, 500 µM and 1000 µM, therefore these concentrations were used for further experiments. The concentration of 5 µM has been chosen in order to compare the effects of all compounds examined.

In some studies, S9 fraction is employed. Fraction S9 is usually used when the compounds studied do not induce any or induce low effects in the examined parameters. In recent study, we have not decided to conduct experiments with S9 mix because Roundup, glyphosate or AMPA exhibited genotoxic potential, and thus, the activation of these substances will the most probably only increase the observed effects.

The cells were incubated with investigated xenobiotics for 24 h. During incubation, the cells were suspended in RPMI medium containing L-glutamine, FBS (10%) and penicillin-streptomycin (0.5%) and incubated with BPA or its analogs in 24-well tissue culture plates in a humidified incubator at 37 °C in 5% CO_2 atmosphere in total darkness.
After incubation, the cells were centrifuged, the compound was discarded, and the cells were resuspended in RPMI medium. Finally cell viability was determined (Table 1). The Trypan Blue dye exclusion test was used to determine the number of viable cells present in a cell suspension. Each experiment concerning DNA damage included a positive control. Hydrogen peroxide at 20 µM was selected to induce DNA SSBs (the cells were incubated with H_2O_2 for 15 min on ice), while DSBs were induced by ionizing irradiation at a dose of 10 Gy (2 Gy/min per 5 min).

2.4. Comet assay

Damage to DNA provoked by Roundup 360 PLUS, glyphosate and AMPA was assessed by means of the single cell gel electrophoresis (comet assay). In this technique, the cells are immersed in low melting point agarose (LMP), placed on microscopic slides, and then lysed. Then, released DNA is submitted to electrophoresis in alkaline conditions (pH > 13). The comet assay enables identification of SSBs and DSBs as well as alkali labile sites (ALSs). Modification of the comet assay with repair enzymes enables to assess purines and pyrimidines lesions.

2.4.1. 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 by 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. Each experiment concerning DNA damage included a positive control. Hydrogen peroxide at 20 µM was selected to induce DNA SSBs (the cells were incubated with H_2O_2 for 15 min on ice).

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 x 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. 6.0 (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.

2.4.2. Neutral version

The neutral version of the comet assay was used to detect DSBs in PBMCs (Singh and Stephens, 1997). The electrophoresis was run in a buffer containing 100 mM Tris and 300 mM sodium acetate at pH 9.0 adjusted by glacial acetic acid. Electrophoresis was conducted for 60 min, after a 20 min equilibrium period, at electric field strength of 0.41 V/cm (50 mA) at 4 ºC. The procedure was then conducted as described in section comet assay ‘‘Alkaline version”. Each experiment concerning DNA damage included a positive control. DSBs were induced by ionizing irradiation at a dose of 10 Gy (2 Gy/min per 5 min).

2.4.3. DNA repair

Control cells and the PBMCs treated with Roundup 360 PLUS, glyphosate or AMPA 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"), 30 min, 60 min, 90 min 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.

2.4.4. DNA repair enzyme treatment – oxidized purines and pyrimidines detection

Detection of oxidative DNA damage was conducted with the comet assay using endonuclease III (Nth) and human 8-oxoguanine DNA glycosylase (hOGG1). Nth and hOGG1 are capable of converting oxidized pyrimidines and purines, respectively into DNA single strand-breaks (SSBs) that can be determined by the comet assay. After the lysis, the slides were washed (three times) using the enzyme buffer (40 mM HEPES-KOH, 0.5 mM Na_2EDTA, 0.1 M KCl, 0.2 mg/mL BSA; pH 8) for 5 min each time. Next, agarose on slides was covered with a volume of 50 µL of buffer consisting of 1 U of Nth or hOGG1 or without the enzyme. Then, the slides were covered with cover glasses and were incubated for 30 min at 37 ºC in a moist chamber. In the next step, the cover glasses were removed and the slides were placed in an electrophoresis unit (Drozdz et al. 2011). DNA was allowed to unwind for 20 min in a solution consisting of 300 mM NaOH and 1 mM EDTA (pH > 13).

We have also performed analysis of oxidized purines and pyrimidines by determining the level of purine and pyrimidine oxidation in the positive control, which referred to the cells incubated with hydrogen peroxide at 20 µM for 15 min on ice and subsequently treated with

the enzymes. The procedure was then conducted as described in section comet assay ‘‘Alkaline version”.

We have not calibrated the enzymes. According to the recommendation contained in the BioLabs protocol in which our experiment based on, dilution of hOGG1 and Nth enzyme should be from 1 : 102 to 1 : 103 and from 1 : 104 to 1 : 105, respectively. It means that 50 µl of enzyme buffer with proper enzyme is equivalent of 0.08-0.8 U for hOOG1 and 0.05-0.5 U for Nth. Based on literature data (Czarny et al., 2015) we have decided to use 1 U of each enzyme per gel, which guaranteed their use in excess (therefore, the calibration curve was not performed).

2.5. Plasmid relaxation assay

pUC19 plasmid was incubated with Roundup 360 PLUS at 0.1, 1 and 10 µM, glyphosate at 100, 500 and 1000 µM and AMPA at 100, 500 and 1000 µM. The plasmid was also exposed to 200 µM H_2O_2 and 20 µM Fe^{+2} for 20 min on ice (Fenton reaction). We have conducted this reaction (positive control) to check the migration of the pUC19 plasmid multimeric forms (supercoiled (SC), open circular (OC) and linear (L)). Hydroxyl radical, which was formed in Fenton reaction, induced strand-breaks in DNA and caused the relaxation of supercoiled plasmid. Structural differences between supercoiled, open circular, and linear forms of the plasmid accounted for their different electrophoretic mobility. Plasmid samples at 250 ng were subjected to 1% agarose gel electrophoresis carried out in TAE (Tris-Acetate-EDTA) buffer. The gel was stained with ethidium bromide (0.5 µg/ml), and plasmid DNA was visualized under ultraviolet light (302 nm) and scanned by a CCD camera. Densitometry analysis of the gel was performed with the GeneTools by Syngene (Cambridge, UK) software (Drozdz et al. 2011).

2.6. Oxidation of H_2DCFDA and HPF

In order to measure the production of reactive oxygen species (ROS), the fluorescence of the probe - 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was measured (Bartosz, 2009). When H_2DCFDA diffuses across the cellular membrane, it is hydrolyzed by membrane esterases to H_2DCF. The increase in fluorescence of DCF (a marker of probe oxidation) was measured by a flow cytometer (LSR II. Becton Dickinson) at excitation/emission wavelengths at 488 nm and 530 nm, respectively. The final concentration of H_2DCF in PBMCs was 5 μM. Positive controls consisted of hydrogen peroxide (2 mM), which was added to the cell suspension.

We have also used 3′-(p-hydroxyphenyl)-fluorescein (HPF) to detect highly reactive oxygen species (mainly hydroxyl radical) (Maćczak et al.2017). The final concentration of HPF in PBMCs suspension was 4 μM. The increase in fluorescence of this dye (a marker of probe oxidation) was measured by a flow cytometer (LSR II. Becton Dickinson) at excitation/emission wavelengths of 490 nm and 515 nm, respectively. Hydroxyl radical formation (positive control) was induced by the addition of ferrous perchlorate(II) (0.1 mM) and hydrogen peroxide (1 mM) to the cell suspension.

After incubation with Roundup 360 PLUS, glyphosate or AMPA, PBMCs were centrifuged (600*g* for 10 min at 4 °C) and diluted with PBS (final density 1×10^6 cells/ml) and then incubated with H_2DCFDA or HPF for 20 min at 37 °C in the total darkness. The analysis of 10,000 cells for both methods was performed.

2.7.Statistical analysis

The experiments have been conducted on blood from three donors, whereas for each donor the experiment was repeated two or three times. Generally, we collected 6 or 9 results for each variable. Therefore, the final n value included in Tukey test was 6 or 9. Certainly, we

checked a normality of distribution using Shapiro-Will test as well as the homogeneity of variance by Brown-Fisher test. Tukey test was used as a post-hoc test. Finally, we checked the 'sample size' and the 'power of test' for all data. Statistical calculations were done using STATISTICA software ver.13.

