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Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the Comet assay and cytogenetic tests

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ABSTRACT

Formulations containing glyphosate are the most widely used herbicides in the world. AMPA is the major environmental breakdown product of glyphosate. The purpose of this study is to evaluate the in vitro genotoxicity of AMPA using the Comet assay in Hep-2 cells after 4 h of incubation and the chromosome aberration (CA) test in human lymphocytes after 48 h of exposition. Potential in vivo genotoxicity was evaluated through the micronucleus test in mice. In the Comet assay, the level of DNA damage in exposed cells at 2.5–7.5 mM showed a significant increase compared with the control group. In human lymphocytes we found statistically significant clastogenic effect AMPA at 1.8 mM compared with the control group. In vivo, the micronucleus test rendered significant statistical increases at 200–400 mg/kg. AMPA was genotoxic in the three performed tests. Very scarce data are available about AMPA potential genotoxicity.

1. Introduction

Glyphosate is already one of the most used xenobiotics in modern agriculture (Peixoto, 2005). Its indiscriminate application arises some concerns regarding the possible health and environmental hazards (Chiu et al., 2008). The main breakdown product of glyphosate in plants, soil, and water is (aminomethyl) phosphoric acid (AMPA).

Reviews on the safety of glyphosate and AMPA have been performed by several regulatory agencies and have concluded that there is no indication of any human health damage (US EPA, 1993; FAO/WHO, 1997; E.U., 2002). However, present researches have shown both are more mobile and persistent in aquatic environments in comparison with former researches (Kolpin et al., 2006). AMPA has a greater environmental persistence than glyphosate, 76–240 and 2–197 days of soil half-life for both, respectively (Battaglin et al., 2005), and there is evidence indicating that AMPA may also pose a risk of groundwater pollution (Landry et al., 2005).

The purpose of this study is to contribute to the evaluation of the genotoxic potential of AMPA, which has only been informed by regulatory agencies in unpublished reports mentioned in reviews. In this study, the Comet assay was used as a preliminary in vitro genotoxicity test and the chromosome aberrations analysis was used to evaluate the response of human cells to AMPA exposition. At last, the in vivo micronucleus test in mice was performed to consider the overall metabolism of an organism.

The single-cell gel electrophoresis assay (Comet assay) is capable of detecting DNA damage with great sensitivity and has been used widely both in vitro and in vivo protocols to identify potentially environmental genotoxins (Moller, 2006). The chromosome aberrations assay in cultured cells has been used for many years, and it has proved to be a great method in the prediction of cancer risk, since there is a connection between the level of chromosome aberrations and some types of cancer (Boffetta et al., 2006). The micronucleus test (OECD, 1997b) has emerged as one of the most successful in vivo methods in the assessment of chromosome damage.

2. Materials and methods

2.1. Chemicals

Analytical grade AMPA, CAS 1066-51-9 (99%), cyclophosphamide, CAS 50-18-0, and mitomycin C, CAS 50-07-7, were purchased from Sigma-Aldrich, Argentina. Eagle's minimum essential medium with Earle's balanced salt solutions (EMEM), L-glutamine, fetal bovine serum, antibiotics, phytohaemagglutinin (Gibco), RPMI with Hepes (Gibco) and Colcemid (Gibco) were purchased from Invitrogen Argentina, Argentina. Normal melting point agarose (NMP) and low melting point

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agarose (LMP) were purchased from Promega, Argentina. All other chemicals and solvents were of analytical grade.

