Food and Chemical Toxicology 135 (2020) 110888

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Evaluation of apoptotic potential of glyphosate metabolites and impurities in human peripheral blood mononuclear cells (*in vitro* study)

Marta Kwiatkowska[a], Jaromir Michałowicz[a], Paweł Jarosiewicz[a], Daria Pingot[a], Paulina Sicińska[a], Bogumiła Huras[b], Jerzy Zakrzewski[b], Monika Jarosiewicz[a], Bożena Bukowska[a,*]

[a] University of Lodz, Faculty of Biology and Environmental Protection, Department of Biophysics of Environmental Pollution, Pomorska 141/143 Str., 90-236, Lodz, Poland

[b] Łukasiewicz Research Network, Institute of Industrial Organic Chemistry, Annopol 6 Str., 03-236, Warsaw, Poland

ARTICLE INFO

Keywords:
Glyphosate
Calcium ions
Mitochondrial transmembrane potential
Caspase activity
Reactive oxygen species

ABSTRACT

Glyphosate is used for cereal, vegetable and fruit crops for reducing or inhibiting the growth of weeds as well as a desiccant for various grain crops. That is why, glyphosate has been shown to be accumulated in humans and animals through ingestion of food of both plant and animal origin.

The study aimed to assessed the effect of glyphosate, its metabolites: aminomethylphosphonic acid (AMPA), methylphosphonic acid and its impurities: PMIDA, *N*-methylglyphosate, hydroxymethylphosphonic acid and bis (phosphonomethyl)amine on apoptosis induction in human peripheral blood mononuclear cells (PBMCs). PBMCs were exposed to the compounds studied at the concentrations ranging from 0.01 to 5 mM for 4 h.

We have observed an increase in reactive oxygen species (including hydroxyl radical) and cytosolic calcium ions levels as well as reduction of transmembrane mitochondrial potential ($\Delta\Psi m$) in PBMCs exposed to the compounds examined. All substances studied changed PBMCs membrane permeability, activated caspase-8, -9, -3 and caused chromatin condensation, which showed that they were capable of inducing apoptosis both via extrinsic and particularly intrinsic pathway.

Generally the study demonstrated that there were no differences between apoptotic changes induced by glyphosate, its metabolites or impurities, and observed changes were provoked by high concentrations of investigated compounds.

1. Introduction

Since 2001 glyphosate (*N*-(phosphonomethyl)glycine) has been constantly on the top of the list of the most commonly used active ingredients in pesticide preparations (Grube et al., 2011). It is contained in over 750 herbicidal preparations all over the world (Guyton et al., 2015).

This substance exhibits an unique mode of action *via* a biochemical pathway that only exists in a small number of organisms utilizing the shikimic acid pathway (inhibits the synthesis of enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase) (Bai and Ogbourne, 2016). Glyphosate is used for cereal, vegetable and fruit crops for reducing or inhibiting the growth of weeds as well as a desiccant for various grain crops (Xu et al., 2019).

The widespread use of glyphosate contributes to the occurrence of this compound in the environment, both in water (0.1–0.7 mg/dm^3), sediments, and soil (0.5–5 mg kg^{-1}) (Peruzzo et al., 2008), as well as in genetically modified soya beans (mean content of 3.3 mg kg^{-1}) (Bøhn et al., 2014).

Glyphosate has been shown to accumulate in humans and animals through ingestion of food of both plant and animal origin (Torretta et al., 2018). In addition, genetically-modified crops (GMO) may contain high amounts of glyphosate residues (Xu et al., 2019).

Glyphosate (from 0.03 mg kg^{-1} to 1.08 mg kg^{-1}) and their metabolite AMPA (from 0.02 mg kg^{-1} to 0.17 mg kg^{-1}) were determined in soy-based infant formula (Brazil). The child population-consuming cereal-based foods is heavily exposed to pesticides because of their developmental phase and higher food consumption per kilogram of

Abbreviations: AMPA, aminomethylphosphonic acid; PBMCs, peripheral blood mononuclear cells; POEA, polyoxyethylene tallow amine; PMIDA, *N*-(phosphonomethyl)iminodiacetic acid

* Corresponding author.
E-mail address: bozena.bukowska@biol.uni.lodz.pl (B. Bukowska).

https://doi.org/10.1016/j.fct.2019.110888
Received 31 May 2019; Received in revised form 24 September 2019; Accepted 14 October 2019
Available online 17 October 2019

body weight in comparison to adults (Rodrigues and de Souza, 2018).

Glyphosate can also be absorbed through inhalation and dermal contact. It mainly concerns farmers spraying fields and habitants of sprayed areas (Torretta et al., 2018).

Due to good water solubility of glyphosate and its main metabolite AMPA, these substances occur in the aquatic environments and their subsequent penetration into ground and surface water may be the source of their presence in human and animal organisms (Xu et al., 2019). In general, all habitats, which are located near the sprayed fields, are exposed to glyphosate (Torretta et al., 2018).

Glyphosate is commonly found in human urine, which is associated with occupational or residential exposure and food intake. Moreover, the available data shows that mean urinary concentrations of glyphosates varies between geographical regions, with higher values found in United States (US) than in Europe, which is due to the differences in agricultural use of glyphosate-based herbicides and the popularity of glyphosate-resistant, genetically modified crops in US (Niemann et al., 2015).

Although glyphosate is not expected to bioaccumulate in living organisms due to its low log K_{OW} value, (-4.59 to -1.70) (Wang et al., 1994), its potential for bioaccumulation has been observed in invertebrates like terrestrial snails (*Helix aspersa*) fed a diet contaminated with glyphosate (Druart et al., 2011), water hyacinth (*Eichhornia crassipes*) exposed to pure glyphosate, and fish, i.e. carp (*Cyprinus carpio*) and tilapia (*Oreochromis mossambicus*) that were exposed to environmentally relevant concentrations of this substance (Wang et al., 1994).

Aminomethylphosphonic acid (AMPA) is a primary metabolite of glyphosate and amino-polyphosphonate (Daouk et al., 2013). Massive use of glyphosate leads to the ubiquity of AMPA in ecosystems, and particularly in aquatic environments (Grandcoin et al., 2017). AMPA has also been found in 45% of the soil samples originating from eleven countries and six crop systems, with the highest concentration of 2 mg kg^{-1} (Silva et al., 2018).

Glyphosate transforms to AMPA under the influence of enzymes. For this reason, this transformation occurs rapidly in soil (a significant amount of microorganisms), and for the same reason only in a minimal extent in water (a small amount of microorganisms) (Rueppel et al., 1977). In water, the half-life of glyphosate and AMPA varies from a few to 91 days, and from 76 to 240 days, respectively (Torretta et al., 2018). The half-lives of glyphosate and AMPA in soil are from 0.7 to 151 days and from 10 to 98 days, respectively, and depends mostly on soil type, pH value, clay and organic carbon content (Bai and Ogbourne, 2016).

Both *in vivo* and *in vitro* studies have demonstrated toxic potential of AMPA. It has been shown that AMPA altered cellular and biochemical parameters of the mussels *Mytilus galloprovincialis* (Matozzo et al., 2018) and caused damage to gills and liver of fish *Poecilia reticulate* (Antunes et al., 2017). Recent study of Martinez and Al-Ahmad (2019) revealed that AMPA affected the blood-brain barrier integrity and changed brain microvascular endothelial cells glucose uptake and neurons metabolic activity.

Human exposure to glyphosate calls for increased control over the use of this compound, and for in-depth studies on the effects of glyphosate and glyphosate-containing products. That is particularly important in the context of multiple reports of toxic effects of glyphosate, associated with its xenoestrogenic and cancerogenic potential (Druart et al., 2017; Zhang et al., 2019). Controversies associated with glyphosate as the world most commonly used herbicide are not weakening. The European Commission did not agree with the statement of the International Agency for Research on Cancer (IARC) on qualification of glyphosate to the 2A group, and decided to extend its usage until the end of 2022. The US Environmental Protection Agency (US EPA) is of the same opinion. In 2019, Zhang and co-workers in their meta-analysis of epidemiological data involving the most recent data of 2018 demonstrated a correlation between increased incidence of Non-Hodgkin Lymphoma (NHL) in humans and the use of glyphosate-based products. Contact with glyphosate-containing products significantly increased the risk of NHL by 41% (meta-RR = 1.41, 95% CI, confidence interval: 1.13–1.75). To support their results indicating the increased risk of NHL in individuals exposed to glyphosate-based products, the authors reviewed available studies on animals and studies on the mechanism of action of this herbicide. Additionally, Zhang et al. confirmed these findings based on studies indicating development of Non-Hodgkin Lymphoma in mice exposed to pure glyphosate as well as research works showing association between exposure of those animals to glyphosate and immunosuppressive, endocrine and genotoxic effects.

That is why, any data confirming or denying toxic effect of glyphosate (including those that may account for determination of the molecular mode of action of this herbicide) is very important. It is also necessary to assess toxic effects of metabolites and production-associated impurities of pesticides including glyphosate, considering the Regulation of the European Parliament and the Council (EC) No. 1107/2009 of 21 October 2009. Pursuant to EU requirements, studies of that kind are very necessary, for example, due to the fact that some contaminations may exhibit stronger toxicity than the active substance itself.

Apoptosis is programmed cells death that is responsible for the removal of aged or damaged cells from the body without inflammatory responses. During apoptosis, biochemical and morphological changes occurs, which leads to cell phagocytosis by macrophages or neighboring cells (Elmore, 2007). It has been shown that apoptosis of blood cells can be enhanced by xenobiotics, which may result in accelerated elimination of these cells from circulation, and finally lead to development of various disorders (Lang and Lang, 2015).