3. Results

3.1. Analysis of DNA damage:

3.1.1. Comet assay – alkaline and neutral version

Under alkaline condition both SSBs and DSBs as well as ALSs can be determined, while in the neutral version of comet assay only DSBs can be detected.

Roundup 360 PLUS, glyphosate and AMPA increased DNA damage (SSBs, DSBs and ALSs formation, alkaline version of comet assay) from the concentration of 5 µM, 250 µM and 500 µM, respectively. We have observed that Roundup caused the strongest SSBs formation (it induced DNA damage even at 50 times lower concentration than glyphosate), following by glyphosate and AMPA (AMPA induced DNA damage at two times higher concentration than glyphosate and 100 times higher concentration than Roundup). Selected comets (alkaline version) originating from DNA of PBMCs exposed to Roundup 360 PLUS are shown in Fig. 1.

It has also been noted that Roundup and glyphosate caused low level of DSBs formation (neutral version) in opposite to AMPA, which even at a very high concentration of 1000 µM did not induce DSBs. Roundup caused a low level of DSBs at 10 µM (2.53% ± 0.11% versus control 1.04% ± 0.11%), while glyphosate changed this parameter only at 1000 µM (2.54% ± 0.11% versus control 1.04% ± 0.11%).

Fig. 2 A-C shows percentage of DNA in the comets tail derived from human PBMCs exposed to xenobiotics studied for 24 h.

3.1.2. Comet assay – damage to purines and pyrimidines

Investigated compounds induced oxidative damage to purines and pyrimidines. Roundup 360 PLUS, glyphosate and AMPA caused DNA bases lesions from the concentration of 5 µM, 250 µM and 500 µM, respectively. It has been shown, that Roundup 360 PLUS (even at the concentration 50 times lower than glyphosate) caused the strongest damage to purines and pyrimidines, following by glyphosate and AMPA. It has also been observed that Roundup 360 PLUS (at 5 µM and 10 µM - $P<0.001$) and glyphosate (at 500 µM $P<0.01$) caused stronger (statistically significant) oxidative damage to pyrimidines than purines. Fig. 3. A-C shows percentage of DNA in the comets tail derived from human PBMCs exposed to investigated xenobiotics for 24 h.

3.1.3. Plasmid relaxation assay

The results obtained my means of electrophoretic separation of plasmid DNA have shown that both Roundup 360 PLUS as well as glyphosate and AMPA did not bind directly with DNA (Fig. 4). In order to assess whether these compounds can directly interact with DNA, we have used the extracellular system – Plasmid relaxation assay. We have assumed that in the case of linking of tested compound to DNA, single or double-stranded DNA breaks may occur. These breaks can cause relaxation of the supercoiled form of the pUC19 plasmid (SC) in the nicked circular form (OC) in the case of a SSBs induction or in the linear form (L) in the case of DNA DSBs formation. Structural differences between supercoiled (SC), nicked circular (OC) and linear form (L) of the plasmid were reflected in their different electrophoretic mobility.

The denstometric analysis did not show any changes in the amount of different forms of the plasmid (Fig. 4) in contrast to positive control. We have observed in positive control

(the pUC19 plasmid exposed to 200 µM H_2O_2 and 20 µM Fe^{+2} for 20 min on ice) transformation forms supercoiled (SC) to open circular (OC) and mainly to linear (L).

We have therefore assumed that neither Roundup 360 PLUS, glyphosate nor AMPA was able to bind directly to DNA.

3.1.4. Analysis of DNA repair

It has been noticed that PBMCs after 120 min post-incubation significantly repaired DNA SBs induced by Roundup at 5 µM - 6.09% (0 min) vs 2.55% (120 min) and 10 µM - 14.30% (0 min) vs 4.80% (120 min) (Fig. 5A-C), glyphosate at 250 µM - 5.99% (0 min) vs 2.35% (120 min) and 500 µM - 11.80% (0 min) vs 2.37% (120 min) and AMPA at 500 µM - 5.25% (0 min) vs 2.28% (120 min) and 1000 µM - 9.19 % (0 min) vs 2.31% (120 min).

It must be underlined that DNA SBs induced by the highest concentration of Roundup 360 PLUS (10 µM) were not totally repaired even after 120 min post-incubation - 4.80% vs control 1.97%. DNA SBs induced by lower concentration (5 µM) of Roundup 360 PLUS were totally repairable after 120 min post-incubation. It has also been noticed that DNA SBs induced by both glyphosate (250 µM and 500 µM) and AMPA (500 µM and 1000 µM) were totally repaired. Fig. 5 A-C shows percentage of DNA in the comets tail derived from human PBMCs exposed for 24 h to investigated xenobiotics.

4. Flow cytometric measurement of ROS

4.1. Oxidation of H_2DCF

The effect of Roundup 360 PLUS, glyphosate and AMPA on ROS production in PBMCs was shown as changes in DCF fluorescence intensity (Fig. 6). The intensity of DCF fluorescence in control PBMCs was referred as 100%. The value for positive control

(hydrogen peroxide 2 mM) was 912.2% of control. The chemicals studied were shown to generate ROS in PBMCs. Statistically significant changes have been observed for Roundup, glyphosate and AMPA from the concentration of 1 µM, 250 µM and 500 µM, respectively. It has also been noticed that Roundup 360 PLUS caused stronger ROS formation than glyphosate and AMPA.

4.2. Oxidation of HPF

We have also investigated the level of highly reactive oxygen species (mainly hydroxyl radical) in PBMCs incubated with chemicals studied. Changes in hydroxyl radical production in PBMCs were shown as fluorescence intensity of oxidized HPF (Fig. 6). The intensity of oxidized probe fluorescence in control cells was referred as 100%. The value for positive control (0.1 mM ferrous perchlorate(II) and 1 mM hydrogen peroxide) was 297.5% of control.

We have observed that Roundup 360 PLUS and glyphosate were able to generate $^{\bullet}OH$ in PBMCs in opposite to AMPA that even at a very high concentration of 1000 µM did not induce hydroxyl radicals formation. Statistically significant changes were observed from the concentration of 10 µM of Roundup and 1000 µM of glyphosate. It has also been noticed that Roundup caused stronger hydroxyl radical formation in comparison to glyphosate.

5. Discussion

In this study we have decided to assess in detail genotoxic mechanism of action of glyphosate, its herbicide formulation (Roundup 360 PLUS) and its main metabolite – AMPA on human PBMCs, the process, which is usually involved in cancer promotion.

It is generally accepted that commercial pesticide preparations containing glyphosate are more cytotoxic than glyphosate itself (Kwiatkowska et al. 2016, Gasnier et al., 2009,

Martinez et al., 2007). These findings may be due to significant toxicity of surfactants and the presence of glyphosate (for example) in form of amine salt in pesticide preparations (Song et al., 2012).

Martinez et al. (2004) have shown that cytotoxic effects caused by Roundup in PBMCs were stronger than those induced by glyphosate. IC_{50} values for glyphosate formulation and pure glyphosate were estimated to be 1.64 mg/L and 56.4 mg/L (9.7 µM and 333.59 µM), respectively.

Similarly, Gasnier et al., (2009) showed that LC_{50} value was significantly lower for pesticide formulations containing glyphosate than for glyphosate alone for human liver HepG2 cells. Gasnier et al., showed that LC_{10} and LC_{50} values for pure glyphosate were 2.56% and 2.78% respectively, whereas LC_{10} and LC_{50} values for R360 preparation were 0.1% and 0.22%, respectively (Almare blue test). Additionally, differences in cytotoxicity were more dependent on the concentration of formulation than on the concentration of glyphosate. LC_{50} values for different Roundup preparations were as follows: R400 (0.0012%) > R450 (0.006%) > R360 (0.22%) > R7.2 (0.8%)

Kwiatkowska et al. (2016) showed that glyphosate was more toxic than AMPA for human PBMCs. They assessed cell viability using flow cytometry and calcein-AM/propidium iodide fluorescent probes and calculated IC_{50} values, which were above 10 mM for both glyphosate and AMPA. They also observed that glyphosate more strongly than AMPA depleted intracellular ATP level as calculated LC_{50} values for glyphosate and AMPA were 9.6 mM and above 10 mM, respectively.

