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Glyphosate Commercial Formulation Causes Cytotoxicity, Oxidative Effects, and Apoptosis on Human Cells: Differences With its Active Ingredient

Gabriela Chaufan[1], Isis Coalova[1], and María del Carmen Ríos de Molina[1]

Abstract

In the present study, the effects on oxidative balance and cellular end points of glyphosate, aminomethylphosphonic acid (AMPA), and a glyphosate formulation (G formulation) were examined in HepG2 cell line, at dilution levels far below agricultural recommendations. Our results show that G formulation had toxic effects while no effects were found with acid glyphosate and AMPA treatments. Glyphosate formulation exposure produced an increase in reactive oxygen species, nitrotyrosine formation, superoxide dismutase activity, and glutathione (GSH) levels, while no effects were observed for catalase and GSH-S-transferase activities. Also, G formulation triggered caspase 3/7 activation and hence induced apoptosis pathway in this cell line. Aminomethylphosphonic acid exposure produced an increase in GSH levels while no differences were observed in other antioxidant parameters. No effects were observed when the cells were exposed to acid glyphosate. These results confirm that G formulations have adjuvants working together with the active ingredient and causing toxic effects that are not seen with acid glyphosate.

Keywords

apoptosis, cytotoxicity, glyphosate, *in vtro*, oxidative stress

Introduction

Glyphosate is a nonselective postemergent herbicide that inhibits 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme of the aromatic amino acid biosynthetic pathway in plants.[1] The main breakdown product of glyphosate in soil is aminomethylphosphonic acid (AMPA), which is broken down further by soil microorganisms.[2]

Half-lives of glyphosate and AMPA in soil range from 2 to 197 days and 76 to 240 days, respectively.[3] They can persist in the environment as residues in soils and crops for up to 3 years.[4] Although humans are not a direct target, they could be in contact with glyphosate due to occupational exposure[5,6] and/or through dietary exposure.[7,8]

Pesticides as active ingredients are combined with other ingredients to create the commercial formulas on the market. Other ingredients include a wide array of compounds; information regarding some of these is considered confidential business information and they are not of public-free access. The toxic effects may be a consequence of the active or other ingredients in the formulation or both.[9,10] The herbicide glyphosate is sold worldwide under a variety of commercial names. Since glyphosate is not applied in the field as a pure active ingredient the toxicity of commercial form should be assayed.[11] Previous studies exposing human cells to glyphosate revealed that glyphosate formulation (G formulation) is more toxic than the active component itself, supporting the idea that additives in commercial formulations play a role in herbicide's toxicity.[12-15]

It has been reported that many pesticides (including herbicides) generate intracellular reactive oxygen species (ROS).[11,16-18] We have demonstrated that an increase in ROS levels triggers oxidative damage to proteins, nucleic acids, and lipids as well as the increase in activity of different antioxidant enzymes.[19,20] Environmental stressors that are well known to induce oxidative stress and alterations to the cellular redox balance have been widely shown as apoptosis regulators. Despite evidence that glyphosate induces cytotoxicity, oxidative damage, and apoptosis in several models,[21-25] the molecular mechanisms and the effects on humans remain largely unknown.

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Liver plays a key role in metabolism of xenobiotics and it is especially sensitive to dietary pollutants.[26] Cultures of human cells offer an option as *in vtro* models to evaluate the potential effect of toxic compounds in humans. Particularly, the HepG2 cell line maintains several physiological functions of intact human liver, so it has served as a model in previous research on toxicity.[27] Herein, we associate the cytotoxic action of glyphosate with oxidative stress, comparing a G formulation, acid glyphosate, and the main break down product of glyphosate, AMPA, in HepG2 cell line.

For this purpose, we determined the cytotoxicity (lethal concentration 50 [LC_{50}]) of glyphosate, AMPA and the G formulation, ROS formation, tyrosine nitration, glutathione (GSH) equivalents content, antioxidant enzymes activity (superoxide dismutase (SOD), catalase (CAT), and GSH-S-transferase (GST)), and caspase 3/7 activity as a marker of apoptosis. We demonstrated that G formulation produces the highest cytotoxicity related to oxidative stress and cellular end points, and these results need to be considered for protection of human health.

Materials and Methods

Reagents

Modified Eagle medium (MEM), MEM vitamin solution, MEM nonessential amino acid solution, and 0.05% trypsin–EDTA were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from BIO-NOS (Buenos Aires, Argentina). The caspase 3/7 colorimetric substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), AMPA, 4′,6-diamino-2-phenylindole dihydrochloride (DAPI), and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Sigma Chemical Co (St Louis, MO, USA). Technical grade acid glyphosate (N-phosphonomethylglycine, 95% purity) was obtained from Monsanto, Buenos Aires, Argentina and the G formulation Roundup UltraMax (Monsanto, Buenos Aires, Argentina) was purchased from a commercial Argentinean retailer. The concentration of G formulation (74.7% v/v monoammonium salt of N-phosphonomethylglycine and 25.3% adjuvants and inert substances) recommended is from 0.5 to 1 kg per 100 L water.[28] No further information was available. Treatment medium was prepared in serum-free medium and adjusted to pH 8.5. Caspase substrates and H_2DCFDA were dissolved in dimethyl sulfoxide (DMSO). Final concentration of DMSO in caspase activity and ROS production assays did not exceed 3%. Dimethyl sulfoxide added to samples did not affect cell viability (assayed by MTT method, $P = 0.15$), morphology, or other parameters tested in this study (cell morphology was verified by optical microscopy).