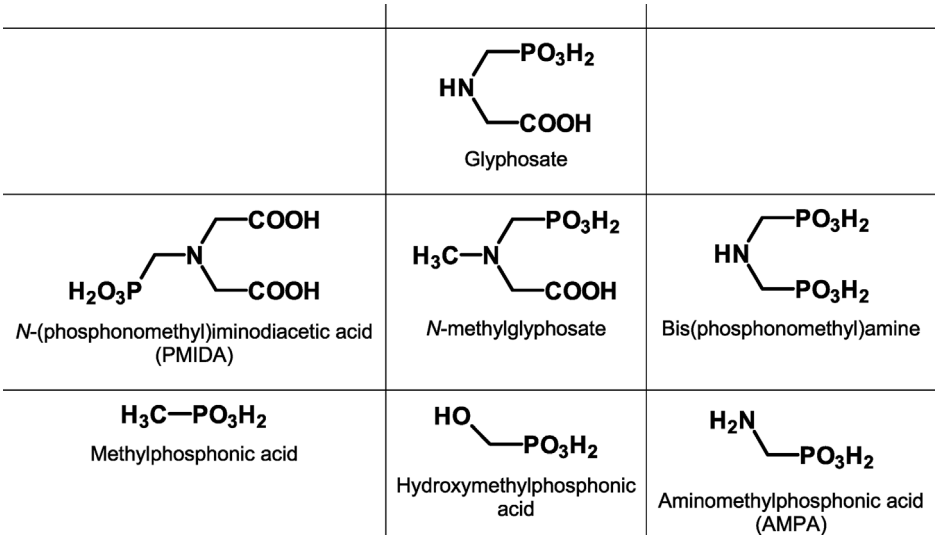
In this study, for the first time, we have investigated apoptotic potential of glyphosate, its metabolites: aminomethylphosphonic acid (AMPA), methylphosphonic acid and its impurities: PMIDA, *N*-methylglyphosate, hydroxymethylphosphonic acid and bis(phosphonomethyl)amine (Scheme 1) (described by Kwiatkowska et al., 2014, 2016) and underlying molecular mechanisms of action of these substances in human peripheral blood mononuclear cells (PBMCs). We have used low concentrations (0.01 mM) as well as high concentrations of these substances that may penetrate into the human organism only as a result of glyphosate formulation poisoning (> 0.5 mM).

2. Materials and methods

2.1. Chemicals

The investigated compounds i.e., aminomethylphosphonic acid (AMPA) (purity 98%), methylphosphonic acid (purity 98%), *N*-(phosphonomethyl)iminodiacetic acid (PMIDA) (purity 98%), *N*-methylglyphosate, hydroxymethylphosphonic acid (purity 98%) and bis-(phosphonomethyl)amine (purity 97%) were synthetized by the Institute of Industrial Organic Chemistry, Warsaw, Poland. Glyphosate [*N*-(phosphonomethyl)glycine] (purity 95%) in acid form was bought from Sigma-Aldrich, USA. The investigated compounds were dissolved in phosphate-buffered saline (pH 7.4). Other chemicals were purchased from POCh (Poland) and Roth (Germany) and were of analytical grade. Different concentrations of glyphosate have been selected in this study:

- **0.01 mM (low concentration)** that is quite similar to the concentration determined in blood of humans who were not directly exposed to this herbicide (0.435 ± 0.167 μM, Aris and Leblanc, 2011),
- **0.05 mM to 0.5 mM (moderate to high concentration)** that corresponds to the concentration that may penetrate into human blood as a result of glyphosate formulation poisoning (medium concentrations determined in blood of patients - 0.45 mM, Zouaoui et al., 2013),
- **5 mM to 10 mM (very high concentration)** that corresponds to the concentration detected in humans after acute poisoning with



Scheme 1. Chemical structure of glyphosate, its metabolites: aminomethylphosphonic acid (AMPA), methylphosphonic acid and impurities: *N*-(phosphonomethyl) iminodiacetic acid (PMIDA), N-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine.

glyphosate formulation (the range of the concentrations from 4.1 to 44.2 mM, determined in blood of patients after a suicide attempt, Zouaoui et al., 2013).

2.2. Cells isolation

PBMCs were isolated from leucocyte-buffy coat obtained from blood collected in Blood Bank in Lodz, Poland. Blood was taken from healthy, non-smoking volunteers who showed no signs of infection disease symptoms at the time the blood samples were collected. The study was approved by the Bioethics Committee of the University of Lodz No. KBBN-UŁ/I/3/2013. PBMCs were diluted with PBS (phosphate-buffered saline (NaCl 137 mmoL/L; KCl 2.7 mmoL/L; Na_2HPO_4 10 mmoL/L; KH_2PO_4 1.8 mmoL/L; pH 7.4) (1:4) and isolated using Lymphocyte Separation Medium (LSM), a mixture of Ficoll® and sodium diatrizoate (Hypaque) (density 1.077 g/ml) by centrifugation at 600*g* for 30 min at 20 °C. PBMCs 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. 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 300 *g* for 15 min. The cells were re-suspended in RPMI medium with L-glutamine, 10% FBS and penicylin-streptomycin (0.5%) and counted in haemocytometer. The final PBMCs density used in the experiments (after addition of glyphosate, its metabolites or impurities) was $2x10^6$ per 1 mL. The viability of the cells was over 95% as determined by flow cytometry.

2.3. Quantitative determination of apoptosis (YO-PRO-1/PI staining)

Apoptosis is characterized by changes in cell membrane permeability. Apoptotic cells that have altered membrane permeability (membrane integrity is maintained) are permeable for a marker YO-PRO-1 (carbocyanine nucleic acid stain), which exhibits green fluorescence, whereas they are not permeable for the dye propidium iodide (PI) that shows red fluorescence (Idziorek et al., 1995).

The cells were treated with glyphosate, its metabolites and impurities in the final concentrations ranging from 0.01 to 10 mM and incubated for 4 h at 37 °C in total darkness. Apoptosis was induced with 10 μM of camptothecin. After the incubation, the samples were centrifuged at 300 *g* for 5 min at 4 °C, the supernatant was removed, and the cells were supplemented with RPMI with L-glutamine and 10% FBS. Then, the mixture of YO-PRO-1 and PI (0.1 μM each) was added to the samples, which were incubated for 20 min on ice in total darkness. The samples were analysed by flow cytometry (LSR II, Becton Dickinson) with excitation maximum at 488 nm to visualize the YO-PRO-1 green fluorescence (520/30 bandpass filter) and PI red fluorescence (610/20 bandpass filter). FMC gate on PBMCs has been established for data acquisition and the data were recorded for a total of 10,000 events per sample.

2.4. Determination of biochemical and morphological hallmarks of apoptosis

2.4.1. Cytosolic calcium ion level

Calcium ion accumulated in mitochondria and endoplasmatic reticulum is an important secondary messenger in controlling apoptotic cell death (Pinton et al., 2008). The level of cytosolic calcium ions was analysed by flow cytometry (LSR II, Becton Dickinson) using a fluorescent probe Fluo-3/AM according to manufacturer's protocol. Fluo-3/AM passes through membrane of living cells, in which it is cleavaged by intracellular esterases to Fluo-3. Inside the cell, Fluo-3 exhibits green fluorescence after complexation with calcium ions. PBMCs treated with glyphosate, its metabolites or impurities in the final concentrations ranging from 0.01 to 10 mM were incubated for 4 h at 37 °C in total darkness. Next, the cells were centrifuged at 300 *g* for 5 min at 4 °C, suspended in Fluo-3/AM (1 μM) solution and incubated at 37 °C for 20 min in total darkness. Then, Hanks' Balanced Salt Solution (HBSS), composed of inorganic salts and supplemented with glucose (with 1% of BSA) was added to the cells suspension, and PBMCs were incubated for 40 min at 37 °C in total darkness. The cells were centrifuged at 300 *g* for 5 min at 4 °C and washed twice with HEPES buffer. After centrifugation, PBMCs were suspended in HEPES buffer and incubated for 10 min at 37 °C in total darkness. The samples were analysed using flow cytometer (LSR II, Becton Dickinson) with excitation at 490/500 nm to visualize the Fluo-3 fluorescence. FMC gate on PBMCs has been established for data acquisition and the data was recorded for a total of 10,000 events per sample.

2.5. Mitochondrial transmembrane potential ($\Delta\Psi m$)

Mitochondrial dysfunction leading to collapse of transmembrane mitochondrial potential has been shown to participate in the induction of apoptosis (Ly et al., 2003). Transmembrane mitochondrial potential was shown as red fluorescence intensity of MitoTracker Red CMXRos (excitation/emission maxima – 579/599 nm). This probe is cell

permeable and contains mildly thiol-reactive chloromethyl moiety for mitochondrial labeling. Nigericin and valinomycin (1 μM) were used to increase and decrease $\Delta\Psi m$, respectively. PBMCs were exposed to glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. Then, the samples were centrifuged at 300 *g* for 5 min at 4 °C, the supernatant was decanted, and the cells were suspended in PBS. The cells were stained with MitoTracker CMXRos in the final concentration of 1 μM for 15 min at 37 °C in total darkness, and then analysed in 96-well plates using a microplate reader (Cary Eclipse, Varian).

2.6. Caspase-8, -9 and -3 activity

Caspases are critical enzymes of apoptosis. The caspases are able to break down peptide bonds *via* cysteine residues in various substrates, and therefore they catalyze the apoptotic cell death irreversibly in mammals including human beings (Nicholson et al., 1995). Analysis of caspase-8 and -3 was executed according to the manufacturers protocols. The assays were based on the hydrolysis of the peptide substrates such as acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC) by caspase-8 and acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, which resulted in the release of the fluorescent 7-amino-4-methylcoumarin (AMC). The excitation and emission wavelengths of AMC were 360 nm and 460 nm, respectively. Caspase-9 colorimetric assay was based on hydrolysis of the substrate acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-pNA), which led to the release of p-nitroaniline (pNA) that absorbance was determined at 405 nm. Camptothecin (10 μM) was used to induce apoptosis. Detection of caspase-3 and -8 activities was executed using fluorescent microplate reader (Fluoroskan Ascent FL, Labsystem) and determination of caspase-9 activity was performed using absorbance microplate reader (BioTek ELx808, Bio-Tek).

2.7. Hoechst 33342/PI staining

Chromatin condensation paralleled by DNA fragmentation is one of the most important criteria, which are used to identify apoptotic cells (Toné et al., 2007). Morphological changes of chromatin in PBMCs were assessed by double staining with Hoechst 33342 and PI. The cells were exposed to glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. After incubation, PBMCs were centrifuged at 200 *g* for 3 min at 4 °C. The supernatant was removed, and the cells were suspended in PBS (0.5 ml). Then the mixture of 1 μl of Hoechst 33342 and 1 μl of PI (1 mg/ml each) was added. After 1 min incubation at 37 °C in total darkness, the cells were analysed by fluorescence microscope (Olympus IX70, Japan) at 400x magnification. PMBCs were classified on the basis of their morphological staining characteristics: viable (blue fluorescence), early apoptotic (intensive bright blue fluorescence), late apoptotic (blue-violet fluorescence) and necrotic (red fluorescence) (Gasiorowski et al., 2001).