2.2. Single-cell gel electrophoresis assay (Comet assay)

Cell line Hep-2, provided by "Asociación Banco Argentino de Células (ABAC, Pergamino, Argentina), mycoplasma and pathogens free, was used in this study. Cells were maintained in EMEM, supplemented with 200 mM L-glutamine, 2% (v/v) fetal calf serum, and antibiotics (penicillin, 100 U/ml, streptomycin 100 and 0.25 μg/ml anfotericine B). Cells were removed from the culture flask and settled in a 96-well tissue culture plate (TPP® Zellkultur testplate 96F Switzerland) (8.25×105 cells/ml; 200 μl/well). Hep-2 cells grew during 24 h at 37 °C in an atmosphere of 5% CO_2 in air with 100% humidity to obtain confluent monolayers; the medium was removed from each well, and replenished in the MEM medium with AMPA 2.5, 4.5, 5.5, 6.5, 7.5, 9 and 10.0 mM. In all cases the cultures were diluted to 200 μl final volume/well and carried out in duplicate. Positive (mitomycin C 0.01 mM) and negative (only MEM medium) controls were included. In preliminary experiments, a 4 h exposure to AMPA was chosen for this assay, because in this condition in 70% of the concentrations tested the viability of Hep-2 cells never decreases below 80%, evaluated through the trypan blue exclusion technique. The protocol followed the general guidelines proposed by Singh et al. (1988), with slight modifications. Volumes of 50 μl of Hep-2 cells from each culture well were added to 100 μl of 0.75% LMP agarose at 37 °C. The mixtures were layered onto slides precoated with 0.75% NMP agarose, covered with a cover slip. The slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris [pH 10], supplemented with 1% Triton X-100 and 10% DMSO just before use) for at least 1 h, immersed in a freshly prepared alkaline buffer pH > 13 and electrophoresis was conducted for 30 min. The slides were fixed in absolute ethanol, stained with ethidium bromide, and scored using a fluorescent microscope. From each treatment, images from 100 "nucleoids" were captured with a camera attached to the fluorescent microscope and linked to the Comet Score 1.5 software. Tail moment (TM), DNA percentage in tail (% of DNA) and tail length (TL) were used to estimate DNA damage (arbitrary units).

2.3. Chromosome aberrations (CA) test

Heparinized human blood samples were obtained from six healthy donors, three females and three males, from 18 to 33 years old. The donors had no history of pesticide exposure and were non-smokers. Lymphocytes were cultured for 72 h at 37 °C according to conventional methods and each treatment was carried out in duplicate (Moorhead et al., 1960). The cultures were exposed to AMPA at concentrations of 0.9 and 1.8 mM during the last 48 h of culture. The herbicide was previously solved in 1 ml of culture medium and adjusted to pH 7.2–7.4. Mitomycin C (0.9 μM) was used as a known CA inductor (OECD, 1997a). Control untreated cultures were established as well. Two thousand cells/treatment were examined for mitotic index (MI), calculated as the number of dividing cells/2000 cells. One hundred metaphases were analyzed for number and type of CA and classified according to the International System of Cytogenetic Nomenclature (ISCN, 1985). Chromatid breaks (ctb) and gaps (ctg), chromosome breaks (csb) and gaps (csg), dicentric chromosomes (dic), acentric fragments (ace) and endoreduplicated (end) cells were considered. The slides were scored blind by two observers.

2.4. Micronucleus test (MNT)

Male and female Balb-C mice of 8–12 weeks old were maintained on rodent diet and water ad libitum, and kept under controlled conditions and in accordance with the criteria established by the Institutional Ethics Committee. Groups with five animals in each were employed for the micronucleus test (MNT) (OECD, 1997b). The groups received i.p. injections of 100 and 200 mg/kg of AMPA. All doses were repeated after 24 h. The positive control group received one cyclophosphamide injection of 20 mg/kg and the negative control group an equivalent volume of saline solution. All the animals were sacrificed 24 h after the second administration and bone marrow smears were prepared from the femoral bones. Smears were stained with May Grunwald–Giemsa as originally described by Schmid (1975). In order to score micronuclei about 1000 erythrocytes per animal were analyzed. To evaluate bone marrow toxicity, 500 erythrocytes were counted and the ratio polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) was calculated.

2.5. Statistical analysis

Statistical analysis was performed using Prism software (PRISM GraphPad, 1997). The mean scores were calculated from the experiments of each duplicate treatment. The Kolmogorov–Smirnov test was performed to verify whether the results follow a normal distribution. The non-parametric Kruskal–Wallis Analysis of Variance on Ranks ($p < 0.05$) test followed by the Dunn's Multiple Comparisons Test were used for comparing the means of each treatment with their negative and positive control in the Comet assessment. Parametric one-way ANOVA and Dunnett as "a posteriori" test were used in CA and MNT. The Pearson statistical test was used to examine possible dose–response effects. In all cases, the level of significance was set at $\alpha = 0.05$.

