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Influence of the spray adjuvant on the toxicity effects of a glyphosate formulation

Isis Coalova, María del Carmen Ríos de Molina, Gabriela Chaufan *

Departamento de Química Biológica, IQUIBICEN – CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires, Argentina

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ABSTRACT

In the present study, the influence of the spray adjuvant on the toxicity effects of a glyphosate formulation was examined in HEp-2 cell line. We determined the median lethal concentration (LC_{50}) of Atanor® (glyphosate formulation), Impacto® (spray adjuvant) and the mixture of both agrochemicals. We also compared the toxicities of the pesticides individually and in mixture and we analyzed the effects on oxidative balance from each treatment.

Our results showed that all the agrochemicals assayed induce dose and time-dependent cytotoxicity and that the toxicity of Impacto® with Atanor® (mixture) was additive on HEp-2 cell line.

All the agrochemicals assayed produced an increase in catalase activity and glutathione levels, while no effects were observed for superoxide dismutase and glutathione-S-transferase activities. We found an important increase in ROS production in cells treated with Atanor® and mixture. Besides, all the agrochemicals used triggered caspase 3/7 activation and hence induced apoptosis pathway in this cell line. In conclusion, our results demonstrated that the addition of adjuvant to glyphosate formulation increase the toxicity of the mixture in cell culture. Furthermore, cell culture exposed to agrochemical mixture showed an increased ROS production and antioxidant defenses.

1. Introduction

An adjuvant is an agent that modifies the effect of other agents. In the particular case of agrochemicals, an adjuvant is generally broadly defined as any substance separately added to a pesticide product that will improve the performance of the pesticide product (US EPA adjuvant). Depending on the usage, adjuvants can be divided into two general types: formulation adjuvants and spray adjuvants. The first type consists of adjuvants which are part of the formulation, while the second type, called tank mix adjuvants, are added in the spray tank along with the pesticide just before application on the field. Adjuvants comprise a large and heterogeneous group of substances, in which surfactants (surface-active substances) and especially non-ionic surfactants make up the largest group (Krogh et al., 2003; Nobels et al., 2011). Surfactants have a hydrophobic and hydrophilic component and they are classified as nonionic, anionic, cationic, or amphoteric depending on the nature of their hydrophilic component (Song et al., 2012a,b).

Due to the extended use of non-ionic surfactants in a wide range of domestic, industrial and agriculture applications these substances and its metabolites have been frequently detected in different environmental compartments (soil, water, sediment) and in our food chain (Chen et al., 2010; Tubau et al., 2010; She et al., 2012; Guenther et al., 2002; Björklund et al., 2009; Soares et al., 2008; Fiedler et al., 2007; Fernández Cirelli et al., 2008). Many studies have reported the toxicity of these substances in several models (Liwarska-Bizukojc et al., 2005; Li, 2008; Jahan et al., 2008; Song et al., 2012a,b). However, there is still much to be done to understand the underlying mechanisms in the case of mixtures with pesticides.

Particularly in Argentina, the major pesticides applied are glyphosate-based herbicides, like the formulation Atanor® (48% glyphosate as isopropylamine salt) which is usually combined with spray adjuvants like Impacto® (alkyl aryl polyglycol ether or alkylphenol ethoxylate) (Table 1) (Romero et al., 2011).

Although glyphosate is known to be minimally toxic to humans (Williams et al., 2000; De Roos et al., 2005; Acquavella et al., 2004) it is well documented that can induce cytotoxicity, oxidative damage and apoptosis in several models (Gasnier et al., 2010; El-Shenawy, 2009; Romero et al., 2011; Alvarez-Moya et al., 2011; Poletta et al., 2011; Sandler and Alavanja, 2005).

* Corresponding author. Address: Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, 2° Pabellón, 4° piso, Ciudad Autónoma de Buenos Aires, CP 1428, Argentina. Tel./fax: +54 11 4576 3342.
E-mail address: gchaufan@qb.fcen.uba.ar (G. Chaufan).

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Table 1
Description of Impacto® surfactant and Atanor® glyphosate formulation.

