In vitro genomic damage caused by glyphosate and its metabolite AMPA

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HIGHLIGHTS

- Glyphosate is the most used herbicide worldwide, with genotoxic properties.
- AMPA, the main metabolite of glyphosate degradation, is ubiquitous in ecosystems.
- Genomic damage caused by glyphosate and AMPA was evaluated by micronucleus assay.
- Both Glyphosate and AMPA showed genotoxic and cytotoxic properties.
- There are evidences of a possible synergistic action of these two compounds.

GRAPHICAL ABSTRACT
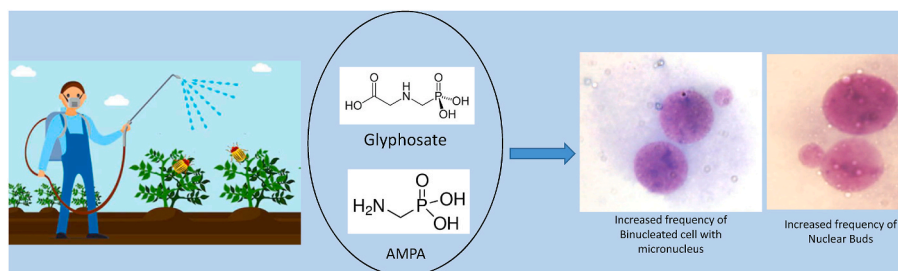
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ABSTRACT

Glyphosate is the most widely used systemic herbicide. There is ample scientific literature on the effects of this compound and its metabolite aminomethylphosphonic acid (AMPA), whereas their possible combined genotoxic action has not yet been studied. With the present study, we aimed to determine the level of genomic damage caused by glyphosate and AMPA in cultured human lymphocytes and to investigate the possible genotoxic action when both compounds were present at the same concentrations in the cultures. We used a micronuclei assay to test the genotoxicity of glyphosate and AMPA at six concentrations (0.0125, 0.025, 0.050, 0.100, 0.250, 0.500 μg/mL), which are more realistic than the highest concentrations used in previous published studies. Our data showed an increase in micronuclei frequency after treatment with both glyphosate and AMPA starting from 0.050 μg/mL up to 0.500 μg/mL. Similarly, a genomic damage was observed also in the cultures treated with the same concentrations of both compounds, except for exposure to 0.0065 and 0.0125 μg/mL. No synergistic action was observed. Finally, a significant increase in apoptotic cells was observed in cultures treated with the highest concentration of tested xenobiotics, while a significant increase in necrotic cells was observed also at the concentration of 0.250 μg/mL of both glyphosate and AMPA alone and in combination (0.125 + 0.125 μg/mL). Results of our study indicate that both glyphosate and its metabolite AMPA are able to cause genomic damage in human lymphocyte cultures, both alone and when present in equal concentrations.

1. Introduction

More and more chemicals are being released into the environment to kill unwanted insects and plants and to increase the yield of agricultural products (Landrigan et al., 2018). Because of this extensive use, residual concentrations of pesticides and their metabolites are found ubiquitously, with negative impacts on human health and ecosystem services (Tresnakova et al., 2021).

Glyphosate (GLY, N-phosphonomethylglycine) is a non-selective, systemic herbicide widely used in agriculture, forestry, parks, and home gardens (Ferreira de Souza et al., 2020). Currently, it is the most commonly used herbicide worldwide, exposing human populations both occupationally and environmentally to its action (for a review, see Talyn et al., 2023). Highly water soluble, it has been detected in all ecosystems (Van Bruggen et al., 2018; Connolly et al., 2020): its residues have been reported to occur in foods (Bai and Ogbourne, 2016), fruits and vegetables (Chen et al., 2013), drinking and surface water (Osten and Dzul-Caamal, 2017), and in sediments (Koskinen et al., 2016).

From an eco-toxicological perspective, glyphosate can affect all biota (Meftaul et al., 2020) and results toxic also to non-target organisms such as phytoplankton (Hernández-García and Martínez-Jerónimo, 2020), fish (Lugowska, 2018), amphibians (Howe et al., 2004; Moore et al., 2012), reptiles (Carpenter et al., 2016), and invertebrates (Santovito et al., 2020; Schleicherová et al., 2023). Its toxicity manifests in mortality, carcinogenicity, reproductive and neurological toxicity. A recent study found that glyphosate toxicity also affects animal behaviour in model systems and in agricultural and environmentally relevant contexts (Talyn et al., 2023).

Moreover, glyphosate has been detected in human biological fluids and in people not directly exposed (Zouaoui et al., 2013; Faniband et al., 2021), resulting in DNA damage, altered reproduction, neurological diseases, and cancer (Mink et al., 2012; Mesnage et al., 2015; Santovito et al., 2018). In particular, glyphosate has been found at a concentration between 1.35 μg/L and 233 μg/L, on average, in farmers and horticulturists (Acquavella et al., 2004; Connolly et al., 2020); the highest urinary concentrations ever reported for occupational exposure (median 292 μg/L; maximum 17.2 mg/L) were detected in a sample of Chinese pesticide production workers (Zhang et al., 2020), while high urinary concentrations were found also in non-farming families probably in relation to residential use of glyphosate-based pesticide products (Curwin et al., 2007).