3. Results

AMPA was first evaluated for its potential genotoxicity in the Comet assay. After 4 h exposure of Hep-2 cells to AMPA, nucleoid images from each treatment were recorded and values of three parameters, Comet TL, % DNA and TM, were obtained. Fig. 1 presents the level of DNA damage in Hep-2 cells (TM 5.42 ± 1.83, TL 6.07 ± 1.05 and % of DNA in tail 3.95 ± 0.53). For AMPA, the TM DNA damage scores increased from 410.20 ± 53.56 to 849.20 ± 95.62 arbitrary units at a range of concentrations from 2.5 to 7.5 mM. These values were significantly different ($p < 0.01$) when compared to negative controls. Mitomycin C 0.01 mM as the positive control induced a significant increase in DNA migration when compared to negative controls ($p < 0.01$) in the Hep-2 cell line (Fig. 1). In the Hep-2 cell line, the viability was measured immediately before the Comet assay. In concentrations above 7.5 mM the viability was lower than 80%; therefore they were not assayed for genotoxicity. The Comet assay was performed for 2.5–7.5 mM because all these concentrations induce cell viability between 81% and 94%. Positive control Hep-2 cells showed cell viability higher than 87%, while in negative control the cell viability was around 91% (data not shown). For the Comet assay, AMPA exhibited dose-dependent genotoxic effect in the three parameters analyzed on Hep-2 cells exposed to AMPA (TM $r = 0.9410$, $p < 0.05$; TL $r = 0.9742$, $p < 0.05$; % of DNA in tail $r = 0.9042$, $p < 0.05$).

AMPA was also tested on 6 different human blood samples with 0.9 and 1.8 mM concentration. The mean of MI ± SEM in untreated cultures was 7.0 ± 3.2 without statistical differences between the different treatments. After 48 h AMPA exposition

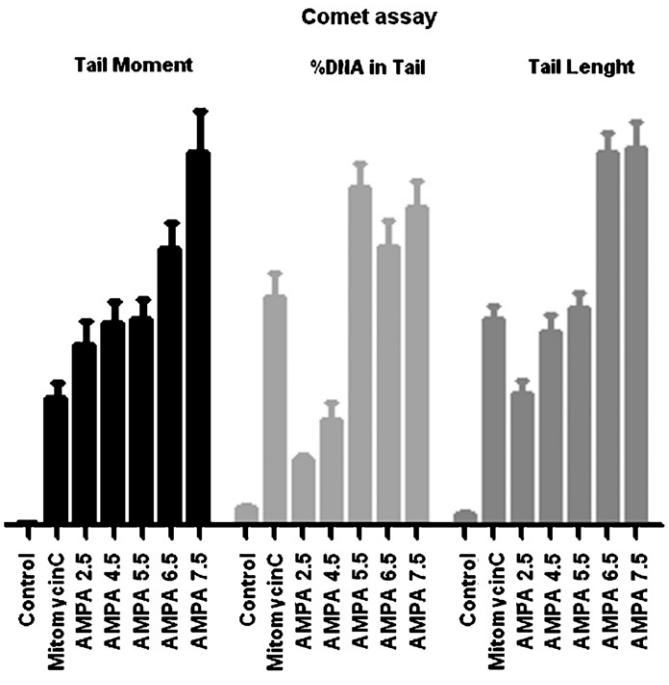

Fig. 1. DNA damage in hep-2 cells exposed to different concentrations of AMPA after 4 h of incubation, by the Comet assay. AMPA 2.5 mM (Not significant), AMPA 4.5 mM ($^{*}p < 0.05$), all other treatments are $^{**}p < 0.01$.