Opposite results were presented by Benachour and Serralini (2009) who suggested that AMPA is more toxic for human cells as compared to glyphosate. They observed that glyphosate and AMPA were capable of altering succinate dehydrogenase and adenylate kinase, which led to mortality of various cell types.

The above inconsistence may be explained by the fact that Serralini and co-workers used percent concentrations (e.g. 0.05%) of the compounds studied, while in our study molar concentrations were used. For instance, the concentration of 250 µM corresponds to a concentration- of 42.5 mg/L and 27.75 mg/L for glyphosate and AMPA, respectively, while the concentration of 500 µM corresponds to concentration of 85 mg/L and 55.5 mg/L for glyphosate and AMPA, respectively. Based on these data, toxic effects of glyphosate and AMPA may be considered to be comparable but surely AMPA is not more cytotoxic than glyphosate for PBMCs.

We have shown that the compounds studied caused DNA SBs, while glyphosate at 1000 µM and Roundup at 10 µM caused DSBs. It is also worth noticing that PMBCs were unable to repair DNA strand-breaks induced by Roundup 360 PLUS at 10 µM, while SBs provoked by glyphosate were effectively repaired after 120 min post-treatment.

The lowest DNA SBs were induced by AMPA, which is the main metabolite of glyphosate. AMPA even at 1000 µM did not cause DSBs formation, while SSBs caused by this chemical were effectively repaired.

As mentioned above, we have noticed that oxidative DNA damage induced by Roundup 360 PLUS in the concentration of 10 µM is not completely repaired (Fig. 5), which may lead to DNA mutation because DNA polymerases during replication may insert incorrect bases against damaged bases. The most frequent DNA lesion provoked by oxidative stress is 8-oxo-7,8-dihydroguanine (8-oxodG). 8-oxodG has high mutagenic potential by misincorporation of an adenine instead of cytosine causing G:C→T:A transversion mutation (Basu et al., 2015). Mutations in oncogenes, tumor-suppressor genes, or genes that control the cell cycle can be the cause of many human diseases, including malignant tumors (Basu, 2018; Turgeon et al., 2018). It has also been shown that oxidative stress-mediated damage to DNA occurs frequently in neurodegenerative diseases, for example Parkinson's disease (Basu et al.,

2015). Parkinson's disease is an age-related progressive neurodegenerative disease associated with mutations in the *SNCA* gene encoding α-synuclein protein. 8-oxodG-mediated transcriptional mutagenesis has been shown to have the potential to alter phenotype of cells through production of mutant pool of α-synuclein (Basu et al., 2015).

Our findings are in agreement with previous studies published by Kwiatkowska et al. (2017) who showed that glyphosate only at high concentration was capable of inducing DNA damage. Kwiatkowska and co-workers observed that glyphosate at 250 µM induced DNA damage in human PBMCs, while DNA lesions were effectively repaired (Kwiatkowska et al. 2017).

In another study, Hong et al. (2017) evaluated toxic potential of glyphosate in crab hematocytes (*Eriocheir sinensi*). The cells were exposed to glyphosate in the concentrations ranging from 4.4 to 98 mg/L (26.02 to 579.64 µM) for 24, 48 and 96 h. They found out that glyphosate at 4.4 mg/L after 24 h incubation caused SSBs and DSBs formation, while elongation of the incubation time caused futher increase in DNA damage (Hong et al. 2017).

Many studies have shown genotoxic action of various glyphosate-based herbicide preparations. Alvarez-Moya et al. (2014) assesed the effect of isopropyl-amino salt of glyphosate in the concentrations range from 0.7 to 700 µM on human lymphocytes. They observed that this substance even at 0.7 µM caused significant DNA damage. In another study, Luaces et al. (2017) reported that glyphosate formulation Roundup Full II® (from the concentration of 280 µmol/l - 47.34 mg/L induced DNA damage in lymphocytes of large hairy armadillo (*Chaetophractus villosus*).

Herbicides are one of the most dangerous substances influencing aquatic organisms. The research conducted by Guilherme et al. (2012) on European eel (*Anguilla anguilla*) confirmed genotoxic potential of Roundup. Fish were exposed to Roundup at environmental concentrations of 58 and 116 µg/L (0.34 and 0.68 µM) during 1 and 3 days. The study

employed modified comet assay with formamidopyrimidine DNA glycosylase in order to assess DNA oxidative bases lesions in liver and gill cells of the species examined. As a result, Guilherme and co-workers observed time-dependent increase in oxidative DNA bases lesions in the cells of animal studied, while liver cells were less susceptible to action of this herbicide.

In another study, Marques and co-workers (2014) exposed European ell to Roundup at the same concentrations for 3 days, and then cultivated fish in water deprived of the herbicide for 1 to 14 days. The experiments showed that DNA lesions caused by Roundup were effectively repaired, while the proteins involved in repair process were susceptible to the effect of higher concentration (116 μg/L - 0.68 μM) of this preparation.

As mentioned above, glyphosate-based preparations that usually contain chemically modified glyphosate and/or glyphosate mixed with other chemical substances reveal different adverse effects. Bonfanti et al. (2018) suggested that differences in GBHs formulations should be carefully considered by the authorities, since sub-lethal and/or long-term effects (e.g. teratogenicity) can be significantly modulated by the active ingredient salt type and concentration of the adjuvants. Similarly, Richard et al. (2005) showed that the presence of adjuvants in Roundup enhanced glyphosate bioavailability and/or bioaccumulation.

That is why, in this study, we have assessed the effect of both glyphosate, AMPA and Roundup on different DNA lesions types paying also attention to the ability of these substances to create adducts with DNA.

Using DNA plasmid in conformational test, we have evaluated changes in conformational structure of DNA, which were due to direct interaction of xenobiotics examined with DNA resulting in adducts formation. As a result, we have noted that none of the compounds studied caused conformational changes in plasmid DNA, therefore glyphosate, Roundup 360 PLUS or AMPA were not able to form adducts with DNA (Fig. 4).

Our results regarding oxidative DNA damage and the ability to induce reactive oxygen species suggest that these compounds caused DNA damage indirectly through ROS-mediated effects.

Apart of direct action of xenobiotics on DNA structure, DNA damage may be induced indirectly by the formation of by-products having oxidative damaging potential. It has been widely accepted that ROS may cause DNA damage (Jena et al. 2012). ROS are usually formed during physiological processes, but an enhanced production of these species is often due to the effect of various xenobiotics on the cell compartments. Hydroxyl radical is the main ROS responsible for oxidative damage to DNA due its strong electrophilic nature that determines the reaction of addition to π bonds of the DNA bases and deoxyribose dehydration (Jena et al. 2012). Within purine bases, addition reactions refers to bonds at C4 and C8, while in pyrimidine bases, the reactions concern bonds at C5 and C6 or are associated with elimination of hydrogen at C5.

Taking the above into consideration, we have assessed the effect of glyphosate, Roundup 360 PLUS and AMPA on oxidative damage to DNA purines and pyrimidines as well as ROS including hydroxyl radical formation in human PBMCs. We have found that glyphosate, Roundup and AMPA induced oxidative damage to purines and pyrimidines and increased ROS including hydroxyl radical level in the incubated cells. Observed changes strongly suggest that the compounds studied induce indirect effect on DNA by ROS production. It is also worth noting that glyphosate and Roundup 360 PLUS in the same concentration (glyphosate at 1000 µM and Roundup 360 PLUS at 10 µM) caused both DSBs formation and increased $^{\bullet}$OH level in the cells studied.