Common name	Chemical abstract name	Molecular formula	Hydrophilic part of the surfactant
Impacto®	Alkyl aryl polyglycol ether or alkylphenol ethoxylate	$R\text{-}C_6H_4\text{-}[OCH_2CH_2]_n\text{-}OH$	Nonionic surfactant
Atanor®	Glyphosate as isopropylamine salt	$C_3H_9N\text{-}C_3H_8NO_5P$	–

Cultures of human cells could offer a good option as in vitro models to evaluate the potential effect of toxic compounds in humans.

In the present study we investigated the influence of the spray adjuvant on the toxicity effects of a glyphosate formulation. Therefore, the objectives of this research included the following: (1) to determine the LC_{50} of Atanor®, Impacto® and the mixture (2) to compare the toxicities of the pesticides individually and in mixture and (3) to analyze the induction of oxidative stress from each treatment.

2. Materials and methods

2.1. Reagents

Modified Eagle's medium (MEM), MEM vitamin solution, MEM non-essential 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-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and 2′,7′dichlorodihydrofluorescein diacetate (H_2DCFDA) were from Sigma Chemical Co. (St. Louis, MO, USA). The commercially available herbicide used in this study was 48% (p/v) Glyphosate (isopropylamine salt of N-phosphonomethylglycine) Atanor® (Atanor, Munro, Buenos Aires province, Argentina) and the surfactant was alkyl aryl polyglycol ether 50% Impacto® (AGROASISTS.R.L., Argentina). The concentration of Impacto® was 2.5% v/v in water or Atanor,® as recommended by the manufacturer. Treatment medium was prepared in serum-free medium, and adjusted to pH. 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%. DMSO 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).

2.2. Cell culture

The human cell line HEp-2 was obtained from the Asociación Banco Argentino de Células (Ciudad Autónoma de Buenos Aires, Argentina) and was cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (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 to 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/0.3 cm^2), and ROS formation detection assay was performed into 24-well plates (0.5 ml; 7.5×10^4/2 cm^2). For the other experiments, cells were replated in cell culture T-Flasks (4 ml; 3×10^6 cells/25 cm^2).

2.3. MTT assay

The method employed was described by Mossman (1983). Briefly, cells were exposed from 24, 48 and 72 h in serum-free medium to different dilutions of glyphosate Atanor®, adjuvant Impacto® alone and mixture (Atanor® plus 2.5% v/v Impacto®), far below agricultural recommendations (sub-agriculture concentrations). Following incubation, different treatment medium were removed; cells were washed with 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 crystals formation, with a 50% decrease in these crystals when media with 5–10% FBS is used (Talorete et al., 2007). The plates with added MTT solution were then placed in the 5% CO_2 incubator for 90 min at 37 °C. 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 substraction 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.

2.4. Antioxidant enzyme activities

For enzyme activities determination (Catalase, *Glutathione-S-transferase and Superoxide dismutase*) cells were grown at confluence with the different treatments for 24 h at concentrations that never induced cell viability bellow 80% (LC_{20}). Determinations were carried out in 11,000*g* supernatants from cells lysates.

Catalase (CAT, EC 1.11.1.6) activity was determined by following hydrogen peroxide decomposition at 240 nm, in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 3 M hydrogen peroxide (Aebi, 1984). Results were expressed as percentage of control (100% of activity).

Glutathione-S-transferase (GST, EC1.11.1.9) activity was measured by Habig et al. (1976) technique. Briefly, standard assay mixture in 100 mM phosphate buffer (pH 6.5) contained: enzymatic sample, 100 mM GSH solution, and 100 mM 1 chloro-2,4 dinitrobenzene (CDNB) in ethanol to a final volume of 0.8 ml. After adding CDNB, change in absorbance at 340 nm was followed for 120 s. 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 (SOD EC 1.15.1.1) activity was measured using a modify procedure in microplate of Beauchamp and Fridovich (1971). The standard assay mixture contained enzymatic sample, 0.1 mM EDTA, 13 mM DL-methionine, 75 μM nitro blue tetrazolium (NBT) and 2 μM riboflavin, in 50 mM phosphate buffer (pH 7.9), to a final volume of 0.3 ml. Samples were exposed to intense cool white light for 5 min. One SOD unit was defined as the enzyme amount necessary to inhibit 50% the reaction rate. Results were expressed as percentage of control.

2.5. GSH equivalents content

GSH levels were measured in HEp-2 cells following the Anderson (1985) procedure, 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 before. For the GSH determination we proceeded as Sabatini et al. (2009). Results were expressed as percentage of control.