Cell Culture

The human hepatoma cell line HepG2 was cultured in Modified Eagle medium (MEM) supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2.5 µg/mL amphotericin B. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2–95% air. Medium was renewed twice a week. After 7 days, cells became confluent and ready for use.

For all experiments, confluent attached cells were removed from cell culture dishes with 0.25% sterile trypsin and diluted with MEM/10% FBS. For MTT assay, cells were replated into 96-well plates (0.2 mL; 2×10^4 cells/well), and ROS formation detection assay was performed in 24-well plates (0.5 mL; 7.5×10^4). For the other experiments, cells were replated in cell culture T-Flasks (4 mL; 3×10^6 cells/flask).

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The method employed was described by Mossman.[29] Briefly, cells were exposed for 24, 48, and 72 hours in serum-free medium to different dilutions of glyphosate, AMPA, and G formulation, below agricultural recommendations for the G formulation (3.39 g/L acid glyphosate; subagriculture concentrations). Following incubation, different treatment media were removed; cells were washed with phosphate-buffered saline (PBS) and replaced with 1 mg/mL of sterilized MTT solution. This MTT solution was freshly prepared in MEM containing no FBS since it has been shown that FBS can dose dependently inhibit formazan crystal formation, with a 50% decrease in these crystals when media with 5% to 10% FBS is used.[30] The plates with added MTT solution were then placed in the 5% CO_2 incubator for 90 minutes at 37°C. The MTT solution was removed, and 200 µL of ethanol was added to each well to dissolve the blue formazan crystals. Optical density was measured at 570 nm with background subtraction at 655 nm in a BIO-RAD Benchmark microplate reader (BIO-RAD Laboratories, Hercules, CA). Results were expressed as percentage of control (100% viability). Each assay involved 8 wells per condition and was performed in triplicate.

Antioxidant Enzyme Activities

For determination of enzyme activities (CAT, GST, and SOD), cells were grown at confluence with the different treatments for 24 hours at concentrations that never induced cell viability below 80% (lethal concentration [LC_{20}]). Determinations were carried out in 11,000× *g* supernatants from cell lysates.

Catalase. Catalase (EC 1.11.1.6) activity was determined by following hydrogen peroxide decomposition at 240 nm in a reaction mixture containing 50 mmol/L potassium phosphate buffer (pH 7.0) and 30 mmol/L hydrogen peroxide.[31] Results were expressed as percentage of control (100% of activity).

Glutathione-S-transferase. Glutathione-S-transferase (EC 1.11.1.9) activity was measured by Habig technique.[32] Briefly, standard assay mixture contained the enzymatic sample, 100 mmol/L GSH solution, and 100 mmol/L 1-chloro-2,4-dinitrobenzene (CDNB)

in ethanol, in 100 mmol/L phosphate buffer (pH 6.5) to a final volume of 0.8 mL. After adding CDNB, change in absorbance at 340 nm was followed for 120 seconds. One GST unit was defined as the amount of enzyme that catalyzes the formation of 1 μmol of GS-DNB per minute at 25°C; results were expressed as percentage of control.

Superoxide dismutase. Superoxide dismutase (EC 1.15.1.1) activity was measured using a modify Beauchamp and Fridovich procedure[33] in microplate. The standard assay mixture contained enzymatic sample, 0.1 mmol/L EDTA, 13 mmol/L DL-methionine, 75 μmol/L nitroblue tetrazolium, and 2 μmol/L riboflavin, in 50 mmol/L phosphate buffer (pH 7.9) to a final volume of 0.3 mL. Samples were exposed 5 minutes to intense cool white light. One SOD unit was defined as the amount of enzyme necessary to inhibit 50% the reaction rate. Samples were measured at 560 nm in a BIO-RAD Benchmark microplate reader (BIO-RAD Laboratories), and results were expressed as percentage of control.

Glutathione Equivalents Content

Glutathione levels were measured in HepG2 cells following the Anderson procedure,[34] with some modifications. Briefly, after being incubated in culture flasks at early confluence (80%-90% confluent) at LC_{20} with the different treatments, cells were collected, washed, and resuspended in PBS. Then, cells were lysed as described previously. To determine GSH, we proceeded as described.[21] Results were expressed as percentage of control.

Detection of ROS

Reactive oxygen species formation was measured using the cell permeable indicator H_2DCFDA. 2′,7′-Dichlorodihydrofluorescein diacetate is a probe widely used to detect several free radicals and other reactive species (OH, $ONOO^-$, H_2O_2, NO, ROO). Cellular esterases hydrolyze the probe to the nonfluorescent 2′,7′-dichlorodihydrofluorescein (H_2DCF), which is retained in the cells. In the presence of ROS and cellular peroxidases, H_2DCF is transformed to the highly fluorescent 2′,7′-dichlorofluorescein[35] that can be easily visualized as strong fluorescence at 525 nm when excited at 488 nm.