2.8. Determination of reactive oxygen species level

2.8.1. Oxidation of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate

ROS play a central role in regulation of the main pathways of apoptosis mediated by mitochondria, death receptors and the endoplasmic reticulum (ER) (Redza-Dutordoir and Averill-Bates, 2003). The rate of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) oxidation was assessed by flow cytometry. 6-Carboxy-H_2DCF-DA is a compound widely used for the detection of intracellular oxidants production. The probe diffuses across cellular membrane where it is hydrolyzed by intracellular esterases to 6-carboxy-2′,7'dichlorodihydrofluorescein (6-carboxy-H_2DCF) that after oxidation, yields highly fluorescent 6-carboxy-2′,7′-dichlorofluorescein (DCF) (Bartosz, 2006). The cells were treated with glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. Next, fluorescent marker was added to PBMCs, which were stained for 15 min at 37 °C in the dark. The final concentration of the fluorescent probe was 10 μM. Positive control consisted of hydrogen peroxide (2 mM). FMC gate on PBMCs has been established for data acquisition, and fluorescence was measured with the excitation and emission maxima of 488 and 530 nm, respectively. The data was recorded for a total 10,000 events per sample.

2.9. Oxidation of hydroxyphenyl fluorosceine

Highly reactive oxygen species (mainly hydroxyl radical) were assessed using flow cytometer (Becton Dickinson, LSR II) and 3-(p-hydroxyphenyl)-fluorescein (HPF). HPF is nonfluorescent until it reacts with hydroxyl radical. As a result of oxidation, the probe exhibits bright green fluorescence (excitation/emission maxima – 490/515 nm) (Setsukinai et al., 2003). Formation of hydroxyl radical was provoked by the addition of the mixture of ferrous perchlorate(II) (0.1 mM) and hydrogen peroxide (1 mM) to PBMCs suspension. Finally, the cells were treated with HPF in the final concentration of 2 μM and incubated for 15 min at 37 °C in total darkness. The data was recorded for a total of 10,000 events per sample.

2.10. Statistical analysis

The statistical analysis was performed with STATISTICA 8 data analysis software (2000 StatSoft, Inc., Tulsa, OK, USA). In this study, one-way analysis of variance (ANOVA) (p and F added to the description of the results) with post hoc multiple comparisons procedure (Tukey test) was used to assess statistical differences in case of normal distribution (significance marked on the charts). The difference was considered to be significant for $P < 0.05$. The individual analysis was performed on blood from 4 to 5 donors, while each experiment was repeated trice.

3. Results

3.1. Apoptotic changes

After 4 h incubation, all compounds studied caused an increase in the number of apoptotic cells. Apoptotic changes were observed in PBMCs treated with 0.5 mM and 5 mM of glyphosate ($F_{6;83} = 20.42$, $P < 0.001$), PMIDA ($F_{6;61} = 14.32$, $P < 0.001$) and hydroxymethylphosphonic acid ($F_{6;80} = 25.33$, $P < 0.001$). Two glyphosate metabolites: AMPA ($F_{6,83} = 9.09$, $P < 0.001$) and methylphosphonic acid ($F_{6;82} = 4.36$, $P < 0.001$) as well as two glyphosate impurities: N-methylglyphosate ($F_{6;60} = 9.14$, $P < 0.001$) and bis-(phosphonomethyl)amine ($F_{4;82} = 13.15$; $P < 0.001$) caused apoptotic changes only at their highest concentration of 5 mM (Fig. 1).

3.2. Morphological changes of chromatin

Cell staining with Hoechst 33342 and PI allowed for the observation of early apoptotic cells (blue color), late apoptotic cells (blue-violet color) and necrotic cells (red color) in the population of PBMCs exposed to the compounds examined for 4 h. Selected photographs showing the presence of individual types of apoptotic and necrotic PMBCs are presented below (Fig. 9).

3.3. Cytosolic calcium ion level

A statistically significant increase in cytosolic calcium ion level was observed after incubation of PBMCs with glyphosate and other compounds studied (except for PMIDA) ($F_{3,32} = 1.01$, $P > 0.05$). Statistically significant changes were observed for the highest concentration (5 mM) of glyphosate ($F_{3;32} = 3.16$, $P < 0.05$), AMPA ($F_{3;32} = 3.41$, $P < 0.05$) and *N*-methylglyphosate ($F_{3;32} = 20.16$, $P < 0.001$). Glyphosate metabolite - methylphosphonic acid

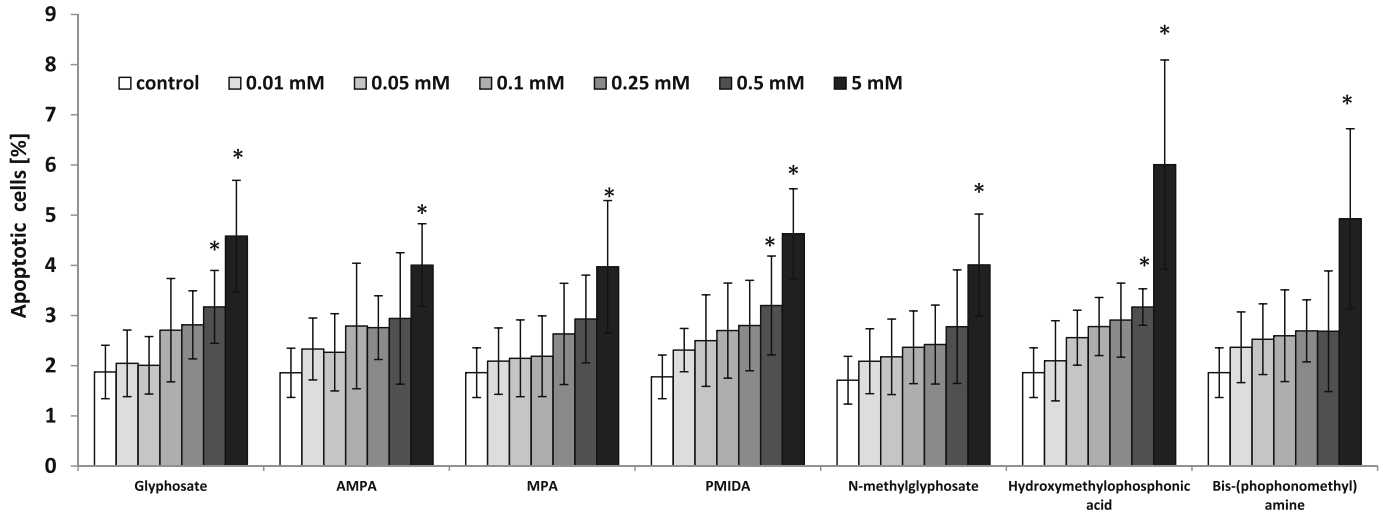


Fig. 1. Changes in the number of apoptotic PBMCs (expressed in per cent) after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to the control ($P < 0.05$).

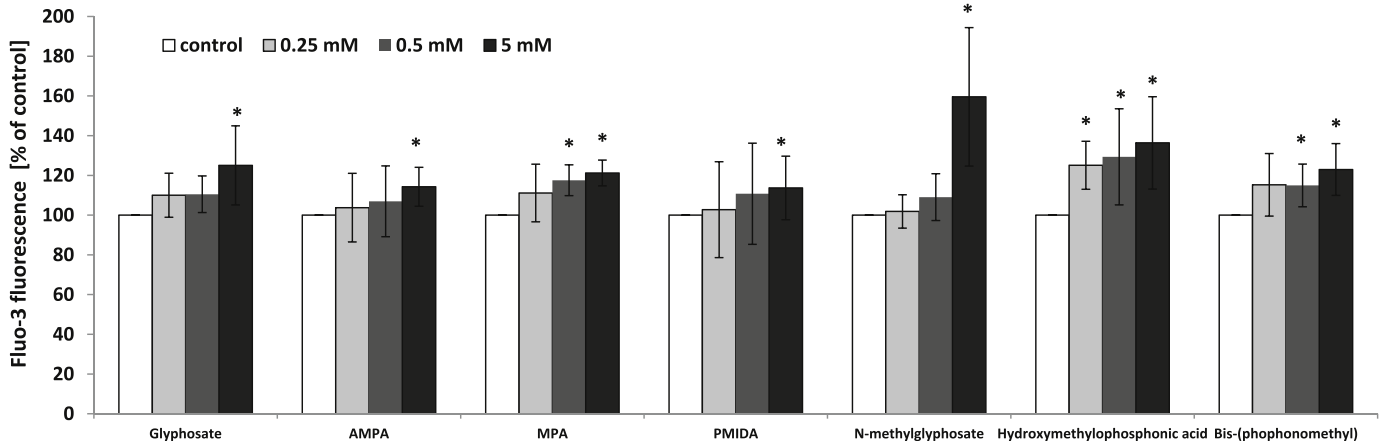


Fig. 2. Changes in cytosolic calcium ion level in control PBMCs and PBMCs incubated with glyphosate, its metabolites and impurities in the concentrations ranging from 0.25 to 5 mM for 4 h (*) Statistically significant changes compared to control ($P < 0.05$).

($F_{3;32} = 9.95$, $P < 0.001$) and glyphosate impurity - bis(phosphonomethyl)amine from the concentration of 0.5 mM ($F_{3;32} = 7.62$, $P < 0.001$) caused an increase in cytosolic calcium ion level, while hydroxymethylphosphonic acid induced changes in the parameter studied from the concentration of 0.25 mM ($F_{3;32} = 7.09$, $P < 0.001$) (Fig. 2).