2.6. Detection of ROS

ROS formation was measured using the cell permeable indicator 2′,7′dichlorodihydrofluorescein diacetate (H_2DCFDA). Cellular esterases hydrolyze the probe to a 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 (DCF) (Halliwell and Whiteman, 2004) which can be easily visualized as strong fluorescence at 525 nm when excited at 488 nm. The stock solution of 1 mM H_2DCF-DA was prepared in DMSO, stored at −20 °C, and protected from light. Briefly, after being incubated for 24 h in 24-well plates at early confluence (80–90% confluent) with different treatments, cells were washed with PBS and incubated with freshly prepared 30 μM H_2DCF-DA solution with serum-free MEM at 37 °C for 60 min. After incubation cells were collected, washed, and resuspended in 1 ml of PBS. After being lysed by sonication for 5 s intervals at 40 V setting in ice, samples were centrifugated at 11,000*g* for 15 min. 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).

2.7. Caspase activity

HEp-2 cells were grown for 24 h and then incubated for 24 h at LC_{20} with the different treatments. After incubation 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. Once lysed as previously described, samples were centrifugated and resultant supernatant was incubated at 37 °C in the dark in 50 mM buffer HEPES (pH 7.4, 10% glycerol, 0.1 mM EDTA and 10 mM DTT) with 7.8 mM substrate Ac-DEVD-pNA for caspase 3/7. Blanks were also run containing cell lysate alone. Caspase-catalyzed chromophore pNA released from substrate was measured at 405 nm in a BIO-RAD Benchmark microplate reader (BIO-RAD Laboratories, Hercules, CA) and results were presented as percentage of control.

Protein concentration was determined by Bradford's method (1976) using bovine serum albumin as a standard.

2.8. Statistical analyses

Each experiment was performed two or three times in different weeks, and each determination was performed at least for quintupled ($n \geqslant 5$). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnet's using significant levels of $p < 0.05$. Normality and homogeneity of variances were tested with Lilliefors and Barlett test, 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.

To assess the mixture toxicity of the compounds, the following formula was used (Loewe 1927, 1928; Marking and Dawson, 1975):

$$S = A_m/A_i + B_m/B_i$$

A_m is the LC_{50} for pesticide *A* in mixture, A_i the L_{C50} for pesticide *A* individually, B_m the LC_{50} of pesticide *B* in mixture, B_i the LC_{50} of pesticide *B* individually, and *S* the sum of biological activity.

Using *S*, then "additive", "less than additive" and "greater than additive" effects for the mixtures were determined from the following formulas:

$$\text{If } S \leqslant 1.0, \text{ then the additive index} = 1/S - 1,$$

$$\text{If } S \geqslant 1.0 \text{ then the additive index} = S(-1) + 1,$$

To determine whether the range for additive indices overlapped zero (simple additive toxicity) the 95% confidence intervals from the LC_{50s} (Table 2) were substituted into the additive formula to establish a range (Marking and Dawson, 1975). Mixture ranges overlapping zero equaled an additive effect (expected action). Mixture ranges less than zero equaled a less than additive effect (antagonism). Mixture ranges greater than zero indicated a greater than additive effect (synergism).

3. Results

3.1. Atanor®, Impacto® and mixture induce dose-dependent cytoxicity

In order to investigate the cytoxicity effects of Atanor®, Impacto® and the mixture Atanor® plus Impacto® HEp-2 cell line was exposed to increasing concentrations of the agrochemicals for 24 h and cell viability was analyzed using MTT assay. All the agrochemicals assayed induced a decrease in cell viability depending on concentration and time exposure (Fig. 1).

The HEp-2 cell viability vs. time was assayed using MTT assay to analyze effects of Atanor®, Impacto® and the mixture Atanor® plus Impacto® at 24, 48 and 72 h. Atanor® LC_{50} had a time dependence decrease but did not show significant differences, while adjuvant Impacto® and mixture LC_{50} showed a significant decrease ($p < 0.05$) at 48 h, remaining constant up to 72 h (Fig. 1). Also, adjuvant addition to Atanor® formulation (mixture) showed higher cytoxicity compared to Atanor® alone (Table 2). While Impacto® exhibited lower LC_{50} than Atanor alone.