In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as "probably carcinogenic to humans" (Group 2A), whereas European and U.S. agencies considered it as non-carcinogenic to humans (European Food Safety Authority (EFSA), 2015; United States Environmental Protection Agency (US EPA), 2016; European Chemical Agency (ECHA), 2017; European Chemical Agency (ECHA), 2022). This discrepancy in classification probably derives from ambiguities surrounding its possible adverse health effects, as well as from the broad variability of *in vivo* and *in vitro* used systems (for a review, see Connolly et al., 2020).

The main metabolite of glyphosate degradation is aminomethylphosphonic acid (AMPA); given the widespread use of glyphosate, AMPA is ubiquitous in ecosystems (Grandcoin et al., 2017), and because it has a longer soil half life than glyphosate, it is 3-6-fold times more persistent (Sun et al., 2019; Tresnakova et al., 2021). Like glyphosate, AMPA can be detected in agricultural areas (Qiao et al., 2020), sediments (Ronco et al., 2016), and surface and ground water (Coupe et al., 2012; Van Stempvoort et al., 2016). Its toxicity has been described for fish (de Brito Rodrigues et al., 2019) and aquatic invertebrates (Matozzo et al., 2018), whereas its toxicity for amphibians has been measured only at concentrations >500 μg/L (Domínguez et al., 2016). AMPA residues have been found in foods, plants, and soil. Since it can leach into watercourses, humans and animals are directly exposed via ingestion of contaminated food and water (Tresnakova et al., 2021).

Both *in vivo* and *in vitro* studies have demonstrated the potential toxicity of AMPA. It can alter the cellular and the biochemical activity of the mussel *Mytilus galloprovincialis* (Matozzo et al., 2018), induce damage to the gills and the liver of *Poecilia reticulata* (Antunes et al., 2017), and alter neuronal metabolic activity and glucose metabolism *in vitro* (Martinez and Al-Ahmad, 2019). Its genotoxicity is controversial and has been poorly investigated to date. It was found to induce mild toxic effects on cultured erythrocytes; clastogenic effects on lymphocytes were observed starting at a concentration of 1.8 mM (Kwiatkowska et al., 2014).

Previous work conducted in the United States, Mexico, Colombia, Germany, Ireland, and Denmark reported urinary AMPA levels within the same concentration range as glyphosate (for a review, see Connolly et al., 2000). Given the ubiquity of AMPA in the environment and the widespread use of the parent compounds (glyphosate and aminopolyphoshonate), it can be assumed that both glyphosate and AMPA pose a potential risk to aquatic and terrestrial organisms. Therefore, it is important to evaluate exposure to AMPA and to glyphosate since AMPA shares a similar toxicological profile with glyphosate (Connolly et al., 2020; Tresnakova et al., 2021; Connolly et al., 2022).

Finally, most previous studies report the genotoxicological effects of single xenobiotics, while the effects of mixtures of compounds have been understudied. This is true also for glyphosate and AMPA: there is abundant literature on the effects of these compounds analysed alone (Grandcoin et al., 2017; de Castilhos Ghisi et al., 2020), whereas no studies to date have investigated their combined genotoxic action. To fill this gap, in the present study we measured the level of genomic damage induced by glyphosate and AMPA separately in cultured lymphocytes and investigated their possible combined genotoxic action when present at the same concentrations as when tested separately.

Our hypothesis is that the known genotoxic properties of glyphosate and AMPA might interact synergistically, causing higher genomic damage and cytotoxicity when both compounds are present together than when they are tested separately.

2. Materials and methods

2.1. Chemicals and reagents

The IUPAC terms for glyphosate (C$_3$H$_8$NO$_5$P, CAS no. 1071-83-6) and AMPA (NH$_2$CH$_2$P(O) (OH)$_2$, CAS no. 1066-51-9) are: N-(phosphonomethyl)glycine and (aminomethyl)phosphonic acid, respectively. Glyphosate and AMPA (Sigma-Aldrich, Milan, Italy) were dissolved in ultrapure water to a final concentration of 0.5 mg/mL (stock solution) and kept at 4 °C until preparation of the final solutions in culture medium.

Gibco RPMI 1640 cell culture media, foetal calf serum, phytohemagglutinin (PHA), and antibiotics were purchased from Invitrogen-Life Technologies, Milan, Italy. Cytochalasin-B, colchicine and mitomycin-C (MMC) were obtained from Sigma-Aldrich. Methanol, acetic acid, Giemsa stain solution, and conventional microscope slides were purchased from Carlo Erba Reagenti, Milan, Italy. Potassium chloride (KCl) and Sörensen buffer were obtained from Merck S.p.A., Milan, Italy. Vacutainer blood collection tubes were from Terumo Europe, Rome, Italy. Ultrapure water was used throughout all experiments.

2.2. Lymphocyte culture

Peripheral venous blood was collected from 20 healthy Italian adults (mean age ± SD, 24.000 ± 2.248), with no current tobacco and alcohol use, not under drug therapy, and no recent history of exposure to mutagens. Signed informed consent was obtained from all subjects. The study was approved by the Ethics Committee, University of Turin (protocol no. 0574348, dated 10-18-2023) and performed in accordance with the ethical standards stated in the 2013 Declaration of Helsinki.