Table 1
Chromosome aberrations in human lymphocytes treated with AMPA

Treatment	ctb	csb	ctg	csg	ace	end	CA/100 metaphases	CA excluding gaps/100 metaphases	MI
Control	1.6±1.5	0.0±0.0	2.0±2.3	0.6±0.5	0.0±0.0	0.2±0.4	4.4±1.4	2.2±0.7	7.0±3.2
Mitomycin (0.9 μM)	23.5±9.2[a]	18.0±15.5[a]	3.0±4.2	4.0±2.8	27.0±36.8	0.0±0.0	71.5±30.2*	68.5±61.5*	3.5±2.1
AMPA (0.9 mM)	1.8±0.8	1.0±1.2	1.8±0.8	1.0±0.0	0.0±0.0	0.6±0.9	6.2±1.3	3.4±0.9	5.8±3.6
AMPA (1.8 mM)	3.2±2.0	0.8±1.6	2.8±2.3	1.7±1.4	0.5±0.8	0.5±0.5	9.5±1.1*	5.0±0.9*	9.7±4.8

CA: chromosome aberrations, MI: Mitotic Index: number of dividing cells/2000 cells. ctb: chromatid break; csb: chromosome break; ctg: chromatid gap; csg: chromosome gap; ace: acentric fragment; end: endoreduplication. n: 6 adult healthy donors of both sexes for each treatment. Data of each treatment are expressed as media±standard error mean (SEM).*$p<0.05$.
[a] Only in mitomycin C cultures cells with multiple breaks were observed.

Table 2
Number of micronucleated erythrocytes (MNE)/1000 analyzed cells

	MNE/1000 analyzed cells	PCE/NCE
Saline solution	3.8±1.8	0.85±0.17
Cyclophosphamide (20 mg/kg)	19.2±3.9**	0.80±0.20
AMPA 200 (2×100 mg/kg)	10.0±1.9**	1.14±0.22
AMPA 400 (2×200 mg/kg)	10.4±3.3**	1.07±0.04

n: 5 animals per treatment. The results are expressed as mean±standard error mean.
**$p<0.01$.

chromosome aberrations were scored by identifying chromatid and chromosome aberrations. The results of the CA analysis in blood exposed in vitro to AMPA are presented in Table 1. The total CA score in the control group was 4.4±1.4 and 2.2±0.7 CA/100 metaphases including and excluding gaps, respectively. In the treatment with AMPA 1.8 mM, a significant increase ($p<0.05$) was observed in the number of CA including gaps, reaching values of 9.5±1.1 CA/100 metaphases. In the chromosome aberrations analysis excluding gaps (5.0±0.9), the difference against control group is still statistically significant ($p<0.05$). Values of 71.5±30.2 and 68.5±61.5 CA/100 including and excluding gaps, respectively, were observed in mitomycin C treatment. No positive clastogenic effect was observed with AMPA 0.9 mM. As it is shown in Table 2 most of the analyzed aberrations after different treatments were of chromatid type, considering gaps and breaks. Cells with multiple breaks were only observed in mitomycin C cultures.

In the MNT it was observed a basal level of 3.8±1.8 micronucleated erythrocytes (MNE)/1000 analyzed cells in the animals of the negative control group. In the treated animals at 200 and 400 mg/kg the number of MNE/1000 analyzed cells was 10.0±1.9 and 10.4±3.3, respectively (Table 2). Both groups and the positive control animals treated with cyclophosphamide (19.2±3.9 MNE/1000 analyzed cells) showed significant differences ($p<0.01$, test de Dunnet) with respect to the control group.

4. Discussion

In this study, the Comet assay using Hep-2 cells has been used for an initial screening of the potential genotoxicity of AMPA. This cell line was previously used by other authors to evaluate genotoxicity through the Comet assay (Andrighetti-Frohner et al., 2006). The high sensitivity of the Comet assay compared to the CA and MNT, and the need for only very small amounts of tested chemicals, makes this assay an alternative tool for the initial screening. The assay showed a clear increase in the TL, % DNA in the tail, and TM, indicators of DNA damage, in cells treated for a 4 h treatment (Fig. 1). AMPA increased the extent of DNA migration in a concentration–response relationship in Hep-2 cells with the employed concentrations in this study. This increase was not associated with the cytotoxicity since the tested concentrations were not toxic to these cells. AMPA concentrations equal to and above 9.0 mM were cytotoxic for Hep-2 cells with a % of viability between 60% and 79%. Mitomycin C 0.01 mM resulted in a correct positive control for the Comet assay in the Hep-2 cell line when compared to negative controls (Fig. 1). Regarding the evaluation of potential genotoxicity of AMPA through Comet assay, no other data have been published so far.