The stock solution of 1 mmol/L H_2DCFDA was prepared in DMSO, stored at −20°C, and protected from light. Briefly, after incubation for 24 hours in 24-well plates at early confluence (80%-90% confluent) with different treatments, cells were washed with PBS and incubated with freshly prepared 30 μmol/L H_2DCFDA solution with serum-free MEM at 37°C for 60 minutes. After incubation, cells were collected, washed, and resuspended in 1 mL of PBS. After lysis by sonication for 5-second intervals at 40 V setting in ice, the samples were centrifugated at 11,000 × *g* for 15 minutes. The supernatant was measured in a white microplate at excitation wavelength of 485 nm and emission wavelength of 520 nm using fluorescence plate reader FLUOstar OPTIMA (BMG Labtech, Germany). For cytoplasmic ROS analysis, the cells were subcultured on glass cover slips in 6-well plates. After G formulation exposure, the cells were washed with PBS and fixed with glacial acetic acid–methanol (1:3 v/v) for 10 minutes at room temperature. Then, the cells were washed twice with PBS, stained with H_2DCFDA solution for 60 minutes, and visualized by fluorescence microscopy using filters B-2A (λ excitation [λexc]: 450-490 nm; λ emission [λem]: 515 nm).

Immunofluorescence

For immunofluorescence analyses, cells were seeded on glass cover slip on 6-well plates and fixed as cytoplasmatic ROS analysis. Then cells were treated with PBS containing 0.25% Triton X-100 for permeabilization 10 minutes at room temperature. Following blockade of nonspecific binding sites by incubation with blocking buffer (PBS, 1% milk) for 30 minutes, the cells were incubated overnight at 4°C with rabbit fraction antinitrotyrosine antibody (Molecular Probes, USA.). Then, the cells were incubated with biotinylated antirabbit IgG (Chemicon, USA) for 1 hour at room temperature, washed twice with PBS, and incubated with Streptavidin Alexa Fluor 488 conjugate. For nuclear counting staining, cells were incubated in DAPI (1 μg/mL) for 10 minutes after immunostaining. After repeated washing with PBS, the slides were mounted and analyzed on an Olympus inverted fluorescent microscope (Olympus Latin America Inc.). Fluorescence pictures were taken with identical exposure settings. Intensities of fluorescence were measured and analyzed using computer-based analysis (ImageJ is supplied freely by National Institutes of Health (NIH)). with the same threshold for all sections.

Cell Death Measurements

Nuclear morphology assessment by fluorescence microscopy. Evaluation of nuclear morphology was performed as described subsequently. In brief, HepG2 cells were subcultured on glass cover slips in 6-well plates. After G formulation exposure, cells were washed with PBS and fixed with glacial acetic acid–methanol (1:3 v/v) for 10 minutes at room temperature. Then, the cells were washed twice with PBS, stained with DAPI (1 μg/mL), washed again with PBS, and examined under fluorescence microscopy (Eclipse E600, Nikon; Nikon Instech Co, Ltd, Karagawa, Japan) using filters for DAPI (λex: 330-380 nm; λem: 435-485 nm). Images were captured with a CoolPix5000 digital camera (Nikon; Nikon Instech Co, Ltd). Digital pictures were analyzed and assembled using Adobe Photoshop CS 4.0 software. Apoptotic cells were scored evaluating the presence of condensed and fragmented nuclei.

Caspase activity. The HepG2 cells were grown for 24 hours and then incubated for 24 hours at LC_{20} with the different treatments. After incubation, the cells were washed once with PBS and removed by trypsinization. Cells were harvested by centrifugation and resuspended in 1 mL of PBS. Samples were stored in freezer at −20°C until use. After lysed as described previously, samples were centrifugated and resultant

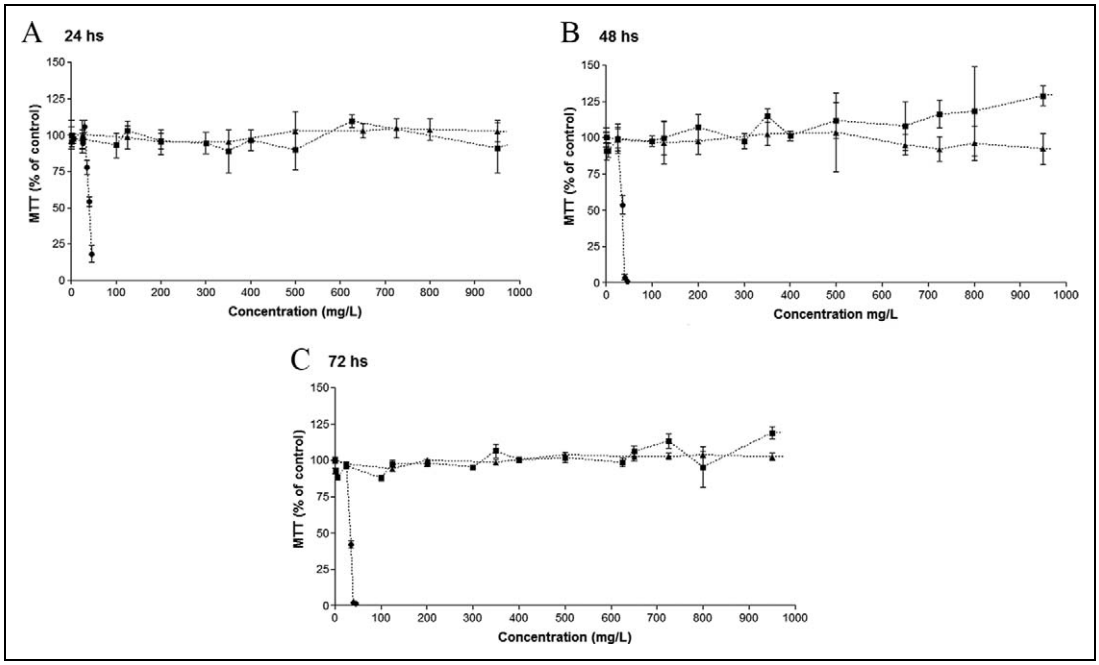

Figure 1. Comparative dose-dependent effects of glyphosate (■), AMPA (▲), and G formulation (●) in HepG2 cells viability after 24 hours (A), 48 hours (B), and 72 hours (C) of exposure, repectively. These effects were evaluated by the MTT test. Data are expressed as mean ± SD (n = 8), relative to control cells (100% viability). AMPA indicates aminomethylphosphonic acid; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; SD, standard deviation.