Kašuba et al. (2017) exposed HepG2 cells to glyphosate at 0.5 mg/L (2.96 µM) (acceptable daily intake), 2.91 mg/L (17.21 µM) (residential exposure level) and 3.5 mg/L(20.7 µM) (occupational daily intake) for 4 h and 24 h. As the result, they observed that

glyphosate even at the lowest concentration of 0.5 mg/L after 24 h incubtation caused DNA oxidative damage in human liver cancer cells.

Our results showed that stronger genotoxic effect was provoked by Roundup 360 PLUS when compared with glyphosate, which was the most probably due to the presence of additional ingredients (i.e. surfactants) in this herbicide preparation.

Similarly, Roundup has been shown to be more toxic than glyphosate in the study of Keller et al. (2012). The research was performed on epithelial cells of cheek (TR146), which were collected from humans exposed to glyphosate and Roundup during spraying operations. Those experiments showed that both Roundup and its active ingredient (from the concentration of 20 mg/L - 118.29 µM) caused an increase in chromosome aberrations in this cell type. The authors of this study, have also proven that glyphosate and Roundup (10-20 mg/L - 59.15-118.29 µM) increased micronucleus formation, which is considered to affect oncogenes activation and cancer promotion.

In another study, Guilherme et al. (2014) showed genotoxic potential of main glyphosate metabolite - AMPA in blood cells of European eel. Fish were exposed to AMPA at environmentally relevant concentrations of 11.8 µg/L and 23.6 µg/L (0.11 µM and 0.21 µM) from 1 to 3 days, and then oxidative damage to DNA and chromosomes of blood cells was assessed using comet assay and erythrocytic nuclear abnormalities assay, respectively. The results showed significant increase in chromosome damage, while oxidative lesions to DNA bases were low in the cells studied (Guilherme et al. 2014).

Taking into consideration the results showing higher toxicity of Roundup in comparison to glyphosate, Myers et al. (2016) suggested that common commercial formulations of GBHs should be prioritized for inclusion in government-led toxicology testing programs such as the U.S. National Toxicology Program, as well as for biomonitoring as conducted by the U.S. Centers for Disease Control and Prevention.

Conclusions

Summing up, Roundup 360 PLUS caused much stronger damage to DNA of human PBMCs in comparison to glyphosate, and AMPA in particular. DNA damage in human PBMCs was induced by Roundup at low concentrations (even at 5 μM), while glyphosate and AMPA were capable of inducing DNA lesions at much higher dosages of 250 μM and 500 μM, respectively.

We may conclude that observed DNA damage was not due to direct interaction of glyphosate, Roundup 360 PLUS or AMPA with DNA as no DNA adducts formation has been observed, while this damage was associated with ROS-mediated effects , e.g. DSBs formation and an increase in $^{\bullet}OH$ level were observed in cells exposed to the same Roundup 360 PLUS (10 μM) and glyphosate (1000 μM) concentration.

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A competing financial interests declaration:

All authors declare they have no actual or potential competing financial interest.

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Fig. 1. Selected photographs of DNA (comets) of human PBMCs incubated with different concentrations of Roundup 360 PLUS for 24 h and PBMCs incubated with hydrogen peroxide (20 µM) for 15 min on ice (positive control) (comet assay, alkaline version). The photos were achieved using fluorescent microscope with 200x magnification.

Fig. 2. The level of DNA strand-breaks in human PBMCs determined by single cell gel electrophoresis (comet assay). DNA damage in PBMCs was induced by Roundup 360 PLUS (A), glyphosate (B) and AMPA (C). The cells were incubated with Roundup 360 PLUS (0.001-10 µM) and glyphosate and AMPA (0.5-1000 µM) for 24 h. DNA damage was measured as percentage of DNA in the comets tail by alkaline version (black bars) and neutral version (gray bars) of the comet assay. Each experiment included a positive control (PC)Hydrogen peroxide at 20 µM was selected to induce DNA SSBs (the cells were incubated with H_2O_2 for 15 min on ice), while DSBs were induced by ionizing irradiation at a dose of 10 Gy (2 Gy/ min per 5 min). The number of cells scored for each slide was 50. Mean ± SD was calculated from 9 individual experiments (450 comets). Statistically different from negative control at *$P < 0.05$; ***$P < 0.001$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Fig. 3. The level of DNA pyrimidines and purines oxidation in human PBMCs (analysis by means of alkaline version of the comet assay with endonuclease III – Nth or human 8-oxoguanine DNA glycosylase – hOOG1). DNA damage in PBMCs was induced by Roundup 360 PLUS (A), glyphosate (B) and AMPA (C). The cells were incubated with Roundup 360 PLUS (0.001-10 µM) and glyphosate and AMPA (0.5-1000 µM) for 24 h. Each experiment included a positive control (PC) which concerned the cells incubated with hydrogen peroxide at 20 µM for 15 min on ice and subsequently treated with the enzymes. The value of DNA in

comet tail in the presence of either enzyme for all concentrations of Roundup 360 PLUS, glyphosate or AMPA was reduced by the value obtained in the comet assay without any enzyme and the value for enzyme buffer only. The number of cells scored from each slide was 50. The mean value for 100 cells analyzed in each treatment in three independent experiments (300 total cells) was recorded. Mean ± SD was calculated for 6 individual experiments. Statistically different from negative control at *P < 0.05; **P < 0.01; ***P < 0.001. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Fig. 4. Plasmid relaxation assay. A: pUC19 plasmid DNA was resolved on a 1% agarose gel, stained with ethidium bromide and visualized in UV light. Line 1 – positive control (Cp) (the plasmid was exposed to 200 µM H_2O_2 and 20 µM Fe^{+2} for 20 min on ice, Fenton reaction); line 2 – negative control (Cn) (pUC19 plasmid); lines 3-9 - pUC19 plasmid incubated with Roundup 360 PLUS, glyphosate or AMPA at indicated concentrations. Structural differences between supercoiled (SC), nicked circular (OC) and linear (L) forms of the plasmid accounted for their different electrophoretic mobility. B: Densitometry analysis of agarose gel was presented below the gel picture. Open circular (OC) (as a consequence of DNA strand-breaks), linear (L) (as a consequence of DNA double strand-breaks) and supercoiled (SC) (undamaged DNA) forms of DNA plasmid are presented as peaks. Densitometry was performed with the GeneTools by Syngene (Cambridge, UK) software.

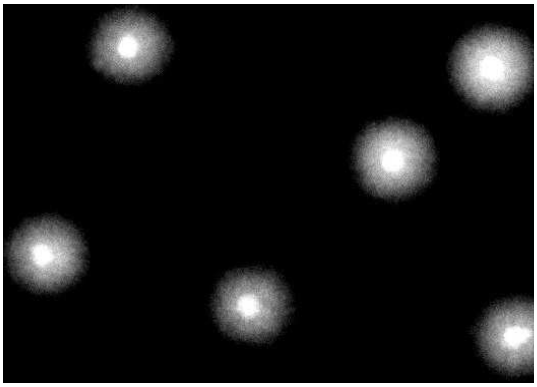
Fig. 5. Time course of the repair kinetics of DNA damage (SBs), measured as DNA in comet tail derived from PBMCs treated for 24 h with Roundup 360 PLUS (5 µM and 10 µM), glyphosate (250 µM and 500 µM) and AMPA (500 µM and 1000 µM) and then for 2 h in medium deprived of these compounds. The number of cells scored from each slide was 50. The mean value for 100 cells analyzed in each treatment in three independent experiments

(300 total cells) was recorded. Mean ± SD was calculated for 6 individual experiments. Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