Blood sample collection, lymphocyte cultures, fixation, and staining procedures, micronuclei assay, and microscope analysis were performed

as described in Santovito et al. (2018). Lymphocyte cultures were treated at six nominal concentrations (0.0125, 0.025, 0.050, 0.100, 0.250, 0.500 μg/mL) separately for glyphosate and AMPA, then combined glyphosate + AMPA at the same nominal concentrations (0.0065 + 0.00625, 0.0125 + 0.0125, 0.025 + 0.025, 0.050 + 0.050, 0.125 + 0.125, 0.250 + 0.250 μg/mL, respectively). The positive control was treatment with mitomycin-C (Mit.C) at 0.100 μg/mL. In particular, 0.500 μg/mL represents the human Acceptable Daily Intake (ADI) concentration established by EFSA (2015) for these compounds, whereas 0.250, 0.100, 0.050, 0.025 and 0.0125 μg/mL are submultiple concentrations selected to evaluate a genotoxicity threshold of these compounds.

2.3. Cytokinesis-block micronucleus assay

Genomic damage was measured by means of micronuclei (MNi) assay, a fast and inexpensive test that can detect the clastogenic and the aneugenic properties of a single chemical or a mixture of compounds (Santovito et al., 2018). MNi are whole chromosomes or chromosome fragments that fail to migrate to anaphase during mitosis, thus resulting visible as extranuclear bodies in interphase nuclei. The MNi assay also consents to evaluate the frequency of nuclear buds (NBUDs), which represent the elimination of amplified DNA or excess chromosomes from

aneuploidy cells (Santovito et al., 2018).

The frequency of micronuclei (MNi) and the number of nuclear buds (NBUDs) were measured in 1000 binucleated lymphocytes with well-preserved cytoplasm per subject, per concentration and per tested compound, for a total of 480,000 scored cells. Similarly, a total of 1000 lymphocytes per donor per concentration were scored to determine the cytokinesis-block proliferation index (CBPI), according to the formula described in Santovito et al. (2018).

2.4. Statistical analysis

Data distribution and normality were tested both graphically and with the Shapiro-Wilk test.

The distribution of both the four nuclear aberrations and the CBPI values was not normal and was not similar among the groups, even after data transformation. Since data consisted of repeated measures on the same subjects, we applied the Friedman test (R package *tidyverse*, function *friedman.test*) and the Conover post hoc test (R package *PMCMRplus*, function *frdAllPairsConoverTest*) to compare the number of aberrations and the CBPI values among the different groups, subjected to different concentrations and compounds.

Statistical analyses were performed using R 4.3.2 (R core team, Vienna, Austria) with the Rstudio interface (RStudio Team, Boston, MA,
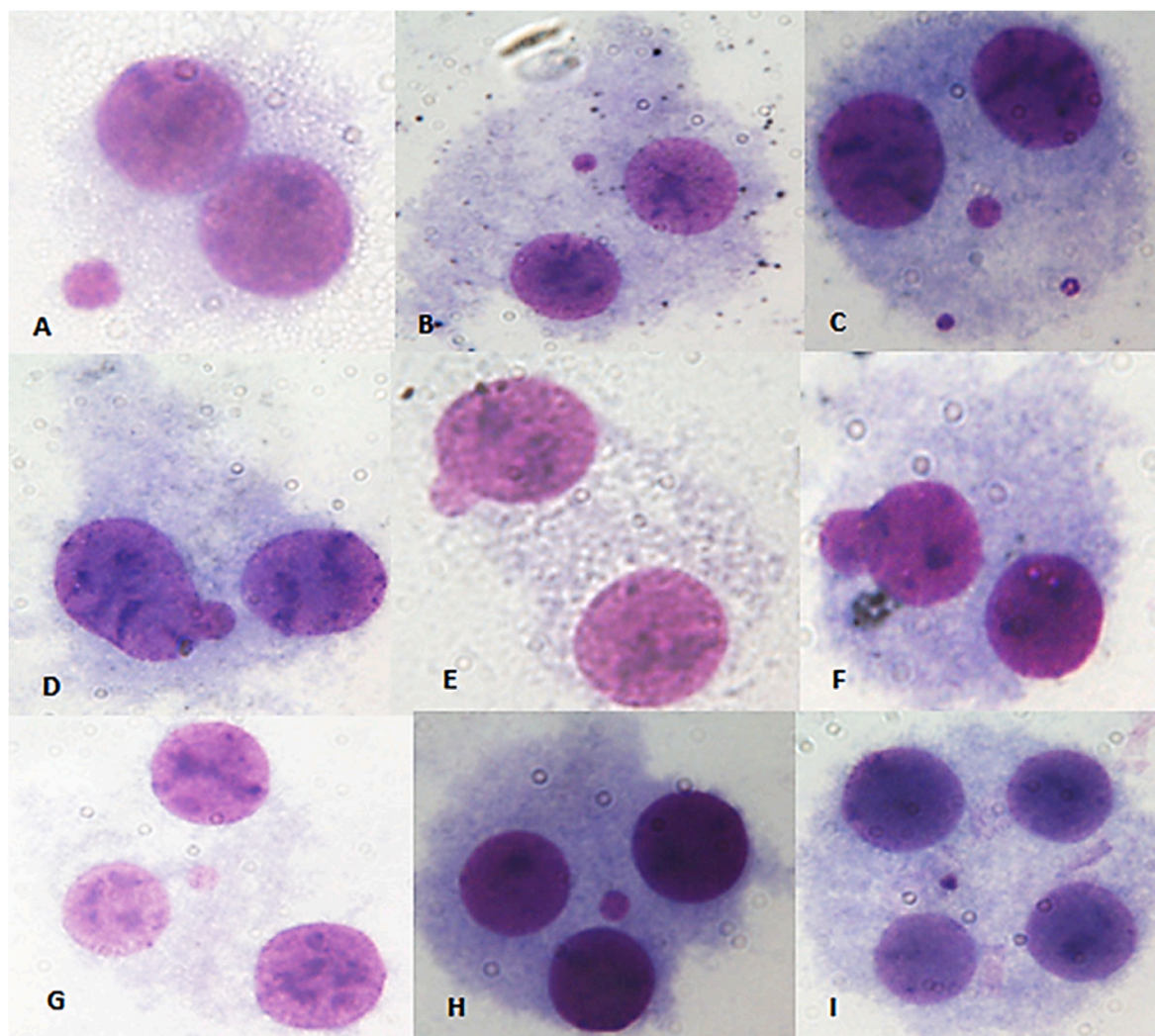
Fig. 1. Micronuclei (A–C), nuclear buds (D–F), tri-nucleated cells with micronuclei (G–H), and tetra-nucleated cell with micronucleus (I) after exposure to the highest concentration of glyphosate and AMPA (1000× magnification, Leitz Dialux 20, Germany). According to standardized procedures, the micronuclei of tri- and tetra-nucleated cells were not scored in the evaluation of total genomic damage.