supernatant was incubated at 37°C in the dark in 50 mmol/L buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4, 10% glycerol, 0.1 mmol/L EDTA, and 10 mmol/L dithiothreitol) with 7.8 mmol/L substrate Ac-DEVD-pNA for caspase 3/7. Blanks were also run containing cell lysate alone. Caspase-catalyzed chromophore pNA release from substrate was measured at 405 nm in a BIO-RAD Benchmark microplate reader (BIO-RAD Laboratories), and results are presented as percentage of control. Protein concentration was determined by Bradford method[36] using bovine serum albumin as a standard.

Statistical Analyses

Each experiment was performed 2 or 3 times in different weeks, and each determination was performed at least 5 times ($n \geq 5$). Statistical analyses were performed using 1-way analysis of variance (ANOVA) followed by Dunnet test using significant levels of $P < 0.05$. Normality and homogeneity of variances were tested with Lilliefors and Bartlett tests, respectively. The LC_{50} value was estimated by nonlinear regression sigmoidal dose-response method. Graph Pad Prism 4 software was used for all statistical analyses.

Results

Glyphosate Formulation Induces Dose-Dependent Cytotoxicity

The HepG2 cell line viability was assayed using MTT assay to examine effects of glyphosate, AMPA, and G formulation at dilutions far below agricultural recommendations; all of them were below 1000 mg/L as mentioned in Materials and Methods section (subagriculture concentrations). Glyphosate and AMPA exposure did not affect cell viability until 1000 mg/L while G formulation treatment induced a rapid decrease in cell viability depending on concentration and duration of exposure (Figure 1). The estimated LC_{50} value was 41.22 (37.65-45.14) mg/L, 35.19 (34.95-35.42) mg/L, and 34.69 (34.08-35.31) mg/L for 24, 48, and 72 hours respectively.

Glyphosate Formulation Modifies the Intracellular Redox Balance

Given the decrease in viability by G formulation, an evaluation of the following parameters related to redox state was performed: antioxidant enzyme activities (SOD, CAT, and GST) and GSH equivalents content, as nonenzymatic antioxidant. The results in Table 1 show a significant increase in SOD activity only in cells treated with G formulation. Significant changes in SOD activity were not observed with glyphosate and AMPA treatments. Catalase activity did not show significant differences between treatments and control. The same result was found for GST activity suggesting that changes in antioxidant enzyme activities are given by the combination of ingredients present in the formulation more than glyphosate itself.

Glutathione participates in detoxification of potentially harmful molecules, such as pesticides or heavy metals. Conjugation process can be accomplished spontaneously or in the

Table 1. Enzymatic and Nonenzymatic Antioxidant and Detoxification Capacity.[a]

	SOD	CAT	GST	GSH
Control	100.0% ± 7.8%	100.0% ± 39.3%	100.0% ± 9.4%	100.0% ± 14.0%
Glyphosate acid	128.7% ± 17.3%	88.1% ± 23.5%	110.2% ± 25.3%	123.6% ± 23.5%
AMPA	148.2% ± 37.0%	100.9% ± 23.0%	121.4% ± 19.2%	181.5% ± 31.3%[b]
G formulation	173.7% ± 66.6%[c]	79.1% ± 15.1%	82.0% ± 19.5%	188.9% ± 36.3%[b]

Abbreviations: AMPA, aminomethylphosphonic acid; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; GST, glutathione-S-transferase; SD, standard deviation; G formulation, glyphosate formulation.
[a] Results are reported as mean ± SD of 5 independent experiments.
[b] Significantly different from control ($P < 0.01$).
[c] Significantly different from control ($P < 0.05$).