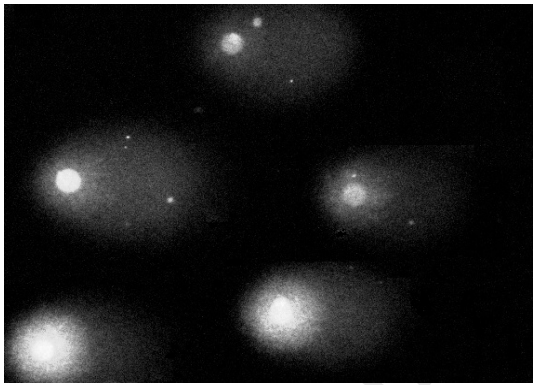
Fig. 6. Changes in reactive oxygen species (DCF fluorescence) and hydroxyl radical level (HPF fluorescence) in human PBMCs incubated with Roundup 360 PLUS (0.001-10 µM) and glyphosate and AMPA (0.5-1000 µM) for 24 h. Mean ± SD calculated from 9 individual experiments. Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Tab. 1. The level in the viability level of human PBMCs determined by Trypan Blue dye exclusion test. PBMCs was induced by Roundup 360 PLUS, glyphosate and AMPA. The cells were incubated with Roundup 360 PLUS (0.001-50 µM) and glyphosate and AMPA (0.5-1000 µM) for 24 h. Mean ± SD was calculated from 9 individual experiments. Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

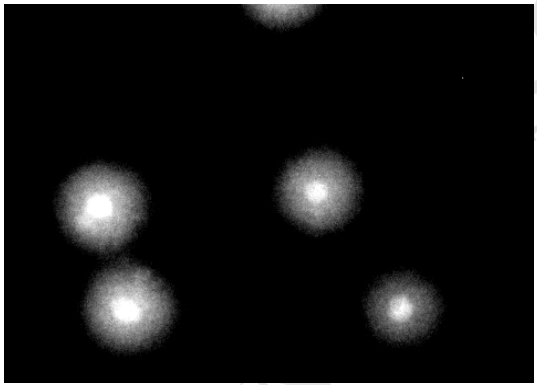
Compounds	**Concentration[µM]**	**Cell viability [%]**	**ANOVA I**
Roundup 360 PLUS	0	99.4 ± 0.8	P < 0.05
	0.001	98.1 ± 1.1	
	0.01	98.8 ± 1.0	
	0.5	98.4 ± 2.0	
	1	97.0 ± 1.3	
	5	96.3 ± 1.7	
	10	96.4 ± 1.0	
	50	**89.4 ± 3,8***	
Glyphosate	0	99.4 ± 0.8	P > 0.05
	0.5	98.45 ± 0.5	
	10	98.9 ± 1.0	
	100	98.9 ± 1.0	
	250	98.7 ±1.3	
	500	98.7 ± 1.2	
	1000	98.8 ±1.2	
AMPA	0	99.4 ± 0.8	P > 0.05
	0.5	99.4 ± 1.0	
	10	99.6 ± 0.7	
	250	99.1 ± 1.6	
	500	98.7 ± 1.3	
	1000	98.8 ± 1.0	