3.4. Transmembrane mitochondrial potential ($\Delta\Psi m$)

Most of the compounds studied caused a decrease in transmembrane mitochondrial potential, while hydroxymethylphosphonic acid (at 0.25 mM) caused statistically significant increase in the parameter examined ($F_{4;65} = 4.13$, $P < 0.001$). Glyphosate ($F_{4;65} = 11.70$, $P < 0.001$) and one of its production impurities: bis(phosphonomethyl)amine ($F_{4;65} = 10.36$, $P < 0.001$) (from the concentration of 0.05 mM) decreased $\Delta\Psi m$. Other glyphosate metabolites: AMPA ($F_{4;65} = 10.75$, $P < 0.001$) and methylphosphonic acid ($F_{4;65} = 8.39$, $P < 0.001$), and two glyphosate impurities: PMIDA ($F_{4;65} = 16.63$, $P < 0.001$) and *N*-methylglyphosate ($F_{4;65} = 14.06$, $P < 0.001$) decreased the parameter studied from the concentration of 0.1 mM (Fig. 3).

3.5. Caspase-8 and -9 activity

Glyphosate, AMPA, PMIDA and bis-(phosphonomethyl)amine increased in caspase-8 activity. The changes were observed in PBMCs treated with 0.5 mM of glyphosate ($F_{2;\ 24} = 20.49$, $P < 0.001$) and AMPA ($F_{2;\ 24} = 19.88$, $P < 0.001$) and with the highest concentration (5 mM) of PMIDA ($F_{2;\ 24} = 6.00$, $P < 0.02$) and bis-(phosphonomethyl)amine ($F_{2;\ 24} = 3.52$, $P < 0.05$). Other compounds studied such as methylphosphonic acid ($F_{2;\ 24} = 1.27$, $P > 0.05$), *N*-methylglyphosate ($F_{2;\ 24} = 1.49$, $P > 0.05$) and hydroxymethylphosphonic acid ($F_{2;\ 24} = 0.64$, $P > 0.05$) did not cause statistically significant changes in caspase-8 activity (Fig. 4).

Glyphosate, its metabolites and impurities caused a substantial increase in caspase-9 activity. Changes in the enzyme activity were observed for the highest concentration (5 mM) of glyphosate ($F_{2;\ 24} = 19.96$, $P < 0.001$), its two metabolites: AMPA ($F_{2;\ 24} = 9.84$, $P < 0.001$) and methylphosphonic acid ($F_{2;\ 24} = 17.54$, $P < 0.001$) and all glyphosate impurities: PMIDA ($F_{2;\ 24} = 12.29$, $P < 0.001$), N-methylglyphosate ($F_{2;\ 24} = 16.20$, $P < 0.001$), hydroxymethylphosphonic acid ($F_{2;\ 24} = 35.95$, $P < 0.001$) and bis(phosphonomethyl) amine ($F_{2;\ 24} = 76.29$, $P < 0.001$) (Fig. 5).

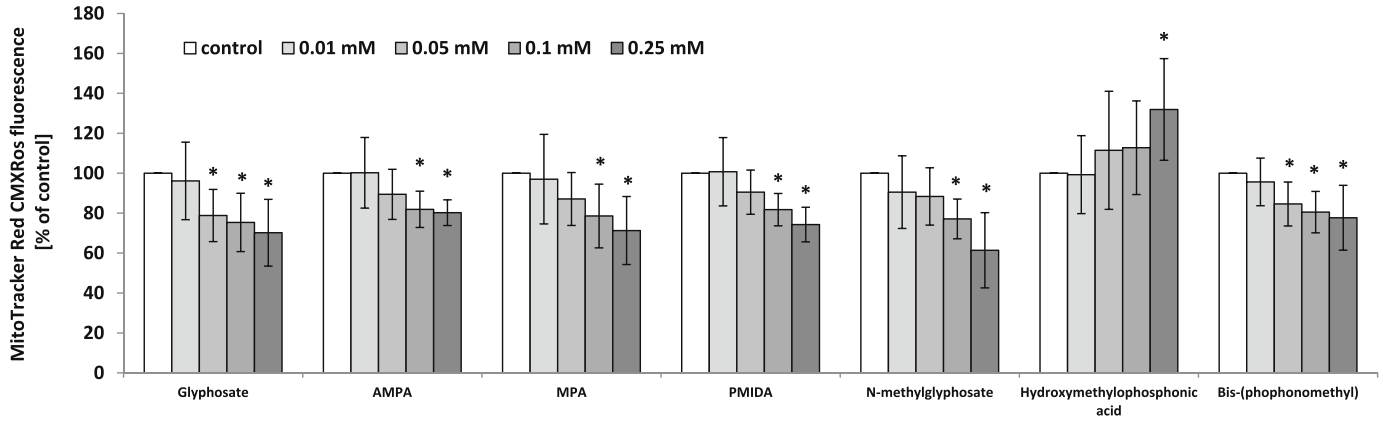


Fig. 3. Changes in transmembrane mitochondrial potential in control PBMCs and PBMCs incubated for 4 h with glyphosate, its metabolites and impurities in the concentrations ranging from 0.01 to 0.25 mM (*) Statistically significant changes compared to control ($P < 0.05$).

3.6. Caspase-3 activity

An increase in caspase-3 activity was noted in PBMCs treated with 0.25 mM of glyphosate ($F_{3;44} = 31.94$, $P < 0.001$) and PMIDA ($F_{3;44} = 10.77$, $P < 0.001$) and with 0.5 mM of AMPA ($F_{3;44} = 6.56$, $P < 0.001$), hydroxymethylphosphonic acid ($F_{3;44} = 10.14$, $P < 0.001$) and bis-(phosphonomethyl)amine ($F_{3;44} = 8.86$, $P < 0.001$). Other compounds tested: *N*-methylglyphosate ($F_{3;44} = 3.63$, $P < 0.05$) and methylphosphonic acid ($F_{3;44} = 5.18$, $P < 0.02$) only at the highest concentration of 5 mM caused an increase in caspase-3 activity (Fig. 6).

3.7. ROS level

It was observed that glyphosate, its metabolites and impurities induced a statistically significant increase in H_2DCFDA oxidation in PBMCs. Changes in ROS level were observed after 4 h of exposure of PBMCs to 0.25 mM of glyphosate ($F_{4;55} = 22.31$, $P < 0.001$), methylphosphonic acid ($F_{4;55} = 10.17$, $P < 0.001$), PMIDA ($F_{4;55} = 13.27$; $P < 0.001$), N-methylglyphosate ($F_{4;55} = 8.27$; $P < 0.001$) and hydroxymethylphosphonic acid ($F_{4;55} = 10.74$; $P < 0.001$). Other compounds studied like AMPA ($F_{4;55} = 24.29$, $P < 0.001$) and bis-(phosphonomethyl)amine ($F_{4;55} = 17.03$, $P < 0.001$) from the concentration of 0.5 mM caused an increase in the H_2DCFDA oxidation (Fig. 7).

3.8. Hydroxyl radical level

Glyphosate, its metabolites and impurities increased highly reactive oxygen species level, including hydroxyl radicals in PMBCs. Statistically significant changes were observed for most of the compounds studied from the concentration of 5 mM. An increase in HPF fluorescence was noted in cells treated for 4 h with the highest concentration (5 mM) of glyphosate ($F_{4;70} = 14.02$, $P < 0.001$), its impurities: *N*-methylglyphosate ($F_{4;70} = 39.12$; $P < 0.001$), hydroxymethylphosphonic acid ($F_{4;70} = 11.67$; $P < 0.001$) and bis-(phosphonomethyl)amine ($F_{4;70} = 88.75$, $P < 0.001$) and metabolites: AMPA ($F_{4;70} = 11.67$; $P < 0.001$) and methylphosphonic acid $F_{4;70} = 63.93$; $P < 0.001$). The strongest changes in HPF oxidation were caused by PMIDA, which changed this parameter from the concentration of 0.5 mM ($F_{4;70} = 125.08$; $P < 0.001$) (Fig. 8).

4. Discussion

Cell death - a final consequence of cellular injury, may be a result of various processes. The best known of them are necrosis and apoptosis, which have distinct roles in development of various diseases (Paduch et al., 2015). Apoptosis participates in maintenance of homeostasis of blood cells and plays a crucial role in the development and function of the immune system, erythropoiesis and maturation of T cells (Baś et al., 2004).

Apoptotic potential of glyphosate has been evaluated in various cell

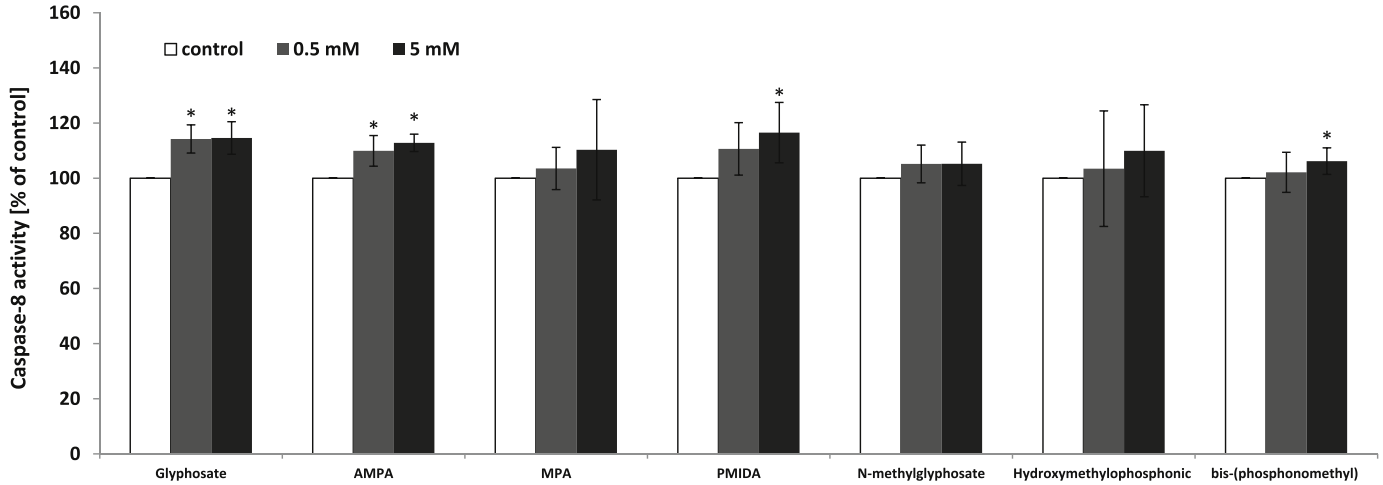


Fig. 4. Changes in caspase-8 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