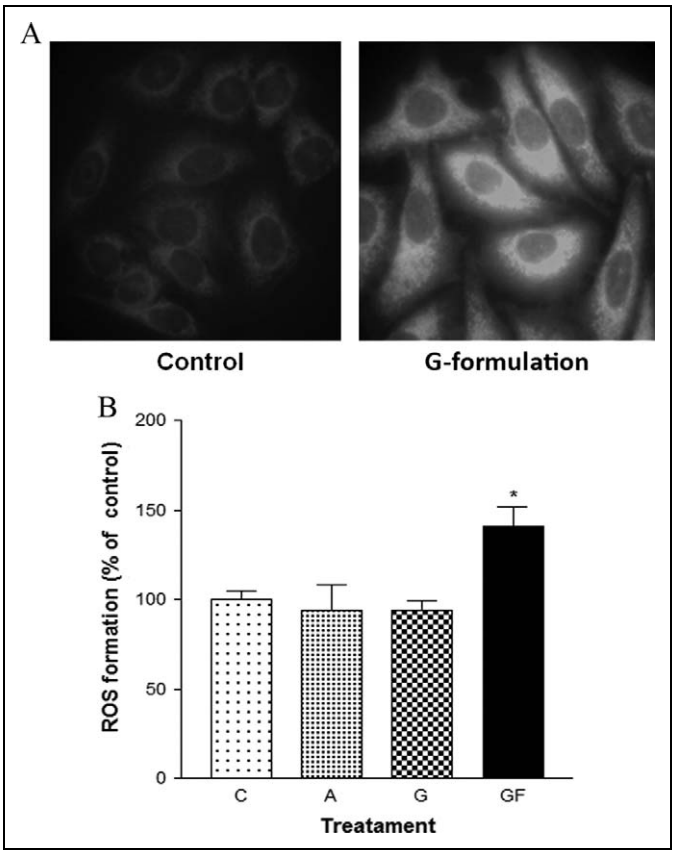

Figure 2. Reactive oxygen species formation by glyphosate (G), AMPA (A), or G formulation (GF) in HepG2 cells. Cultures were exposed to 900 mg/L of glyphosate, 900 mg/L of AMPA, or 40 mg/L of G formulation for 24 hours. A, Cytoplasmic ROS analysis. The ROS production was visualized by fluorescence microscopy using filters B-2A (λexc: 450-490 nm; λem: 515 nm). Control and G formulation treatment: 100×. B, ROS production is presented as percentage compared to untreated cells. C, Results were expressed as mean ± SD (n = 6) and analyzed as FUs per milligram of protein. Significant differences between treatments and control are indicated by *$P < 0.01$. AMPA indicates aminomethylphosphonic acid; exc, excitation; em, emission; ROS, reactive oxygen species; FUs, fluorescence units; SD, standard deviation.

presence of GST. In this work, we found a significant increase in GSH content in cells exposed to G formulation and AMPA treatments compared to control, while no differences were found in glyphosate treatment (Table 1). Altogether, these results indicate that G formulation modifies the intracellular redox balance.

Glyphosate Formulation Induces ROS Formation

Since G formulation-induced cell viability decreased and modified the intracellular redox balance on HepG2 cell line, ROS production was investigated as a possible cytotoxicity mechanism.[37] We worked with the LC_{50} for G formulation treatment because we found that exposure to this concentration resulted in more ROS production by cell number (118.53 ± 5.73, 140.5 ± 11.53, and 116.06 ± 6.56, ROS production for LC_{20}, LC_{50}, and LC_{70} respectively). For AMPA and acid glyphosate treatments, we worked at 900 mg/L. As shown in Figure 2, G formulation LC_{50} treatment showed significantly higher levels of ROS (140% of control) than that of the control. In contrast, neither glyphosate nor AMPA treatment caused differences in ROS formation (Figure 2).

Glyphosate Formulation Induces Tyrosine Nitration

To identify whether nitrosative stress was involved, an experiment testing peroxynitrite formation by G formulation was performed. As shown in Figure 3, G formulation treatment resulted in significant increases in nitrated proteins (140% of control).

Since it is known that peroxynitrite mediates oxidation of cellular targets, significant increases of peroxynitrite protein concentration in cells incubated with nitrite plus hydrogen peroxide were expected (Figure 3). Instead, neither glyphosate nor AMPA treatment caused differences in peroxynitrite formation.

Glyphosate Formulation Induces Apoptotic Cell Death Involving Caspase 3/7 Activation

As G formulation causes cell death at low concentrations, we investigated the contribution of apoptosis through caspase-dependent pathway on this observation. First, we performed nuclear staining with DAPI fluorescent dye to assess changes in nuclear shape and chromatin integrity by fluorescence microscopy. In control cultures, nuclei exhibited normal shape