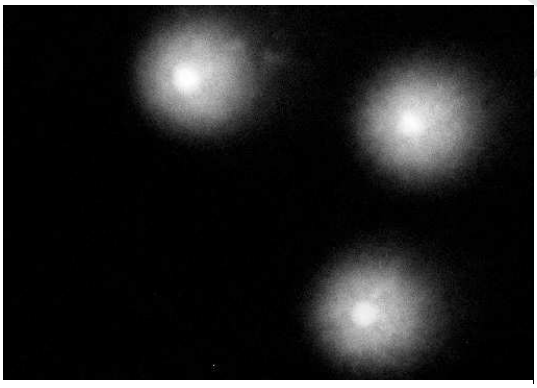
Control



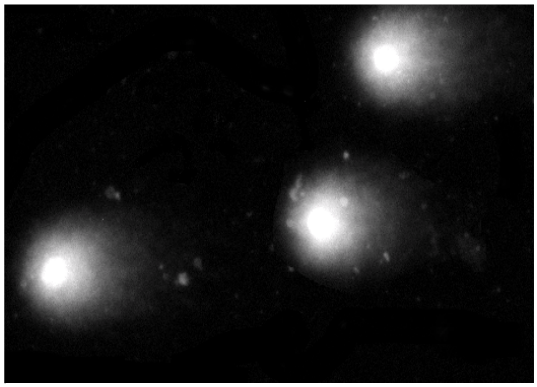
H_2O_2 20 µM



Roundup 1 µM

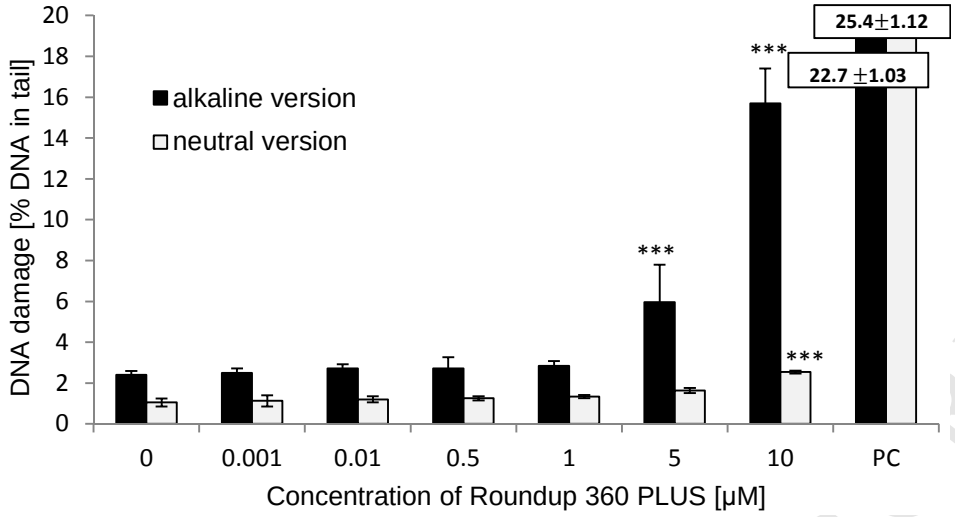


Roundup 5 µM

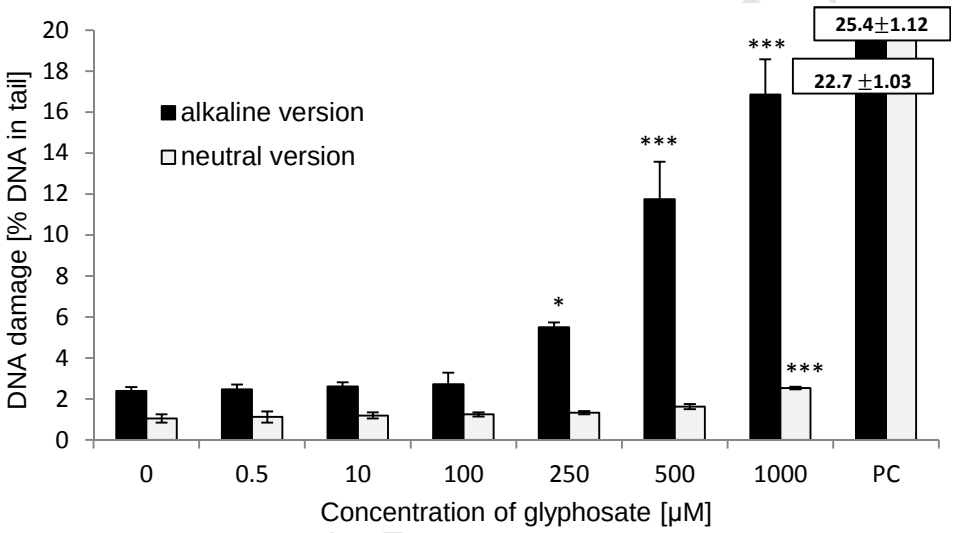


Roundup 10 µM

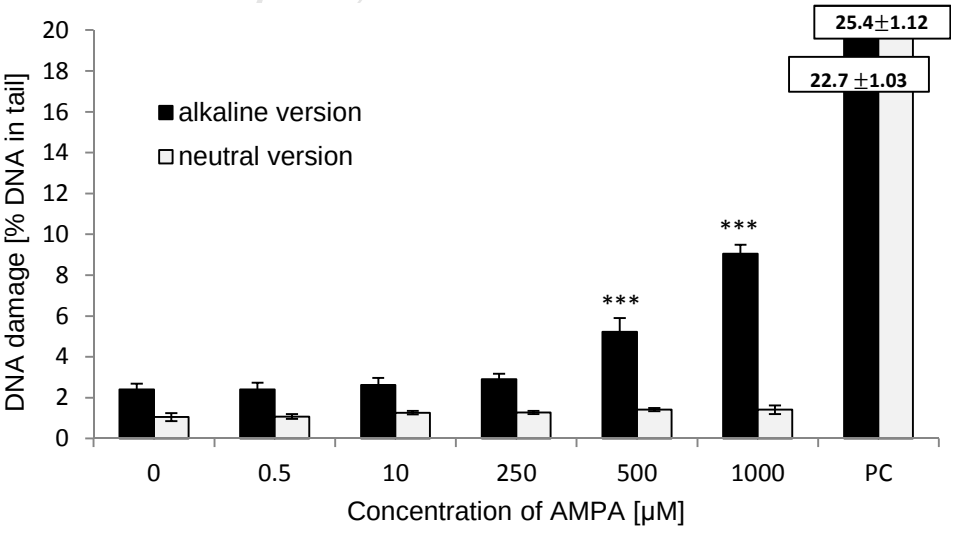
A


DNA damage [% DNA in tail]
alkaline version
neutral version
25.4±1.12
22.7 ±1.03
***
***
***
***
0
0.001
0.01
0.5
1
5
10
PC
Concentration of Roundup 360 PLUS [µM]

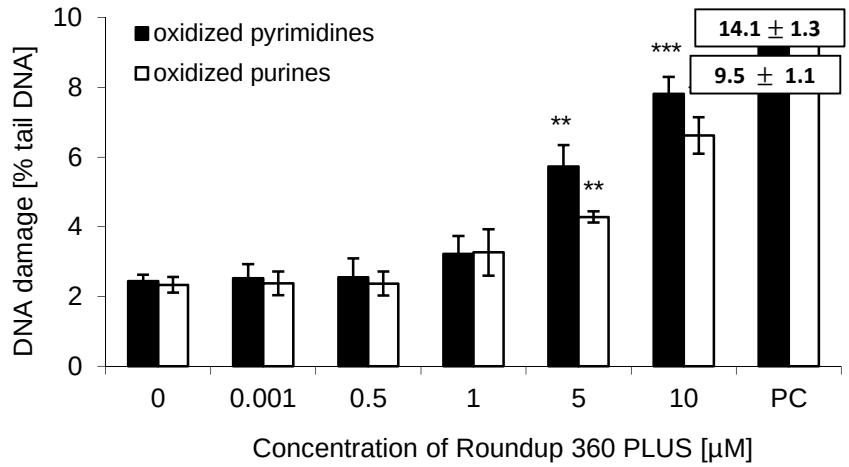
B


DNA damage [% DNA in tail]
alkaline version
neutral version
25.4±1.12
22.7 ±1.03
***
***
*
***
***
0
0.5
10
100
250
500
1000
PC
Concentration of glyphosate [µM]

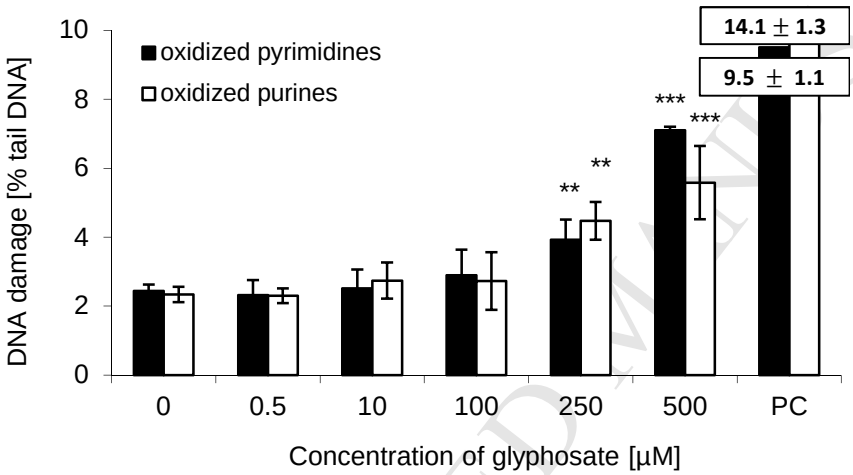
C


DNA damage [% DNA in tail]
alkaline version
neutral version
25.4±1.12
22.7 ±1.03
***
***
0
0.5
10
250
500
1000
PC
Concentration of AMPA [µM]