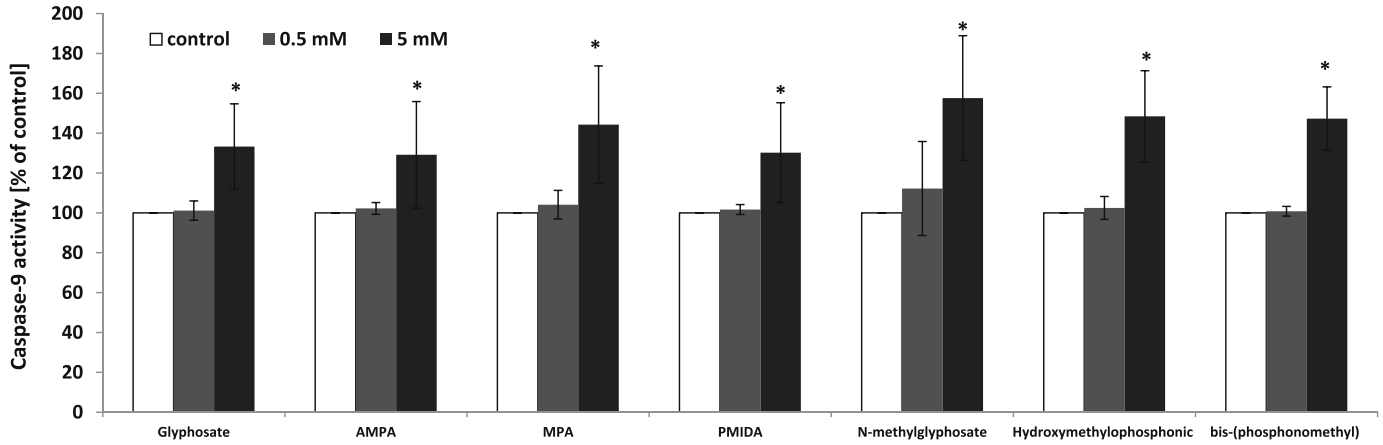


Fig. 5. Changes in caspase-9 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

types; however the effect of glyphosate, its metabolites (including AMPA and methylphosphonic acid) and its impurities on apoptotic changes in human leucocytes has not been assessed.

This study has described apoptotic potential of glyphosate, its metabolites and impurities in human PBMCs. Besides a quantitative determination of apoptotic cells (staining with YO-PRO-1/PI fluorescent probes) the analysis concerned evaluation of the mechanism of action of these substances by measurement of a variety of parameters involved in the programmed cell death. The activities of caspases, of both initiator caspase-8 and -9, as well as executor caspase-3 were determined. Alterations in cytosolic calcium ion and ROS levels were also analysed. Moreover, changes in transmembrane mitochondrial potential and chromatin condensation were assessed. PBMCs were exposed to tested compounds for 4 h, the time necessary to observe apoptotic changes (Michałowicz and Sicińska, 2009).

Flow cytometry analysis has demonstrated a statistically significant increase in the number of apoptotic cells exposed to all compounds studied. Apoptotic changes induced by glyphosate, PMIDA and hydroxymethylphosphonic acid were observed from the concentration of 0.5 mM, while those induced by AMPA, methylphosphonic acid, *N*-methylglyphosate and bis(phosphonomethyl)amine from the concentration of 5 mM.

A slight increase in the number of apoptotic cells (5.76%) treated with glyphosate was observed, compared to the control (1.22%). In another study, Martini et al. (2012) analysed induction of apoptosis by glyphosate preparation in 3T3-L1 cell line (fibroblasts) using annexin-V and PI. The authors found that a commercial product containing glyphosate at the concentration of 1.55 mM caused an increase in the number (percentage) of apoptotic cells up to 11%, compared to the control (1.6%). Kim et al. (2013) demonstrated a comparable increase in the number (11.3%) of apoptotic rat cells (line H9C2) treated with a mixture of glyphosate (10 μM) and the POEA surfactant (5 μM). Those results were obtained with much lower glyphosate concentrations compared to those of pure glyphosate (as used in our study), which indicated induction of apoptosis by glyphosate combined with a surfactant (POEA in that case). Luo et al. (2017) also assessed cytotoxicity of the glyphosate-based Roundup preparation on hepatocytes (line L-02) and showed pro-apoptotic potential of that product. The authors analysed ROS level, the activity of superoxide dismutase, permeability of the mitochondrial membrane, release of the apoptosis-inducing factor (AIF), and cytosolic Ca^{2+} level, demonstrating that Roundup reduced the activity of the antioxidative system, caused damage to mitochondria and DNA and impaired the integrity of the cellular membrane, which led to apoptosis of the cells studied.

Other researchers have also analysed this issue. Zhao et al. (2013) studied the effect of pure glyphosate on apoptosis induction of murine Sertoli cells. Sertoli cells were exposed to different concentrations of glyphosate (60–180 mg/L) for 24 h. The authors observed that glyphosate inhibited cell proliferation and induced apoptosis of Sertoli cells.

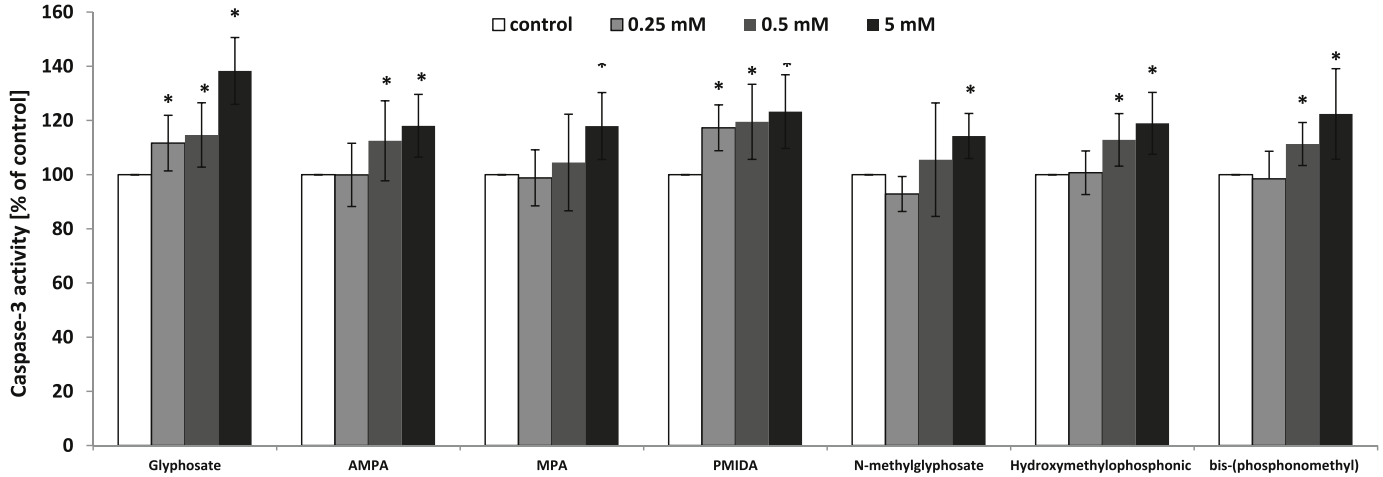


Fig. 6. Changes in caspase-3 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

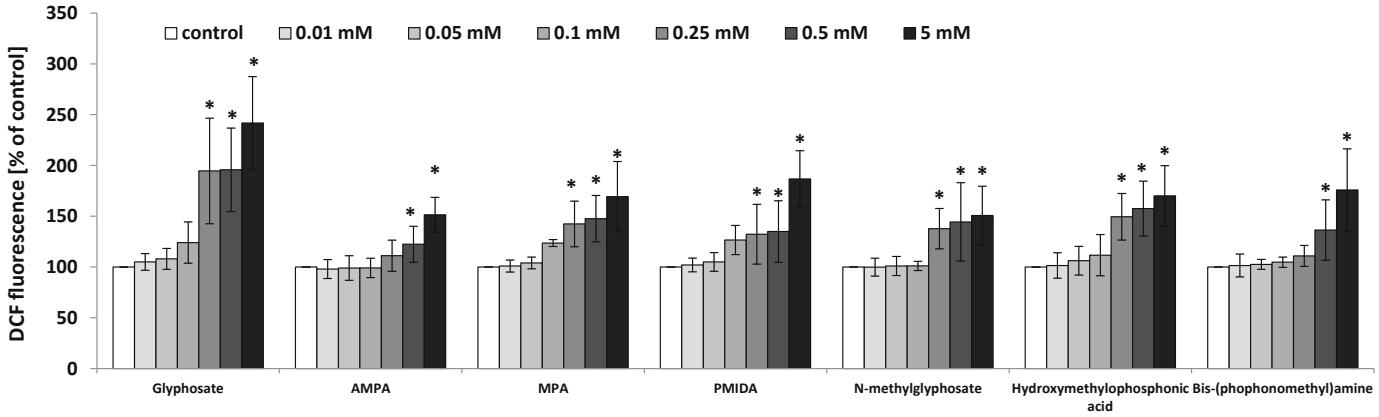


Fig. 7. Changes in total ROS level in PMBCs incubated with glyphosate, its metabolites and impurities for 4 h (*) Statistically significant changes compared to the control ($P < 0.05$).

Many research works have shown that the process of apoptosis is closely associated with changes in the cytosolic calcium ion level.

Calcium has been shown to be an important regulator of apoptosis at all its stages, from initiation to final phagocytosis of apoptotic bodies. An increase in cytosolic calcium ion level has been shown to trigger apoptosis *via* different pathways. It has been proven that excessive amount of Ca^{2+} in cytosol stimulates an increase of mitochondrial calcium uptake, which results in opening of mitochondrial permeability transition pore (MPTP) and finally causes apoptotic cell death. Calcium ions may also activate calpains, which cleave several members of BCL-2 family, including anti-apoptotic BCL-2 and BCL-2-like protein 1 (Artem Kondratskyi et al., 2015).

It was observed that methylphosphonic acid at lower concentration (0.25 mM) (in comparison to other analysed compounds) caused a statistically significant increase of cytosolic calcium ion level in PBMCs. Other compounds such as methylphosphonic acid and bis(phosphonomethyl)amine induced apoptosis from the concentration of 0.5 mM, while glyphosate, AMPA and *N*-methylglyphosate from 5 mM. It was also observed that aminomethylphosphonic acid did not cause any statistically significant increase in this parameter.