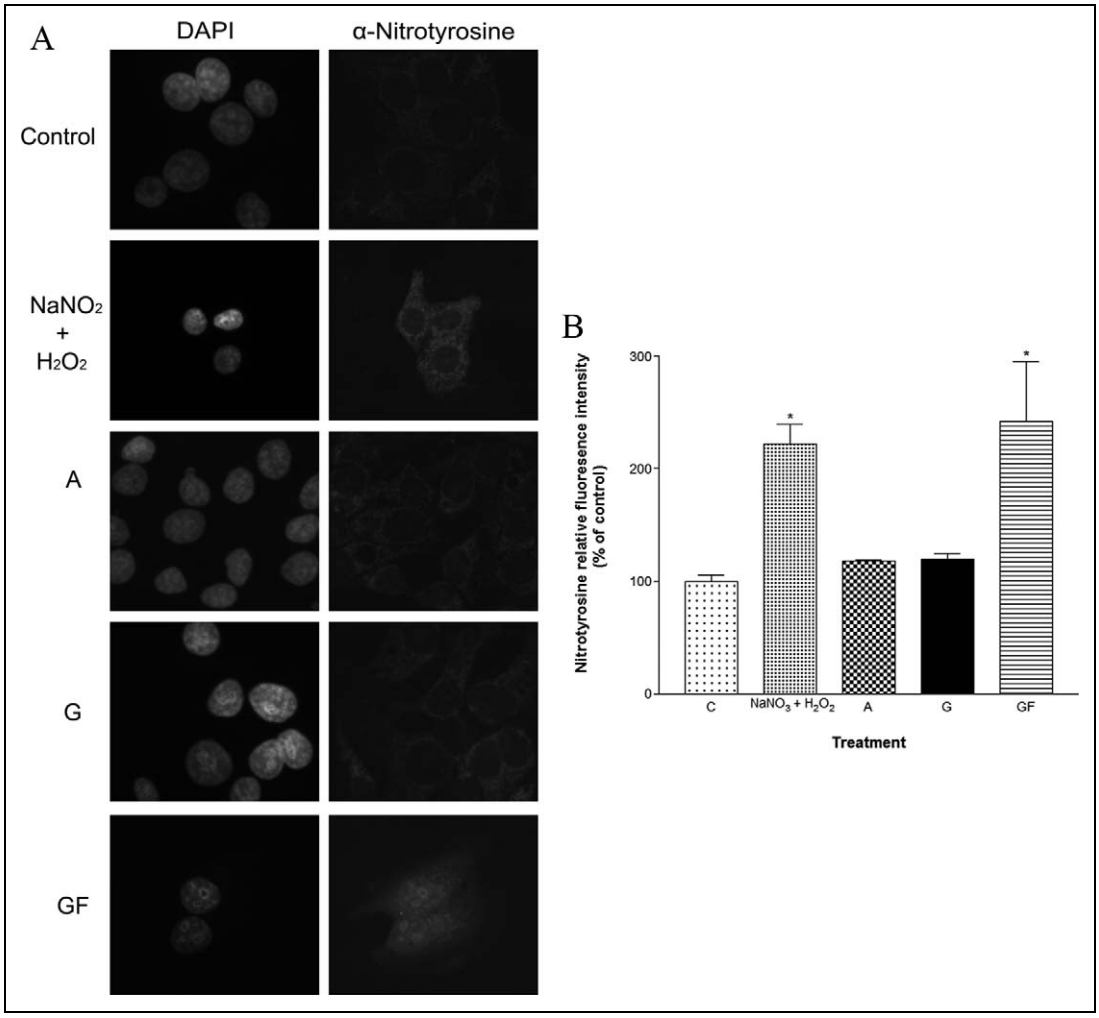

Figure 3. Tyrosine nitration by glyphosate (G), AMPA (A), or G formulation (GF) in HepG2 cells. Cultures were exposed to 900 mg/L of glyphosate, 900 mg/L of AMPA, or 35 mg/L of G formulation for 24 hours. Peroxynitrite proteins (positive control) were induced by 24-hour exposure of cells to 5 mmol/L sodium nitrite plus 1 mmol/L hydrogen peroxide ($NaNO_2 + H_2O_2$). A, Tyrosine nitration was visualized by inverted fluorescence microscopy (Olympus IX71) using a 60×/1.25 NA oil objective and camera. Images were acquired using Micro-Manager software. B, Tyrosine nitration was presented in percentage comparatively to nontreated cells. Results were expressed as mean ± SD (n = 3). Significant differences between treatments and control are indicated by $^*P < 0.01$. AMPA indicates aminomethylphosphonic acid; SD, standard deviation.

and uniformly stained chromatin (Figure 4A). However, when cells were exposed to G formulation, a 23.5% of the nuclei showed condensed and fragmented chromatin ($P < 0.01$), a typical feature of apoptotic cell death (Figure 4B). In the same way, we found that caspases 3/7 are significantly activated with LC_{20} concentration of G formulation (35 mg/L), up to 100% while its activity was not modified by glyphosate alone or its main breakdown product, AMPA (Figure 5). These results are consistent with cell death obtained in MTT assay, and all these data together suggest that cell death induced by G formulation is predominantly through a caspase-dependent apoptotic pathway.

Discussion

In order to provide evidence of glyphosate-induced cytotoxicity on hepatic cells, we compared the action of glyphosate, AMPA, the main breakdown product, and a G formulation. We found that G formulation induces dose-dependent cytotoxicity, while we did not find toxic effects with acid glyphosate and AMPA at assayed concentrations. These results are consistent with the concept that additives in commercial formulations play a role in toxicity attributed to herbicides.[10] Furthermore, the G formulation LC_{50} value determined in HepG2 cells in this work was 100 times below the agriculture concentration (3.73 mg/L, Roundup UltraMax[28]). Also, Benachour and Séralini[13] found that, for all Roundup formulations assayed, human umbilical cell, embryonic cell, and placental cell mortalities were not linearly linked to glyphosate concentration. In addition, these authors tested the effect of the supposed inert product polyethoxylated tallow amine present in the formulation of Roundup and they found that this compound produced cytotoxicity. Benachour and Séralini[13] found that AMPA and glyphosate concentrations 10 times higher than those used in this work promote cell death. Other recent works have also