USA), while graphs were created with GraphPad Prism 8 (GraphPad Software, https://www.graphpad.com, accessed on June 26, 2024)

3. Results

Examples of binucleated cells with MNi and NBUDs in cultures treated with glyphosate and AMPA are shown in Fig. 1. Tables presenting MNi, NBUDs, apoptotic and necrotic cells frequencies are given in Supplementary Material 1.

3.1. Glyphosate

Compared to negative control, the frequency of MNi was significantly higher in lymphocyte cultures exposed to mitomycin C (positive control) and to glyphosate at concentrations of 0.050, 0.100, 0.250, and 0.500 μg/mL, whereas no significant differences in MNi frequency were observed between negative control and lymphocyte cultures exposed to the concentrations of 0.0125 and 0.025 μg/mL of glyphosate (Conover's post-hoc test) (Fig. 2). There was a significant difference in the number of NBUDs only at the highest concentration (0.500 μg/mL) and for the positive control, compared with the negative control (Conover's post-hoc test) (Fig. 2). Finally, the CBPI was significantly lower at the concentrations of 0.100, 0.250 and 0.500 μg/mL and for the positive control compared with the negative control (Conover's post-hoc test) (Fig. 5).

3.2. AMPA

The frequency of MNi was significantly higher in lymphocyte cultures exposed to mitomycin C (positive control) and AMPA at the concentrations of 0.050, 0.100, 0.250, 0.500 μg/mL than the negative control, whereas there was no significant difference in MNi frequency between lymphocyte cultures exposed to 0.0125 and 0.025 μg/mL. *Vice*

versa, no significant differences were found in the frequency of NBUDs at all tested concentrations of AMPA, compared with the negative control (Conover's post-hoc test) (Fig. 3). The CBPI was significantly lower at the three highest concentration (0.500, 0.250 and 0.100 μg/mL) and for the positive control compared with the negative control (Conover's post-hoc test) (Fig. 5).
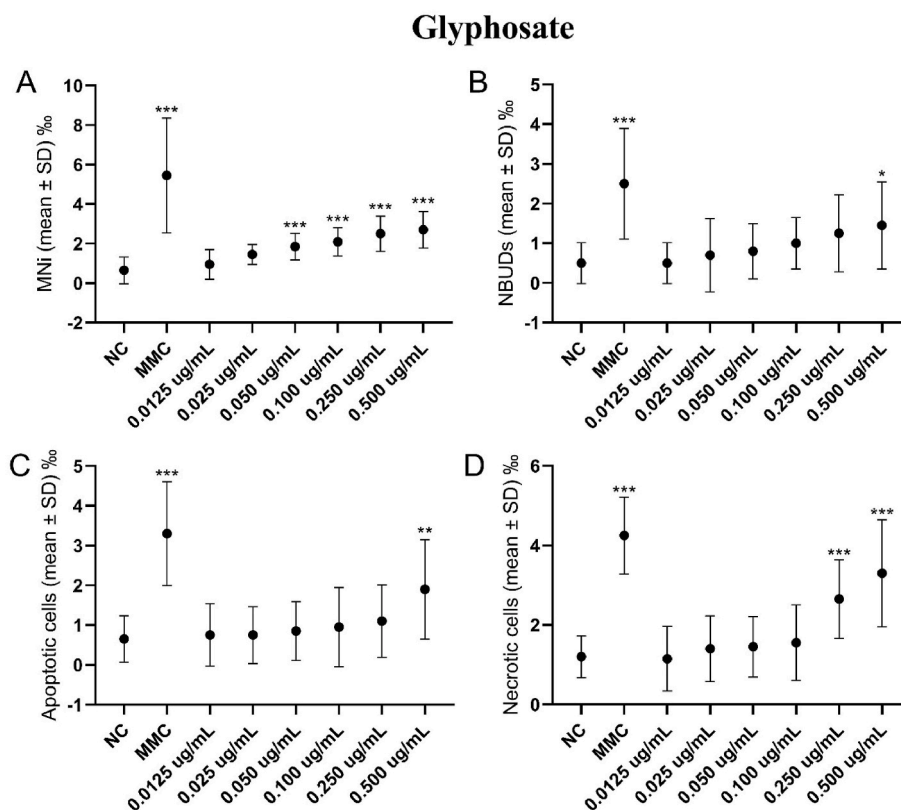
3.3. Glyphosate + AMPA