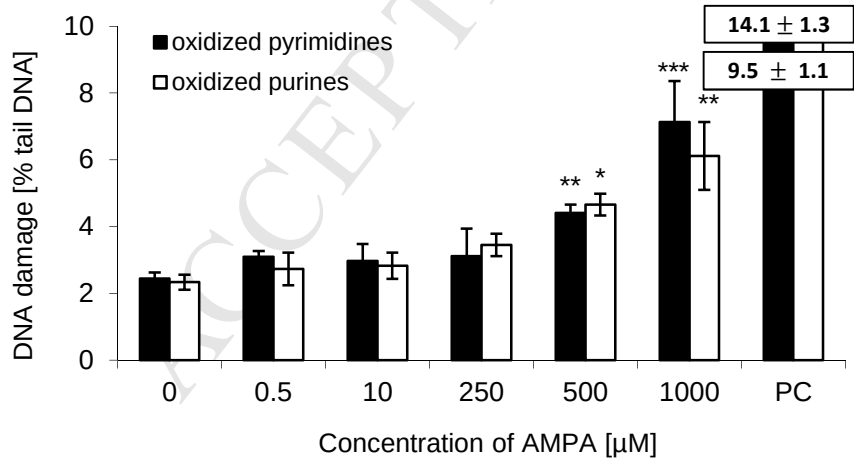
A

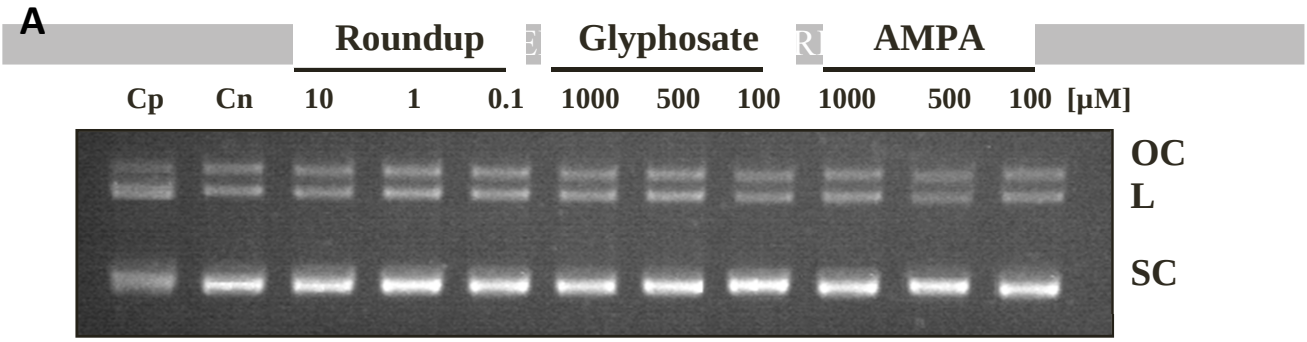


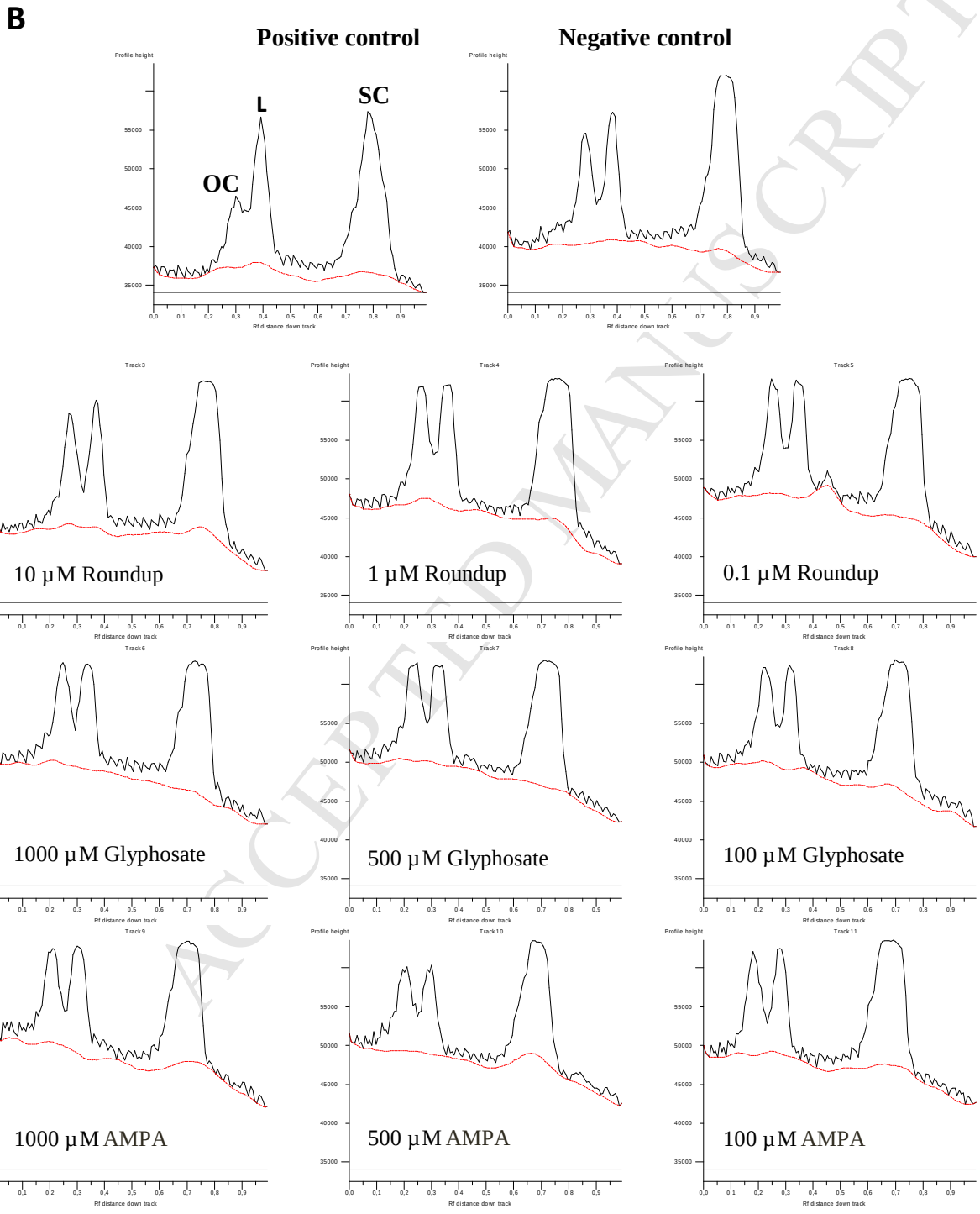
B



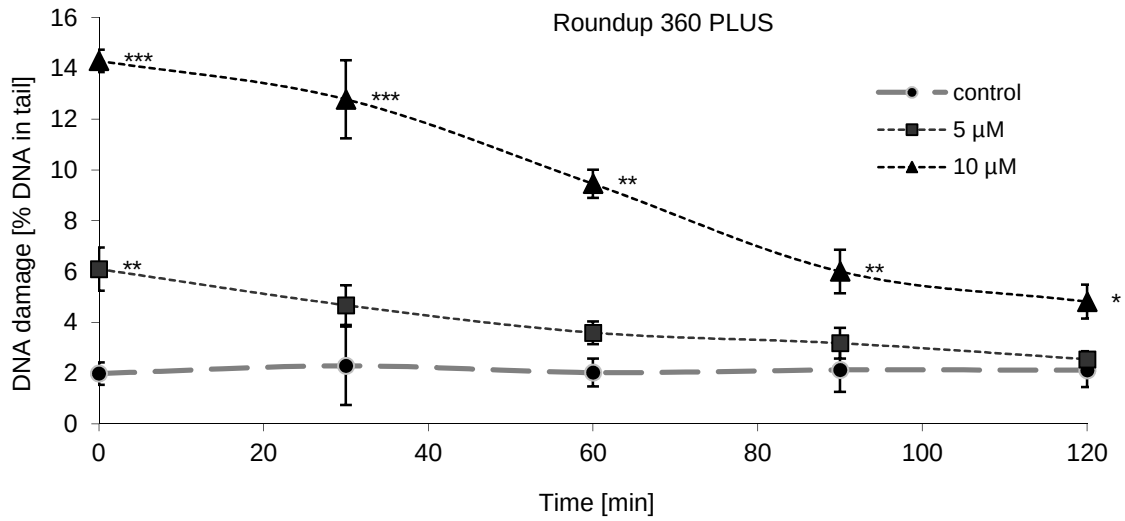
C



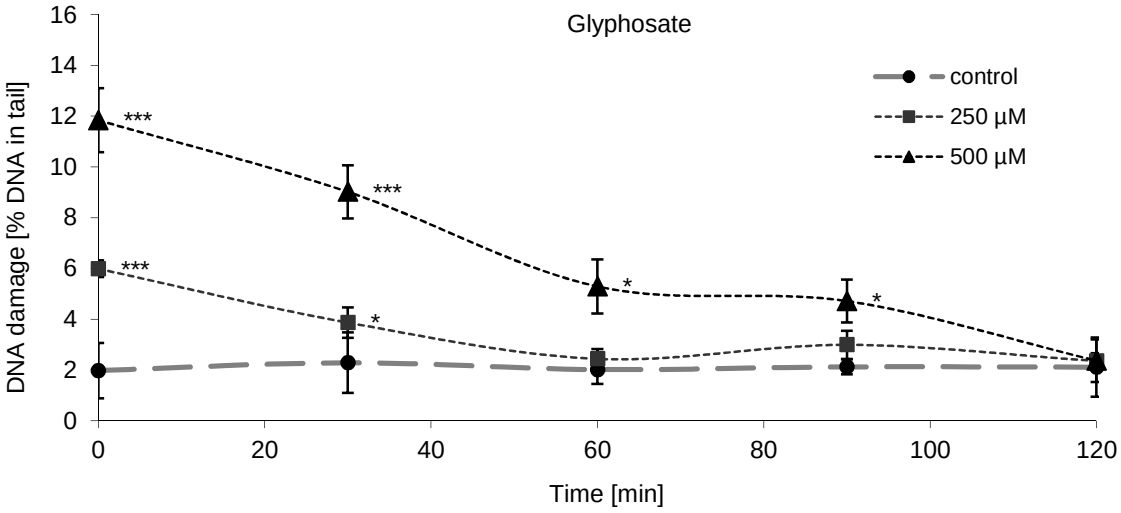




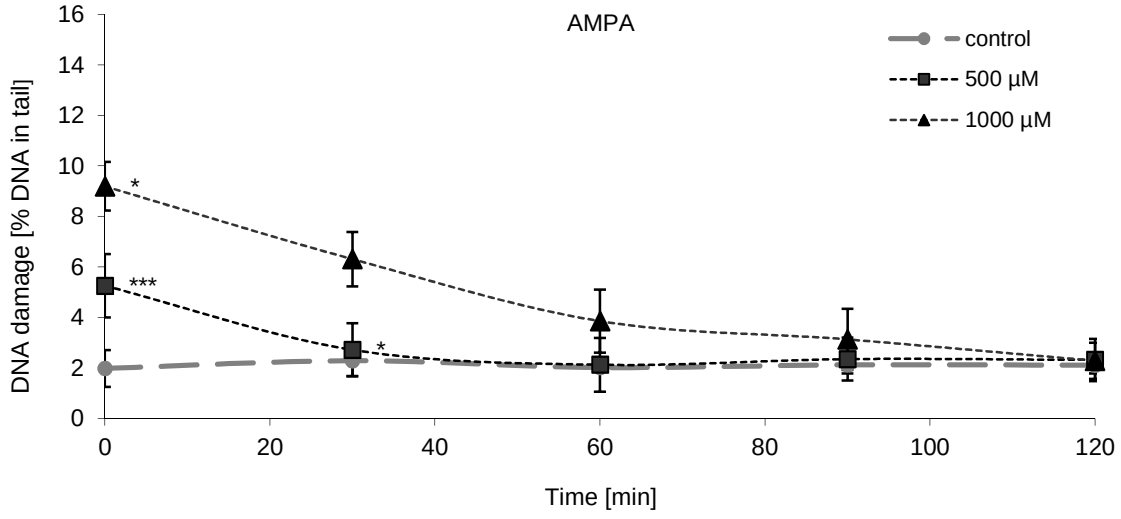
A


Roundup 360 PLUS
DNA damage [% DNA in tail]
Time [min]
control
5 µM
10 µM
***
***
**
**
**
*

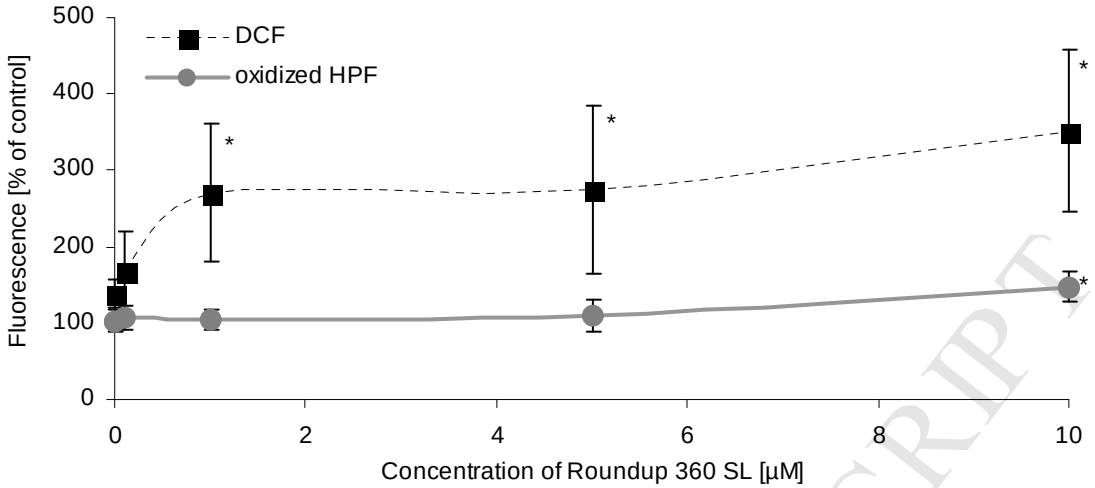
B


Glyphosate
DNA damage [% DNA in tail]
Time [min]
control
250 µM
500 µM
***
***
***
*
*
*

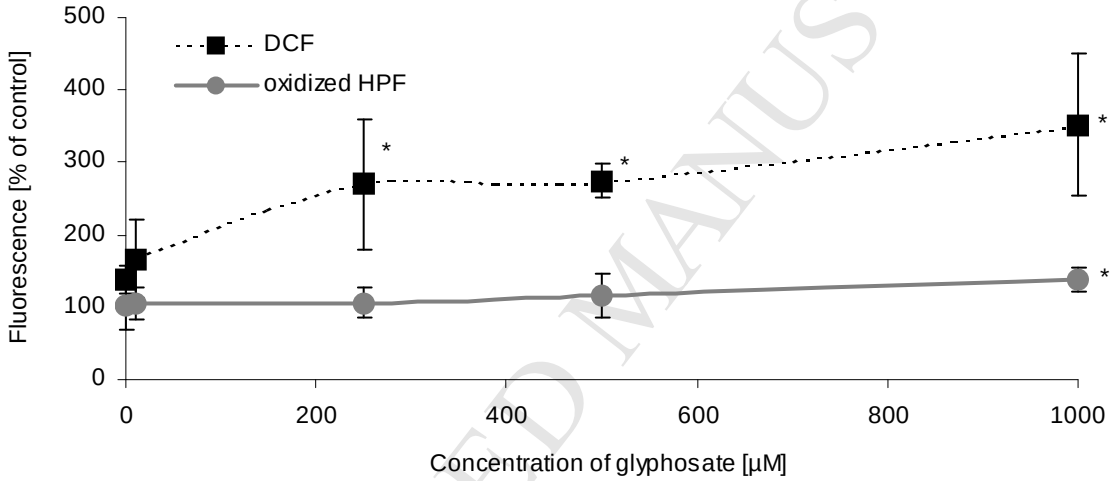
C


AMPA
DNA damage [% DNA in tail]
Time [min]
control
500 µM
1000 µM
*
***
*

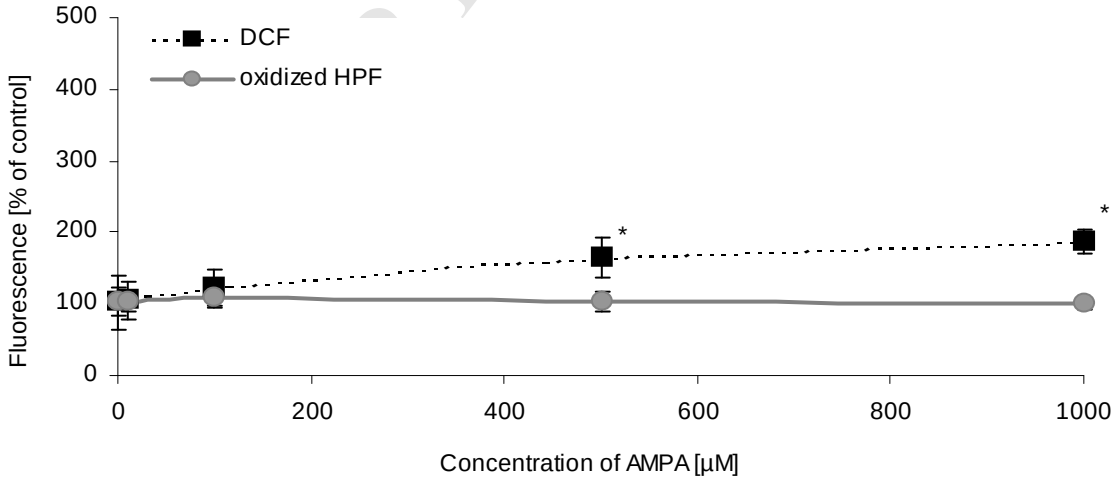
A

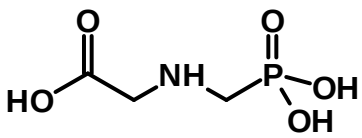


B

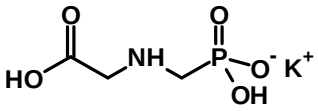


C

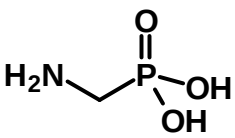




Glyphosate



Glyphosate in a form of potassium salt



AMPA

Scheme 1. Chemical structures of *N*-(phosphonomethyl)glycine (glyphosate), N-(phosphonomethyl)glycine in a form of potassium salt (contained in Roundup 360 PLUS) and aminomethylphosphonic acid (AMPA).

- Glyphosate, Roundup 360 PLUS and AMPA induced DNA single and double strand-breaks.
- The compounds studied caused purines and pyrimidines oxidation.
- Glyphosate, Roundup and AMPA increased ROS level in PBMCs.
- Glyphosate and Roundup increased $^{\bullet}OH$ level in peripheral blood mononuclear cells.
- The compounds examined were not capable of creating adducts with DNA.