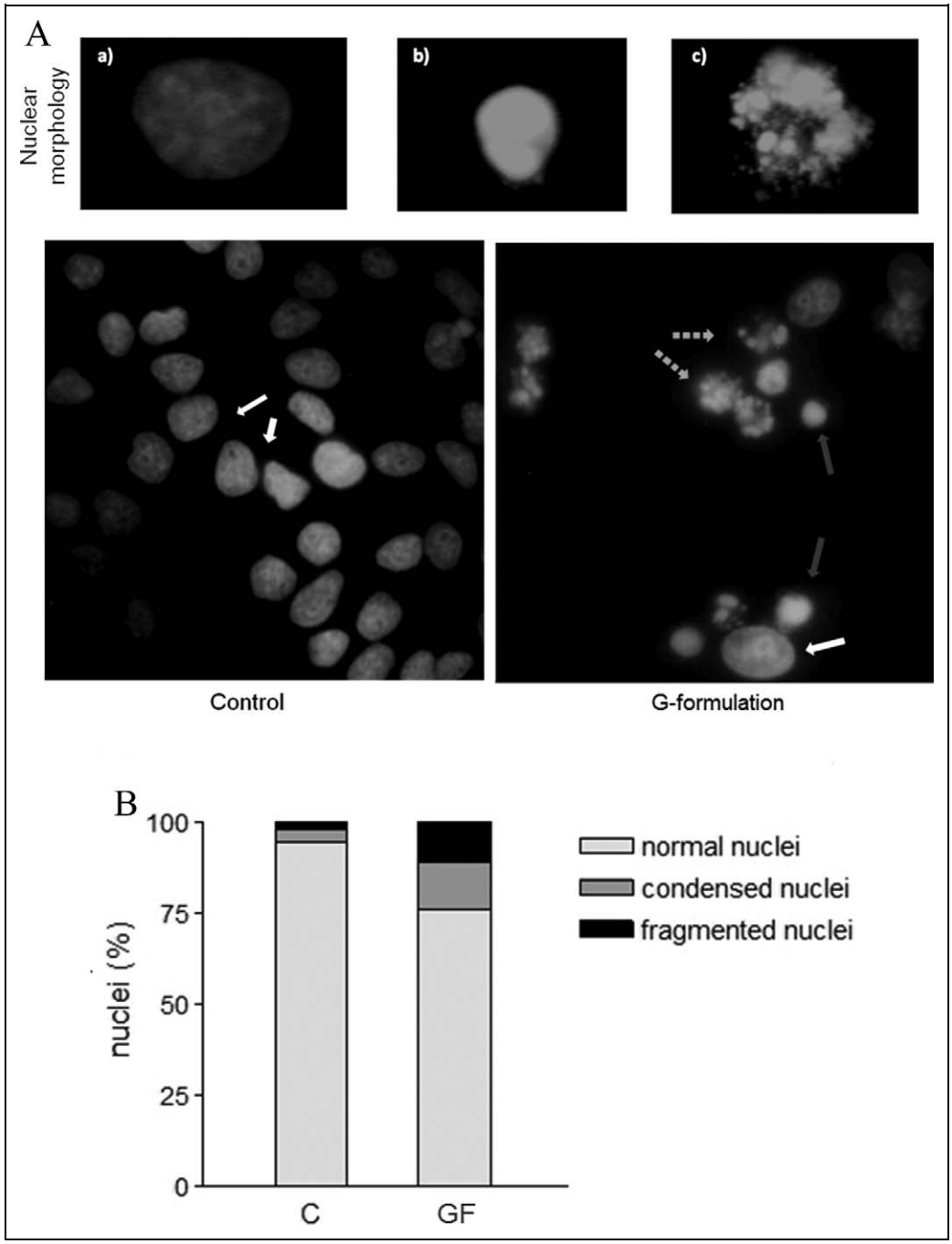

Figure 4. HepG2 cells were exposed to vehicle (C) or LC_{20} of G formulation (GF) for 24 hours. A, Nuclear morphology analysis. Nuclear DNA was stained with DAPI dye and visualized by fluorescence microscopy using filters for DAPI (λexc: 330-380 nm; λem: 435-485 nm). Apoptotic nuclei were visualized as hyperfluorescent, more condensed and smaller than normal nuclei (magnification 1000×). White arrows show normal chromatin, gray arrows show condensed chromatin, and the dotted arrows show fragmented nuclei. B, Percentages of nuclei in each state (normal, condensed, and fragmented) function of treatments (control or G formulation). The results are given as mean of 7 independent experiments ($n = 7$); the standard deviation is evaluated for each cell percentage. LC_{20} indicates lethal concentration 20; DAPI, 4′,6-diamino-2-phenylindole dihydrochloride; exc, excitation; em, emission.

showed that G formulations toxicity is dependent on adjuvants present in commercial mixtures.[14,15,21]

Then, we evaluated the involvement of oxidative stress in cytotoxicity. It has been reported that many pesticides (including herbicides) generate intracellular ROS.[11,16-18] We demonstrated that the increased ROS trigger oxidative damage to proteins, nucleic acids, and lipids as well as the increase in activity of different antioxidant enzymes.[19,20] Free radical scavengers with antioxidant properties in animal cells compensate for damaging effects caused by reactive free radicals. Antioxidant enzymes

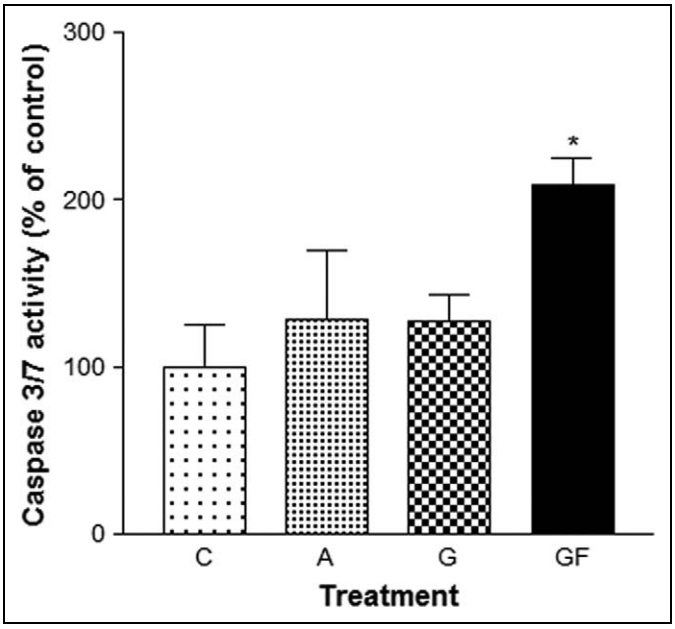

Figure 5. Caspase 3/7 activity. HepG2 cells were exposed to vehicle (C) or LC_{20} of G formulation (GF) for 24 hours. The effect was evaluated by the substrate Ac-DEVD-pNA for caspase 3/7, and the results are presented in percent compared to untreated cells (% of control). Results were expressed as mean $\pm$ SD ($n = 4$). Significant differences between treatments and control are indicated by $^*P < 0.01$. Ac-DEVD-pNA indicates acetyl-Asp-Glu-Val-Asp-p-nitroanilide; LC_{20}, lethal concentration 20; SD, standard deviation.