Similar results were obtained by Cavalli et al. (2013) who demonstrated a statistically significant increase of Ca^{2+} level in testis and Sertoli cells (isolated from Wistar rats) exposed to glyphosate. They demonstrated a statistically significant increase of this parameter in Sertoli cells treated with glyphosate (36 μg/ml) and indicated that this compound increased cytosolic calcium ion level by opening calcium channels.

It has been shown that uptake and accumulation of Ca^{2+} in mitochondria results in ROS production and subsequent reduction of transmembrane mitochondrial potential ($\Delta\Psi m$) (Kaufman et al., 2002). Mitochondrial dysfunction has been shown to participate in the induction of apoptosis in a number of different cell types including PBMCs and has been suggested to be central to the apoptotic pathway (Ly et al., 2003).

Our study demonstrated that all compounds analysed caused reduction of transmembrane mitochondrial potential. Glyphosate and its impurity - bis(phosphonomethyl)amine, from the concentration of 0.05 mM, caused reduction of $\Delta\Psi m$, while other compounds analysed induced the same changes from the concentration of 0.1 mM (except for hydroxymethylphosphonic acid, that caused reduction of the discussed parameter at the concentration of 0.25 mM).

Glyphosate-induced reduction of $\Delta\Psi m$ was observed by Astiz et al. (2009). The authors demonstrated that administration of pesticides, including pure glyphosate (IP 1/250 LD_{50}, 3-times a week for 5 weeks) to Wistar rats led to a reduction of $\Delta\Psi m$ and cardiolipin content in cells of the dark matter of the brain, with simultaneous increase of lipid peroxidation.

In 2005 Peixoto studied the effect of glyphosate and its commercial preparation Roundup on mitochondria isolated from rat liver. The findings of that study are opposite to the results obtained in this work because they demonstrated that glyphosate even at the concentration of 15 mM caused no reduction of $\Delta\Psi m$, whereas Roundup reduced this parameter in a concentration-dependent manner, (with the maximum effect at 10 mM). The most probably, the differences in methodology of the analysis could account for discrepancy of those results. Peixoto measured $\Delta\Psi m$ using a TPP + electrode, while our study used the fluorimetric method with the MitoTracker Red CMXRos stain.

Also Kim et al. (2013), similarly to Peixoto (2005), showed that

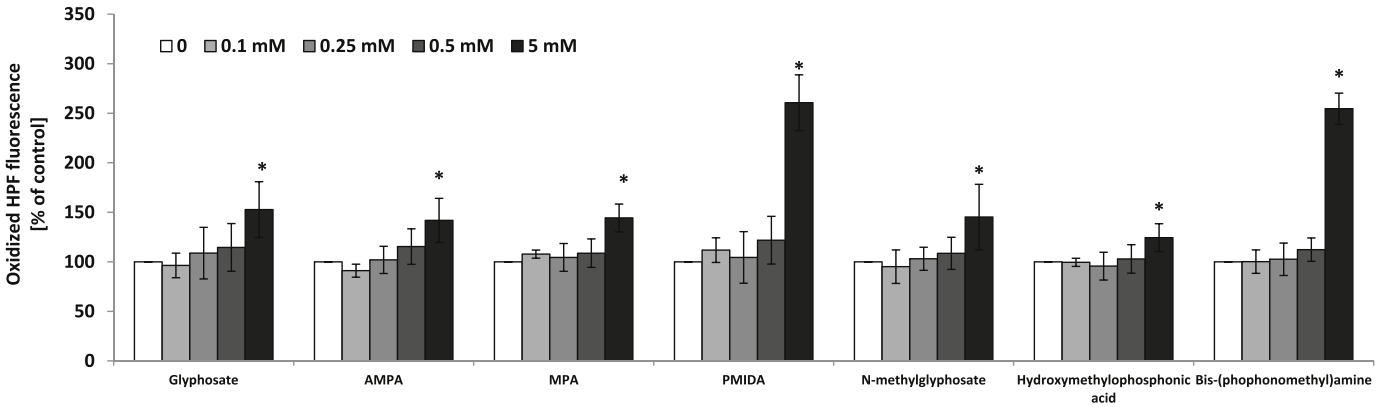


Fig. 8. The level of highly reactive oxygen species (mainly hydroxyl radical) in human PMBCs incubated with glyphosate, its metabolites and impurities for 4 h (*) Statistically significant changes compared to the control ($P < 0.05$).

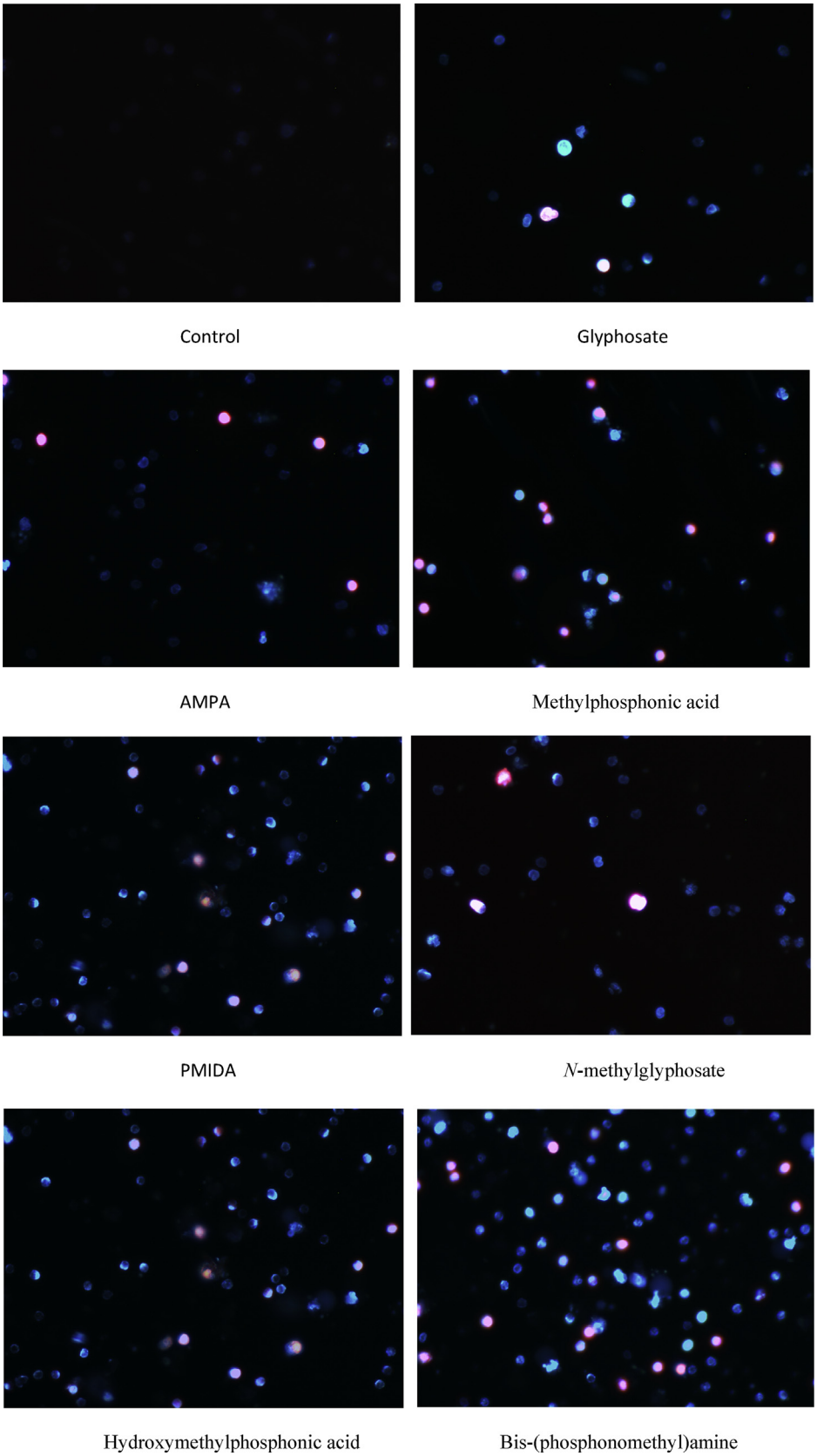


Photo 1. The representative photomicrographs of Hoechst 33324/PI-stained PBMCs pretreated with PBS (control) and with 5 mM of glyphosate, AMPA, methylphosphonic acid, PMIDA, *N*-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine. Viable cells (blue fluorescence), early apoptotic cells (intensive bright blue fluorescence), late apoptotic cells (blue-violet fluorescence) and necrotic cells (red fluorescence).

increased activity of caspases and reduction of $\Delta\Psi m$ occurred only when the cells were exposed to a mixture of the TN-20 surfactant and glyphosate (while separately both compounds caused no changes). The authors suggested that in this process TN-20 interfered with the integrity of the cellular membrane, allowing glyphosate entering the cell. Therefore, it may be stated that the TN-20 surfactant intermediates toxicity of glyphosate.

Decreased mitochondrial potential and opening of mitochondrial mega-channels causes a release of various pro-apoptotic factors including cytochrome *c*, apoptosis inducing factor (AIF), smac/DIABLO or procaspase-9 from intermembrane space into the cytosol (Ferri and Kroemer, 2001; Giorgi et al., 2012). Initiatory caspase-9, similarly to caspase-8 can activate executory caspase-3, which is crucial in apoptotic cell death induction.

It was found that glyphosate and PMIDA from the concentration of 0.25 mM caused an increase in caspase-3 activity, while AMPA, hydroxymethylphosphonic acid and bis(phosphonomethyl)amine induced changes in this parameter from the concentration of 0.5 mM. Other analysed compounds such as *N*-methylglyphosate and methylphosphonic acid only from high concentration of 5 mM increased caspase-3 activity.