The MNi frequency was significantly higher in the lymphocyte cultures exposed to mitomycin C (positive control) and to the concentrations of 0.025 + 0.025, 0.050 + 0.050, 0.125 + 0.125, 0.250 + 0.250 μg/mL of both compounds, than the negative control, whereas there was no significant difference in micronuclei between lymphocyte exposed to the lower concentrations (0.0065 + 0.0065 μg/mL and 0.0125 + 0.0125 μg/mL) compared with the negative control (Conover's post-hoc test) (Fig. 4).

No significant differences were found in the frequency of NBUDs at all tested concentrations of the mix Glyphosate + AMPA, compared with the negative control (Conover's post-hoc test) (Fig. 4). The CBPI was significantly lower at the highest concentrations (0.125 + 0.125 and 0.250 + 0.250 μg/mL) and for the positive control compared with the negative control (Conover's post-hoc test) (Fig. 5).

Finally, when we compared the level of genomic between the mix of glyphosate + AMPA and glyphosate and AMPA alone, no synergistic action was observed (Conover's post-hoc test, $p > 0.05$).

3.4. Apoptotic and necrotic cells

The frequency of apoptotic cells was significantly higher in lymphocyte cultures exposed to mitomycin C (positive control, $p < 0.01$), to the highest concentration (0.500 μg/mL, $p < 0.01$) of both

Glyphosate

Fig. 2. Box plots of micronuclei (MNi) (A), nuclear buds (NBUDs) (B), apoptotic and necrotic cells (C and D, respectively), expressed as mean ± standard deviation (SD) ‰ in lymphocytes exposed to glyphosate. Asterisks indicate significant differences according to Dunnett's multiple comparison test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). NC = negative control; MMC = mitomycin C (positive control).
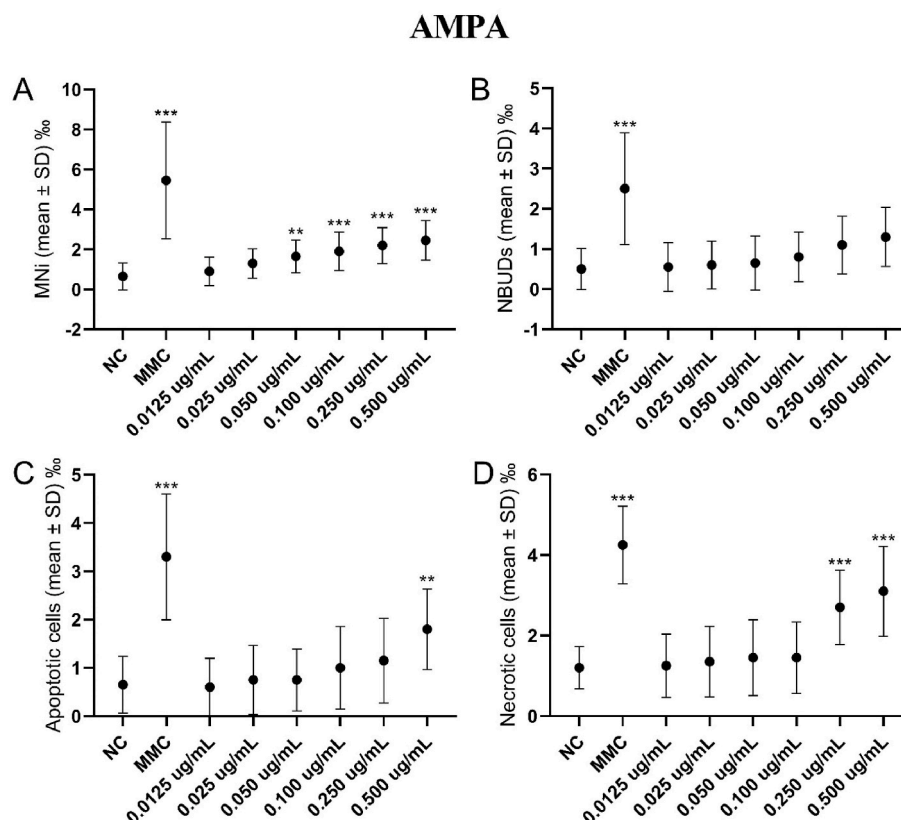
AMPA

Fig. 3. Box plots of micronuclei (MNi) (A), nuclear buds (NBUDs) (B), apoptotic and necrotic cells (C and D, respectively), expressed as mean $\pm$ standard deviation (SD) ‰ in lymphocytes exposed to AMPA. Asterisks indicate significant differences according to Dunnett's multiple comparison test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). NC = negative control; MMC = mitomycin C (positive control).

glyphosate and AMPA, and the highest concentration (0.250 + 0.250 μg/mL, $p < 0.001$) of glyphosate + AMPA compared to the negative control (Conover's post-hoc test) (Figs. 2–4).