AMPA was also evaluated for its potential genotoxicity through the chromosome aberration test in human blood cells. We have found a statistically significant increment in the CA levels with AMPA 1.8 mM (Table 1). In this culture, the MI did not present statistical differences against the other treatments; thus it can be concluded that the clastogenic effect observed with AMPA is not due to cytotoxicity. We have not found any report of the CA test for AMPA. In this study, we observed a two-fold increase in the number of CA ($p<0.05$) with AMPA 1.8 mM in comparison with the control group. To observe a result that showed twice the damage than in the basal level is a weak response, especially if these results are compared with the response observed in the Comet assay. This may be due to the fact that the Comet assay is a particularly sensitive test. Concerning the MNT test, it is known that many compounds may produce negative in vitro results and positive in vivo results because of their indirect action. In this study the response was higher in the in vivo test, perhaps due to a difference of sensitivity in both systems in response to the AMPA effect.

The results obtained with AMPA are important because it is the main environmental breakdown product of glyphosate. In this way, at least three previous researches have shown that herbicide metabolites are often detected more frequently than their parent compound in both, streams (Battaglin et al., 2005; Kolpin et al., 2006) and groundwater (Kolpin et al., 2004). Some regulatory agencies have determined that AMPA is not of toxicological concern and do not include it in risk assessments (US EPA, 1993). Nevertheless, relatively little is known about the biological activity of this compound, which can be found as a pollutant in the environment.

In a *Salmonella typhimurium* mutation test performed on AMPA, no mutagenic activity was observed (Shirasu, 1980). In an in vitro unscheduled DNA synthesis repair in rat hepatocytes exposed to AMPA, no genotoxic effects were observed (Bakke, 1991) (all unpublished reports cited in Williams et al., 2000).

At last, the micronucleus test in mice was performed as an in vivo method. The MNT allows the detection of small fragments of

chromosomes in the cytoplasm of immature erythrocytes, and it has the potential to detect clastogenic and aneugenic chemicals effects (OECD, 1997b). Micronucleated erythrocytes were significantly positive in both concentrations assayed: 200 and 400 mg/kg of AMPA. Kier and Stegeman (1993) reported that at 100 mg/kg body weight, a significant increase in nucleated polychromatic erythrocyte was seen after 72 h in females only; thus the increase was considered not to be related to the treatment (unpublished reports cited in FAO/WHO, 1997). Surprisingly, in this study, in the micronuclei test in Balb-C mice treated with AMPA by i.p. injection at doses of 200 and 400 mg/kg, we have observed a duplication of the control levels of MNE/1000 cells. The 400 mg/kg administration of analytical grade of AMPA has not been previously assayed in vivo in genotoxicity evaluations. So far, no other data have been available on the potential genotoxicity of AMPA. In this study, the effects observed in the Comet assay with cells exposed to AMPA are confirmed by in vitro chromosome aberration and in vivo micronucleus test. The Comet assay could be considered a biomarker of genotoxic exposure and the CA test a biomarker of genotoxic effect. It is not possible to discuss the potential genotoxicity of AMPA because of the lack of information about this compound. After its first genotoxic evaluation, in a few systems, reported by the regulatory agencies, no further information was produced.

Conclusion: The use of glyphosate in agriculture continues to expand. Its present widespread and frequent use also involves vegetable production, ornamentals, lawns, turf, greenhouses. As it was exposed, its environmental breakdown product has been found in Midwestern streams and wastewater treatment plants. Thus, ongoing risk assessment of AMPA through a battery of short-term tests with different end-points is of importance. Its environmental detection, along with glyphosate, is a matter of concern. The suitable, controlled, and regular use of this herbicide is recommended, to obtain its beneficial effects without polluting the environment and without leaving their residues in water and food sources with potential risk on human health.

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The authors of this manuscript assure that the test in vivo performed in mice was conducted in accordance with institutional guidelines for the protection of animal welfare.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2008.09.019.

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