Benachour and Séralini (2009) showed that glyphosate and glyphosate-based herbicides may cause activation of executor caspase-3 and -7 in human embryonic kidney 293, placental JEG3 and umbilical (HUVEC) cell lines. Umbilical cells were 60–160 times more susceptible to glyphosate compared to the embryonic 293 line and placental JEG3 line. The authors demonstrated that glyphosate and Roundup increased the activity of caspases in umbilical cells even at the concentration of 50 μg/ml. Interestingly, glyphosate induced apoptosis of umbilical cell line even at the concentrations of 500–1000 times lower than those used in agriculture. Our observations are also consistent with the study of Martini et al. (2012) who showed a substantial increase in caspase-3 activity following cell treatment with glyphosate.

Also Clair et al. (2012) studied the effect of pure glyphosate and its commercial mixture (at concentrations found in the environment: from 1 to 10000 μg/ml) on the course of apoptosis or necrosis in mammalian reproductive cells, particularly testicular cells. Glyphosate in high concentration of 5000 μg/ml caused an increase of caspase activity in embryonic cells and Leydig cells following a 48-h incubation, but in Sertoli cells it caused no increase of caspases activity. It was once again shown that the commercial product Roundup was more toxic than its active substance alone, causing an increase in caspase-3 and -7 activities in all analysed cell types, contrary to pure glyphosate.

In opposite to the studies mentioned above, Chaufan et al. (2014) did not observe any changes in caspases activities following exposure of HepG-2 cell line to pure glyphosate and AMPA. The authors also assessed the effect of Roundup UltraMax preparation (74.7% glyphosate + 25.3% adjuvants) and found a significant increase in caspase-3 and -7 activity in HepG-2 line cells. Similarly, Kim et al. (2013) did not observe changes in caspase-3, -7 and -9 activities in rat H9c2 cells treated with pure glyphosate (up to 10 μM). The authors found that only a mixture of 5 μM of glyphosate and 2.5 μM of POEA caused a significant increase in the activity of all analysed enzymes.

In order to determine the pathway on which apoptosis is induced in PBMCs, the activities of caspase-8 and -9 were assayed. Moreover, changes in the total ROS and of highly reactive oxygen species (mostly hydroxyl radical) levels were assessed. Activation of caspase-8 is associated with the induction of extrinsic apoptotic pathway by cellular receptors, while caspase-9 is involved in the activation of the intrinsic (mitochondrial) pathway.

It was found that glyphosate, AMPA (from the concentration of 0.5 mM) as well as PMIDA and bis(phosphonomethyl)amine (from the concentration of 5 mM) caused an increase in caspase-8 activity. It was also noted that other examined compounds did not increase the activity of the enzyme studied. Contrary to changes in caspase-8 activity, it was demonstrated that all analysed compounds (from the concentration of 5 mM) substantially increased the activity of caspase-9, which showed that mainly mitochondrial pathway was involved in apoptosis of human PBMCs.

At higher concentrations, ROS such as H_2O_2 have been shown to play a central role in the main pathways of apoptosis mediated by mitochondria, death receptors and the endoplasmic reticulum (ER). For instance ROS can activate caspase-8, -9 and -3 and change expression of p53, the protein playing a crucial role in apoptotic cell death (Redza-Dutordoir and Averill-Bates, 2003).

An increased ROS production was observed in PBMCs exposed with all analysed compounds. The obtained results are consistent with data published by Woźniak et al. (2018) who observed ROS formation in human PBMCs treated with glyphosate, AMPA and particularly glyphosate preparation - Roundup 360 PLUS.

Similarly, Lioi et al. (1998) suggested that glyphosate (17–170 μM) induced changes in metabolism of bovine lymphocytes, most probably by generating ROS. The authors observed a reduction of intracellular glutathione level and activation of glucose-6-phosphate dehydrogenase in those cells. In another study, Chaufan et al. (2014) using the same analytical method (oxidation of the H_2DCF probe) did not observe any increase in ROS levels in HepG2 cell line treated with glyphosate and its metabolite AMPA (900 mg/l), while they observed that the glyphosate-based preparation caused an increase in ROS level up to 140% compared to the control. The Authors of this study also determined a high level of nitrotyrosine - a substance known of its ability to alter proteins structure and function.

Hydroxyl radical is the most reactive free radical possessing a low substrate specificity (Gutowicz, 2011). It has been shown that the increase in ROS level, and hydroxyl radical in particular may contribute to DNA damage, and thus apoptotic cell death (Schuessel et al., 2005). For that reason, the formation of highly reactive oxygen species (including hydroxyl radical) was assessed in PBMCs treated with glyphosate, its metabolites and impurities. It has been noted that glyphosate, its impurities: *N*-methylglyphosate, hydroxymethylphosphonic acid and bis(phosphonomethyl)amine as well as its metabolites: AMPA and methylphosphonic acid at the concentration of 5 mM caused an increase in hydroxyl radical level. The highest increase in this parameter was noted in PBMCs exposed to PMIDA (from 0.5 mM).

Similar results were obtained by Woźniak et al. (2018). These researches observed an increase in total ROS level, and the level of highly reactive oxygen species in human PBMCs exposed to glyphosate at 1 mM, while they did not observe the formation of hydroxyl radical under influence of AMPA (up to 1 mM). Similarly, our study showed that AMPA at lower concentration did not induce hydroxyl radical formation, and the increase in discussing parameter was noted only at its highest level of 5 mM.

The proteolytic activity of caspases involves cleavage of their substrates at aspartate residues. There are at least 1000 substrates of effector caspases such as caspase-3, which are located in the cytosol, cytoskeleton and nucleus. Caspases play also a key role in the apoptotic nuclear disassembly leading to chromatin condensation (Toné et al., 2007). It has been shown in animal cells that DNA laddering and subsequent chromatin condensation is associated with irreversible proteolytic degradation of the nuclear lamins that are believed to play a role in nuclear envelope integrity and the organization of interphase chromatin (Lazebnik et al., 1995).

In this study we have observed that glyphosate, its metabolites and impurities caused chromatin condensation in human PBMCs.

Based on our own research, and also on studies of other authors it may be suggested that glyphosate, its metabolites and impurities induce apoptosis both *via* the intrinsic pathway (evidenced by the observed increase in total ROS and hydroxyl radical levels, a decrease in transmembrane mitochondrial potential and an increase in caspase-9 activity), and - to a lesser extent - *via* the extrinsic pathway (evidenced by changes in caspase-8 activity) (except for methylphosphonic acid, *N*-methylglyphosate and hydroxymethylphosphonic acid).

5. Conclusions: toxicity of pure glyphosate versus its metabolites and impurities

The results obtained in this study do not indicate an essential role of metabolites and production impurities of glyphosate in toxic (pro-apoptotic) action of glyphosate and possibly glyphosate-based preparations. Research presented in this paper indicated that both AMPA and MPA exerted a smaller effect on ROS and hydroxyl radical formation than glyphosate. For other analysed parameters, no significant differences have been demonstrated between glyphosate and its metabolites activities. Studies on toxicity of glyphosate metabolite - MPA - are even more limited, while our results clearly demonstrated a relatively low toxicity of this compound. The study also revealed that some of glyphosate impurities were characterized by a slightly stronger pro-apoptotic potential than the parent compound. They constitute, however, a minor impurities of glyphosate and should not significantly increase toxicity of *N*-(phosphonomethyl)glycine-based products (contrary to surfactants).

Obtained results clearly indicate low proapoptotic properties of all analysed compounds. Initial clear apoptotic effects are associated with their highest analysed concentrations, which correspond to the concentrations to which a human organism could be exposed only as a result of acute or subacute poisoning with glyphosate.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Polish National Science Centre (Contract-2013/11/N/NZ7/00371) and also by a Polish Ministry of Science and Higher Education and statutory research admitted for Department of Biophysics of Environmental Pollution, University of Lodz.

References

Antunes, A.M., Rocha, T.L., Pires, F.S., Alves de Freitas, M., Leite, V.R., Arana, S., Paulo César Moreira, P.C., Sabóia-Morais, S.M., 2017. Gender-specific histopathological response in guppies *Poecilia reticulate* exposed to glyphosate or its metabolite aminomethylphosphonic acid. J. Appl. Toxicol. 37, 1098–1107.

Aris, A., Leblanc, S., 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in eastern townships of Quebec, Canada. Reprod. Toxicol. 31, 528–533.

Astiz, M., de Alaniz, M.J., Marra, C.A., 2009. Effect of pesticides on cell survival in liver and brain rat tissues. Ecotoxicol. Environ. Saf. 72, 2025–2032.

Bai, S.H., Ogbourne, S.M., 2016. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ. Sci. Pollut. Control Ser. 23 (19), 18988–19001.

Bartosz, G., 2006. Use of spectroscopic probes for detection of reactive oxygen species. Clin. Chim. Acta 368, 53–76.

Baś, M., Cywińska, A., Sokołowska, J., Krzyżowska, M., 2004. Apoptoza – programowana śmierć komórki. Część III. Rola apoptozy w procesach fizjologicznych i patologicznych. Życie Weterynaryjne 79, 671–675.

Benachour, N., Séralini, G.E., 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem. Res. Toxicol. 22, 97–105.

Bøhn, T.M., Cuhra, M., Traavik, T., Sanden, M., Fagan, J., Primicerio, R., 2014. Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans. Food Chem. 153, 207–221.

Cavalli, V.L., Cattani, D., Rieg, C.E.H., Pierozan, P., Zanatta, L., Parisotto, E.B., Filho, D.W., Mena Berreto Silva, F.R., Pessoa-Pureur, R., Zamoner, A., 2013. Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. Free Radic. Biol. Med. 65, 335–346.

Chaufan, G., Coalova, I., Ríos de Molina, M., 2014. Glyphosate commercial formulation causes cytotoxicity, oxidative effects, and apoptosis on human cells: differences with its active ingredient. Int. J. Toxicol. 33, 29–38.

Clair, E., Mesange, R., Travert, C., Seralin, G., Seralini, E., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. In Vitro 26, 269–279.

Daouk, S., Copin, P.J., Rossi, L., Chevre, N., Pfeifer, H.R., 2013. Dynamics and environmental risk assessment of the herbicide glyphosate and its metabolite AMPA in a small vineyard river of the Lake Geneva catchment. Environ. Toxicol. Chem. 32 (9), 2035–2044.

Druart, C., Millet, M., Scheifler, R., Delhomme, O., de Vaufleury, A., 2011. Glyphosate and glufosinate-based herbicides: fate in soil, transfer to, and effects on land snails. J. Soils Sediments 11, 1373–1384.