Finally, the frequency of necrotic cells was significantly higher in lymphocyte cultures exposed to mitomycin C (positive control, $p < 0.001$) and to the two highest concentrations of glyphosate (0.250 and 0.500 μg/mL, $p < 0.001$), AMPA (0.250 and 0.500 μg/mL, $p < 0.001$), and glyphosate + AMPA (0.125 + 0.125 and 0.250 + 0.250 μg/mL, $p < 0.001$) compared to the negative control (Conover's post-hoc test) (Figs. 2–4).

4. Discussion

The massive use of non-specific systemic herbicides poses a serious environmental problem. Due to their non-specific action, these chemicals can harm non-target organisms and reduce biodiversity (Tresnakova et al., 2021; Connolly et al., 2022).

In this study, we evaluated for the first time the genotoxic potential of glyphosate and its metabolite AMPA alone and combined. In agreement with our previously published work (Santovito et al., 2018), glyphosate showed genotoxicity from the concentration of 0.050 μg/mL up to the ADI value of 0.500 μg/mL. Similarly, Khan et al. (2021) noted genotoxicity, as evaluated by comet assay, of glyphosate in human blood cells at concentrations from 0.1 to 50 mM (about 17 μg/mL to 8.4 x 10³ μg/mL). Alvarez-Moya et al., 2022; Alvarez-Moya and Reynoso-Silva, 2023 reported on the genotoxic effect of glyphosate in human blood cells within this range and at the lower concentration of 0.0007 mM (corresponding to 0.12 μg/mL).

The importance of our findings resides in our having tested glyphosate at concentrations comparable to those used by other studies in different environmental matrices. For example, in surface waters of the

southern Ontario (Canada), glyphosate was detected at a concentration of about 0.040 μg/mL (Struger et al., 2008, 2015). Concentrations ranging from 0.10 to 0.70 μg/mL were detected in water samples from a transgenic soybean cultivation area located in the north of the Province of Buenos Aires (Argentina) (Peruzzo et al., 2008). In Italy, the average use of glyphosate is over 1000 tons/year. It has been detected at 39.7% of surface water monitoring points at concentrations above the limits of environmental water quality standards in some cases (Matozzo et al., 2018). This means that humans are directly and indirectly exposed, as suggested by Connolly et al. (2022) who performed a human biomonitoring study assessing glyphosate and AMPA exposure in farming and non-farming families. The study findings suggested potential exposure from residential co-exposure or living with a pesticide user.

AMPA shares similar genotoxic properties with glyphosate and can induce genomic damage starting at a concentration of 0.050 μg/mL. Since AMPA is the primary metabolite of glyphosate, our results hold relevance for public health. Regulatory agencies have declared that AMPA is of no toxicological concern and so do not include it in risk assessments. However, these statements are based on old studies that produced negative results, such as those conducted on *Salmonella typhimurium* (Shirasu, 1980), and on rat hepatocytes (Bakke, 1991). More recently, despite the lack of epidemiologic data, human cell-based studies provide evidence that AMPA is potentially genotoxic and may exert toxic effects also at low concentrations (International Agency for Research on Cancer (IARC) Working Group, 2015). In their study on human lymphocyte cultures, Mañas et al. (2009) reported on the clastogenic properties of AMPA (e.g., increased frequency of chromosomal aberration starting at 1.8 mM, corresponding to 199.87 μg/mL), as well as increased frequency of micronuclei in fish at 200 and 400 mg/kg (corresponding to 200 and 400 μg/mL), which are hundred times higher than the concentrations we tested (Mañas et al., 2009). Kwiatkowska