such as SOD and CAT constitute the major defensive system against ROS formation.[20] Glutathione-S-transferase is also recognized as an important catalyst in xenobiotic biotransformation, including drugs, environmental pollutants, and by-products of oxidative stress.[38]

A significant increase in SOD activity and GSH levels (for G formulation but not for acid glyphosate) was observed. No differences between control and treatment groups were observed for CAT and GST activities in HepG2 cells. Our results agree with previous findings[39-42] that observed a decrease or no alteration in CAT activity when cells were treated with G formulation. These results could be explained at least in 2 ways, first, inactivation of CAT activity resulting in accumulation of intracellular ROS or second, CAT is a less sensitive biomarker for oxidative stress than SOD when HepG2 cells were exposed to G formulation. In the present work, cells exposed to the G formulation showed no variation in GST activity, which might indicate that the metabolism of the compounds present in G formulation occurs by other biotransformation pathways. Thus, although CAT and GST activities showed no alteration, the hypothesis that exposure to G formulation generates ROS cannot be discarded, since other parameters of the antioxidant response have been affected in this work. It was demonstrated that increases in SOD activity and GSH levels induced by oxidative stress may be linked to adaptive responses.[43-45] These adaptive responses could depend on the studied system, the glyphosate concentrations, and formulations. Glutathione increase found in the cell line could be related to an induction of γ-glutamylcysteine synthetase, the enzyme that controls the biosynthesis of GSH, or to an increase in the levels of reduced GSH as a result of an increase in activity of GSH reductase.

We evaluated for the first time whether exposure to G formulation increases ROS formation. We found an important increase in ROS production (140% of control) in cells treated with G formulation. Neither acid glyphosate nor AMPA treatment caused differences in ROS formation. There is a single recent work in the literature, which measures ROS formation as a result of pure glyphosate exposure using the H2DCFDA dye method.[46] These authors observed that glyphosate provokes ROS production in a dose dependent manner. It should be taken into account that the concentrations of glyphosate used in this study were at least 2 times higher than those used in that study.

In addition to the increase in ROS, it was observed high levels of nitrotyrosine when cells were treated with G formulation as measured by immunofluorescence. The formation of 3-nitrotyrosine represents a specific peroxynitrite-mediated protein modification. Peroxynitrite is a strong oxidant formed by reaction of nitric oxide with superoxide. As known, high levels of nitrotyrosine can lead to loss or alteration in protein function and are associated with a large numbers of diseases.

Reactive oxygen species play a critical role in apoptosis signalling. Glutathione acts as a major antioxidant against free radicals and it was shown that GSH is involved in apoptosis induction modulation.[47,48] Also, increase in GSH at earlier stages can be considered as a cell response (protective) mechanism.

Increases in GSH levels in AMPA treatment could be an adaptive single response since no other parameter was affected. In this work, the increase in GSH, induced by G formulation, triggers caspase 3/7 activation and hence induced apoptosis pathway. However other works showed that this activation was switched on by depletion of GSH.[45,49] In this report, we demonstrate that cell death induced by G formulation is predominantly apoptotic, as determined by DAPI staining and caspase-3/7 activation. We found a 23.5% increase in morphological changes (condensed and fragmented nuclei) consistent with apoptotic cell death using G formulation at LC_{20}. These results agree to those found by Gui et al[50] who demonstrated that glyphosate induced both apoptotic and autophagic cell death in PC12 cells as a neuronal model.

Aminomethylphosphonic acid only caused an adaptative response that was not observed in glyphosate treatment at the same doses. However, Benachour and Séralini[13] found that AMPA is more toxic than glyphosate in human umbilical, embryonic, and placental cells, targeting their cell membranes. Furthermore, it was demonstrated that AMPA has a potential genotoxicity.[51] The comparative study of the breakdown products and their parent compounds is important because some metabolites present higher toxic effects than their parent compounds, and these are often detected in the environment.[3]

Although it is known that *in vtro* studies do not take into consideration the toxicokinetics of chemicals (absorption,

distribution, metabolism, and excretion), these studies are useful to evaluate the mechanism of action of G formulation.

In conclusion, our results clearly demonstrated that G formulation induced cytotoxicity, ROS production, antioxidant defense induction, and apoptosis at subagriculture concentrations. This indicates that G formulations have adjuvants that, together with the active ingredient, cause toxic effects not observed with glyphosate itself. Considering that G formulation LC_{50}, obtained in this work, was 100 times lower than the concentration used in agricultural praying, agricultural workers and rural populations are the group at greatest risk. Taking into account the great deal of controversy about the risk of glyphosate-containing herbicide exposure,[5,6,51-53] more investigation on this area should be necessary to understand the effect of chronic exposure on human health. The effects observed by G formulation on HepG2 cells may provide evidence of cytotoxicity related to oxidative stress and cellular end points.

Declaration of Conflicting Interests

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