Druart, C., Gimbert, F., Scheiffer, R., de Vaufleury, A., 2017. A full life-cycle bioassay with *Cantareus aspersus* shows reproductive effects of a glyphosate-based herbicide suggesting potential endocrine disruption. Environ. Pollut. 226, 240–249.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35 495–51.

Ferri, K.F., Kroemer, G., 2001. Organelle-specific initiation of cell death pathways. Nat. Cell Biol. 3, E255–E263.

Gasiorowski, K., Brokos, B., Kulma, A., Ogorzalek, A., Skorkowska, K., 2001. A comparison of the methods applied to detect apoptosis in genotoxically-damaged lymphocytes cultured in the presence of four antimutagens. Cell. Mol. Biol. Lett. 6, 141–159.

Giorgi, C., Baldassari, F., Bononi, A., Bonora, M., De Marchi, E., Marchi, S., Missiroli, S., Petergnani, S., Rimessi, A., Suski, J.M., Więckowski, M.R., Pinton, P., 2012. Mitochondrial Ca $^{(2+)}$ and apoptosis. Cell Calcium 52, 36–43.

Grandcoin, A., Piel, S., Baurès, E., 2017. Aminomethylphosphonic acid (AMPA) in natural waters: its sources, behavior and environmental fate. Water Res. 117, 187–197.

Grube, A., Donaldson, D., Kiely, T., Wu, L., 2011. Pesticides Industry Sales and Usage. US EPA, Washington, DC.

Gutowicz, M., 2011. The influence of reactive oxygen species on the central nervous system. Adv. Clin. Exp. Med. 65, 104–113.

Guyton, K.Z., Loomis, D., Grosse, Y., Ghissassi, F.E., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Mattock, H., 2015. Carcinogenicity tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. Lancet Oncol. 16, 490–491.

Idziorek, T., Estaquier, J., De Bels, F., Ameisen, F.C., 1995. YO-PRO-1 permits cytofluorimetric analysis of programmed cell death (apoptosis) without interfering with cell viability. J. Immunol. Methods 18, 249–258.

Kaufman, R.J., Scheuner, D., Schröder, M., 2002. The unfolded protein response in nutrient sensing and differentiation. Nat. Rev. Mol. Cell Biol. 3, 411–421.

Kim, Y., Hong, J., Gil, H., Song, H., Hong, S., 2013. Mixtures of glyphosate and surfactant TN20 accelerate cell death via mitochondrial damage-induced apoptosis and necrosis. Toxicol. In Vitro 27, 191–197.

Kondratskyi, A., Kondratska, K., Skryma, R., Prevarskaya, N., 2015. Ion channels in the regulation of apoptosis. Biochim. Biophys. Acta 1848, 2532–2546.

Kwiatkowska, M., Huras, B., Bukowska, B., 2014. The effect of metabolites and impurities of glyphosate on human erythrocytes (*in vitro*). Pestic. Biochem. Physiol. 109, 34–43.

Kwiatkowska, M., Jarosiewicz, P., Michałowicz, J., Koter-Michalak, M., Bukowska, B., 2016. The impact of glyphosate, its metabolites and impurities on viability, ATP level and morphological changes in human peripheral blood mononuclear cells. PLoS One. https://doi.org/10.1371/journal.pone.0156946.

Lang, E., Lang, F., 2015. Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. Semin. Cell Dev. Biol. 39, 35–42.

Lazebnik, Y.A., Takahashi, A., Moir, R.D., Goldmam, R.D., Poirier, G.G., Kaufmann, S.H., Earnshaw, W.C., 1995. Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. Proc. Natl. Acad. Sci. U.S.A. 92, 9042–9046.

Lioi, M.B., Scarfi, M.R., Santoro, A., Barbieri, R., Zeni, O., Salvemini, F., Berardino, D., Ursini, M.V., 1998. Cytogenetic damage and induction of pro-oxidant state in human lymphocytes exposed *in vitro* to glyphosate, vinclozolin, atrazine, and dpx-e9636. Environ. Mol. Mutagen. 32, 39–46.

Luo, L., Wang, F., Zhang, Y., Zeng, M., Zhong, C., Xiao, F., 2017. *In vitro* cytotoxicity assessment of roundup (glyphosate) in L-02 hepatocytes. J. Environ. Sci. Health Part B 52 (6), 1–8.

Ly, J.D., Grubb, D.R., Lawen, A., 2003. The mitochondrial membrane potential ($\Delta\psi m$) in apoptosis; an update. Apoptosis 8, 115–128.

Martinez, A., Al-Ahmad, A., 2019. Effects of glyphosate and aminomethylphosphonic acid on an isogeneic model of the human blood-brain barrier. Toxicol. Lett. 304, 39–49.

Martini, C.N., Gabrielli, M., Vila, M.C., 2012. A commercial formulation of glyphosate inhibits proliferation and differentiation to adipocytes and induces apoptosis in 3T3-L1 fibroblasts. Toxicol. In Vitro 26, 1007–1013.

Matozzo, V., Marin, M.G., Masiero, L., Tremonti, M., Biamonte, S., Viale, S., Finos, L., Lovato, G., Pastore, P., Bogialli, S., 2018. Effects of aminomethylphosphonic acid, the main breakdown product of glyphosate, on cellular and biochemical parameters of the mussel Mytilus galloprovincialis. Fish Shellfish Immunol. 83, 321–329.

Michałowicz, J., Sicińska, P., 2009. Chlorophenols and chlorocatechols induce apoptosis in human lymphocytes (*in vitro*). Toxicol. Lett. 191, 246–252.

Nicholson, D., Ali, A., Thornberry, N., Vaillancourt, J., Ding, C., Gallant, M., Gareau, Y., Griffin, P., Labelle, M., Lazebnik, Y., Munday, N., Raju, A., Smulson, M., Yamin, T.T., Yu, V., Miller, D., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37–43.

Niemann, L., Sieke, C., Pfeil, R., Solecki, R., 2015. A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers. Journal für Verbraucherschutz und Lebensmittelsicherheit 10 (1), 3–12.

Paduch, R., Klatka, M., Klatka, J., 2015. Rodzaje śmierci komórki. Pomeranian J. Life Sci. 61, 411–418.

Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61, 1115–1122.

Peruzzo, P.J., Porta, A.A., Ronco, A.E., 2008. Levels of glyphosate in surface waters, sediments and soilsassociated with direct sowing soybean cultivation in northpampasic region of Argentina. Environ. Pollut. 156, 61–66.

Pinton, P., Giorgi, C., Siviero, R., Zecchini, E., Rizzuto, R., 2008. Calcium and apoptosis:

ER-mitochondria Ca^{2+} transfer in the control of apoptosis. Oncogene 27, 6407–6418.
Redza-Dutordoir, M., Averill-Bates, D.A., 2003. Activation of apoptosis signaling pathways by reactive oxygen species. Biochim. Biophys. Acta 1863, 2977–2992.
Rodrigues, N.R., de Souza, A.P.F., 2018. Occurrence of glyphosate and AMPA residues in soy-based infant formula sold in Brazil. Food Addit. Contam. Part A Chem Anal Control Expo Risk Assess 35 (4), 723–730.
Rueppel, M.L., Brightwell, B.B., Schaefer, J., Marvel, J.T., 1977. Metabolism and degradation of glyphosate in soil and water. J. Agric. Food Chem. 25, 517–528.
Schuessel, K., Schfer, S., Bayer, T.A., Czech, Ch, Pradier, L., Franz, M., Muller-Spahn, F., Muller, W.E., Eckert, A., 2005. Impaired Cu/Zn-SOD activity contributes to increased oxidative damage in APP transgenic mice. Neurobiol. Dis. 18, 89–99.
Setsukinai, K., Urano, Y., Kakinuma, K., Majima, H.J., Nagano, T., 2003. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J. Biol. Chem. 278, 3170–3175.
Silva, V., Montanarella, L., Jones, A., Fernández-Ugalde, O., 2018. Distribution of glyphosate and aminomethylphosphonic acid (AMPA) in agricultural topsoils of the European Union. Sci. Total Environ. 621, 1352–1359.
Toné, S., Sugimoto, K., Tanda, K., Suda, T., Uehira, K., Kanouchi, H., Samejima, K., Minatogawa, Y., Earnshaw, W.C., 2007. Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis. Exp. Cell Res. 313, 3635–3644.
Torretta, V., Katsoyiannis, I.A., Viotti, P., Rada, E.C., 2018. Critical review of the effects of glyphosate exposure to the environment and humans through the food supply chain. Sustainability 10 (4), 950.
Wang, Y.S., Yen, J.H., Hsieh, Y.N., Chen, Y.L., 1994. Dissipation of 2,4-D, glyphosate and paraquat in river water. Water, Air, Soil Pollut. 72, 1–7.
Woźniak, E., Sicińska, P., Michałowicz, J., Woźniak, K., Reszka, E., Huras, B., Zakrzewski, J., Bukowska, B., 2018. The mechanism of DNA damage induced by Roundup 360 PLUS, glyphosate and AMPA in human peripheral blood mononuclear cells – genotoxic risk assessment. Food Chem. Toxicol. 120, 510–522.
Xu, J., Smith, S., Smith, G., Wang, W., Li, Y., 2019. Glyphosate contamination in grains and foods: an overview. Food Control 106 106710.
Zhang, L., Rana, I., Shaffer, R.M., Taioli, E., Sheppard, L., 2019. Exposure to glyphosate-based herbicides and risk for non-hodgkin lymphoma: a meta-analysis and supporting evidence. Mutat. Res. Rev. Mutat. Res. https://doi.org/10.1016/j.mrrev.2019.02.001.
Zhao, W., Yu, H., Zhang, J., Shu, L., 2013. Effects of glyphosate on apoptosis and expressions of androgen-binding protein and vimentin mRNA in mouse Sertoli cells. Nan Fang Yi Ke Da Xue Xue Bao 33, 1709–1713.
Zouaoui, K., Dulaurent, S., Gaulier, J.M., Moesch, C., Lachâtre, G., 2013. Determination of glyphosate and AMPA in blood and urine from humans: about 13 cases of acute intoxication. Forensic Sci. Int. 226 (1), 20–25.