Glyphosate + AMPA
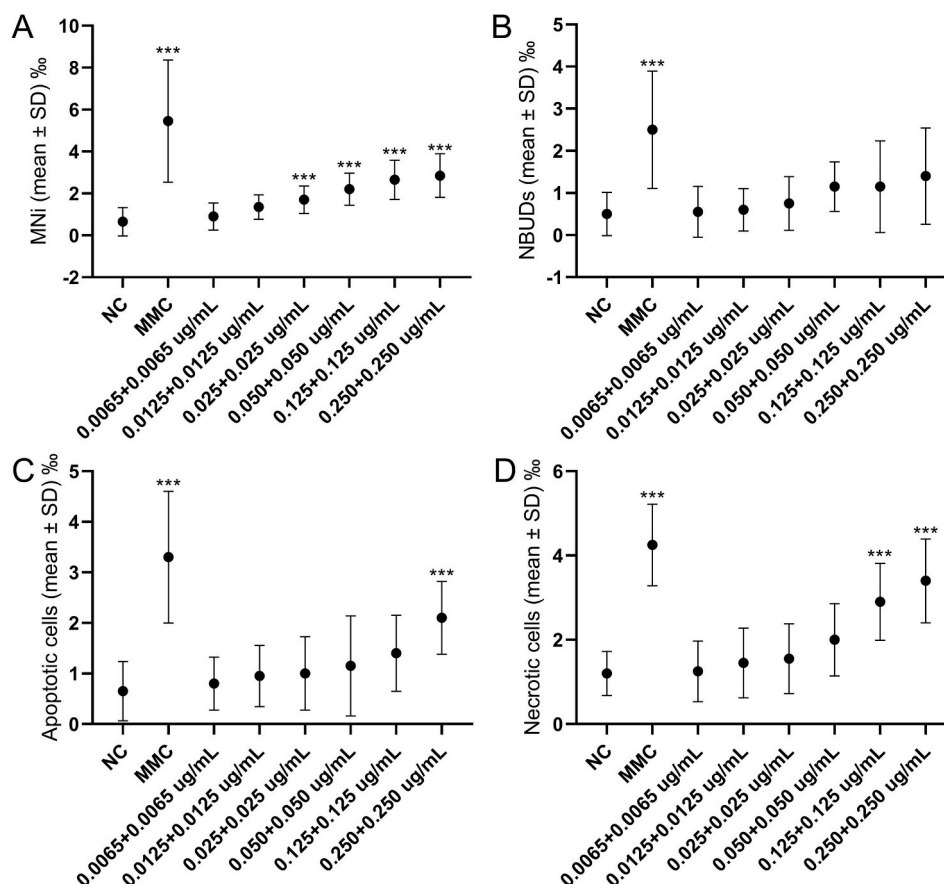
Fig. 4. Box plots of micronuclei (MNi) (A), nuclear buds (NBUDs) (B), apoptotic and necrotic cells (C and D, respectively), expressed as mean ± standard deviation (SD) ‰ in lymphocytes exposed to Glyphosate + AMPA. Asterisks indicate significant differences according to Dunnett's multiple comparison test (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). NC = negative control; MMC = mitomycin C (positive control).

et al. (2014) showed that AMPA at a concentration of 0.25 mM (corresponding to 27.76 µg/mL) can induce haemolysis in human erythrocytes and raise the methaemoglobin level in blood.

At genetic level, both glyphosate and AMPA were found to induce DNA single-strand breaks, oxidation of purines and pyrimidines (Woźniak et al., 2018), and alterations in human epigenome and in global DNA methylation in particular (Kwiatkowska et al., 2017; Duforestel et al., 2019). In their study investigating the effect of glyphosate and AMPA on the expression of genes involved in chromatin architecture in human peripheral blood mononuclear cells, Woźniak et al. 2020a; 2020b showed that both compounds changed the expression of *DNMT1* and *HDAC3* genes and the methylation pattern of *P16* and *TP53* suppressor gene promoters.

The molecular mechanisms by which glyphosate and AMPA can induce genomic damage are still not completely understood. However, it has been observed that peripheral blood mononucleated cells significantly repaired glyphosate-induced DNA damage, but they were unable to repair completely DNA strand-breaks, which can lead to mutations that may cause genetic instability. Moreover, glyphosate at a high concentration was observed to activate retrotransposable sequences, which may induce genomic damage by insertion and/or homologous recombination (Kwiatkowska et al., 2017).

We observed that combined treatment of glyphosate and AMPA induced genotoxic effects starting at a concentration of 0.025 µg/mL of both compounds. This may suggest that glyphosate and AMPA contribute equally to causing genomic damage. Of note is the importance of testing not single xenobiotics alone but rather mixtures as found

in environmental matrices. For example, both glyphosate and AMPA have been found in agricultural soil at concentrations from 35 to 1502 µg/kg and from 299 to 2256 µg/kg, respectively, which include the concentrations we tested in the present study (Aparicio. et al., 2013).

The CBPI analysis indicated that AMPA is more cytotoxic than Glyphosate, and that there is a possible combined cytotoxic action of these two xenobiotic compounds when present together in the same cultures (Tables 1–3 supplementary material, Figs. 2–4). Interestingly, Li et al. (2013) reported that glyphosate and AMPA inhibit the growth of cancer cells but not that of healthy cells; they also suggested the hypothesis of an anticancer therapy based on glyphosate and AMPA (Li et al., 2013; Grandcoin et al., 2017).

Finally, a significant increase in apoptotic cells was observed in cultures treated with the highest concentration of tested xenobiotics, while a significant increase in necrotic cells was observed also at the concentration of 0.250 µg/mL of both glyphosate and AMPA alone and in combination (0.125 + 0.125 µg/mL). Numerous factors can induce apoptosis and necrosis, some of them contribute to both processes, whereas others play a role in only one of these processes. The DNA damage induced by toxicants is at the basis of both processes (McConkey, 1998), whereas oxidative stress seems to play an important role in apoptosis (Kwiatkowska et al., 2020). In particular, glyphosate and AMPA have been found to promote an excessive cellular production of reactive oxygen species, as a consequence of inflammation mechanisms (Martínez et al., 2020). Prolonged oxidative stress is capable of inducing damage to cellular proteins, lipids, and DNA and activating apoptotic and autophagic pathways (Kwiatkowska et al., 2020).
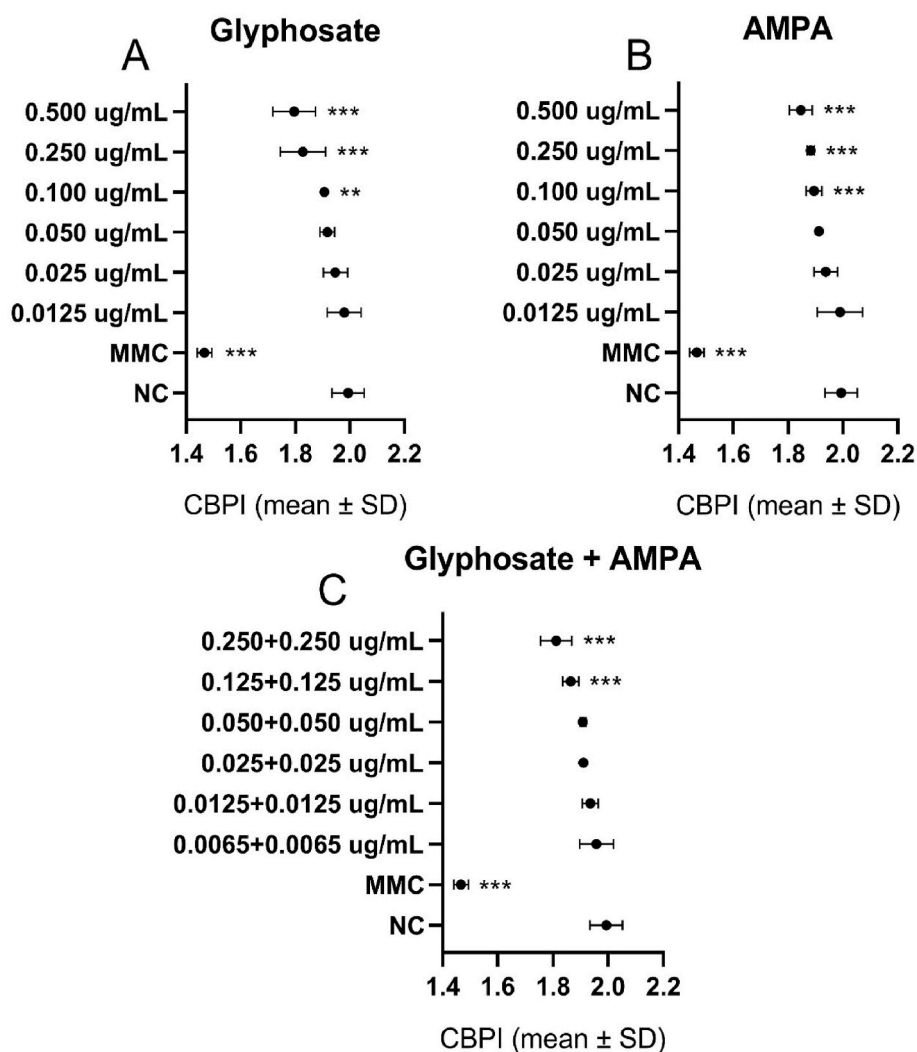
Fig. 5. Box plots of cytokinesis-block proliferation index (CBPI), expressed as mean ± standard deviation (SD) in lymphocytes exposed to Glyphosate, AMPA and Glyphosate + AMPA. Asterisks indicate significant differences according to Dunnett's multiple comparison test (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). NC = negative control; MMC = mitomycin C (positive control).

5. Conclusions

Assessment of the genotoxic potential of glyphosate and AMPA exposure *in vitro* and in exposed populations is a critical public health measure. The IARC classifies glyphosate as "probably carcinogenic to humans". The present study provides evidence for the genotoxic and cytotoxic action of glyphosate and AMPA on cultured human lymphocyte. The two compounds were tested at concentrations from 0.0125 to 0.500 μg/mL, which are more realistic than the higher concentrations used in other published papers. Our findings underline the utility of testing not single xenobiotics alone but rather combined in a mixture with their respective metabolites. Such a procedure can more accurately determine genotoxicity and reveal possible interactions between the compounds and their metabolites.

Finally, our data should be read in a chronic exposure scenario, as the consequences of exposure will be observed in the long term, in which they may well be greater than those observable *in vitro* or in the short term.

CRediT authorship contribution statement

Alfredo Santovito: Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization.

Alessandro Nota: Investigation, Formal analysis, Conceptualization, Writing – review & editing, Data curation. **Paolo Pastorino:** Formal analysis, Validation, Writing – review & editing. **Claudio Gendusa:** Investigation, Methodology, Validation. **Enrico Mirone:** Formal analysis, Investigation, Validation. **Marino Prearo:** Conceptualization, Investigation, Validation. **Dasa Schleicherová:** Data curation, Investigation, Validation, Writing – review & editing.

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.

Data availability

No data was used for the research described in the article.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142888.

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