3.2. Mixture toxicity tests

To understand the role of adjuvant cytotoxicity in the mixture (Athanor® plus Impacto®), we proceeded to calculate the additive index and the 95% confidence intervals as we explained in Section 2 (Table 3). Mixture resulted in significantly higher toxicity to cell cultures compared to each agrochemical alone suggest additive effect. With $S = 0.867$, 0.933 and 0.892 (24, 48 and 72 h respec-

Table 2
Toxicity values (mg/L) for HEp-2 cell line exposed to Atanor®, Impacto® and mixture Atanor® + Impacto® for 24, 48 and 72 h.

Agrochemicals	LC_{50} (mg/L)			NOEC (mg/L)		
	24 h	48 h	72 h	24 h	48 h	72 h
Atanor® alone	376.4 (356.1–398.0)	323.1 (306.4–340.9)	267.0 (259.2–274.8)	300	200	133.3
Impacto® alone	12.1 (10.1–14.5)	4.8 (4.5–5.2)	4.8 (4.7–5.0)	1.6	3.7	3
Atanor® in mixture[a]	180.2 (155.0–210.0)	110.2 (95.5–127.2)	98.6 (88.6–111.7.1)	91.7	75	75
Impacto® in mixture[a]	4.7 (4.0–5.5)	2.9 (2.4–3.3)	2.6 (2.3–2.9)	2.4	1.9	2.0

LC_{50} indicates median lethal concentration. NOEC indicates no observed effect concentrations. LC_{50} was estimated by nonlinear regression sigmoidal dose–response method. NOEC was derived from no observed statistically effect concentration. LC_{50} and NOEC were presented as mean of three independent analyses.

[a] Atanor® in mixture or Impacto® in mixture, represent the concentration values corresponding to Atanor® or Impacto® in the mixture (Atanor® plus 2.5% v/v Impacto®) of such compounds.

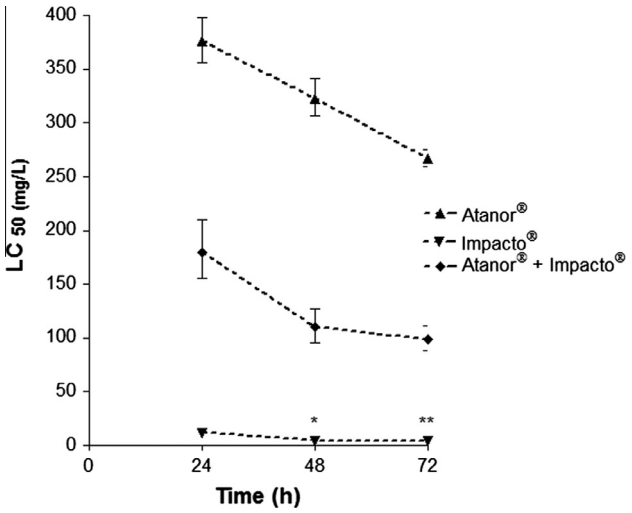

Fig. 1. Time dependence of LC_{50} values for HEp-2 cells exposed to Atanor®, Impacto® and mixture. These effects were evaluated by the MTT test. Data are expressed as mean + SD ($n = 8$), relative to control cells (100% viability).

tively) and an index range encompassing 0, the Atanor®/Impacto® mixture was considered to be additive (Table 3).

3.3. Intracellular redox state

Since the agrochemicals induced cell viability decreased, parameters related to redox state was performed: antioxidant enzyme activities (SOD, CAT, and GST) and GSH equivalents content, as nonenzymatic antioxidant (Table 4). SOD and GST activities did not show significant differences between treatments and control. The results in Table 4 show a significant increase in CAT activity for all the treatments. We also found a significant increase in GSH content in cells exposed to the different treatments compared to control (Table 4). These results indicate that agrochemical treatment modifies the intracellular redox balance reflected in GSH and CAT activity increase, without affecting SOD or GST activities.

3.4. Induction of ROS formation

Given the decrease in viability and the additive effect by the addition of adjuvant to the mixture, ROS production was investigated as a possible source of toxicity. We worked with the LC_{50} for all agrochemicals treatment because we found that exposure to this concentration resulted in more ROS production by cell number. As shown in Fig. 2, Atanor® formulation and mixture treatment showed significantly higher levels of ROS (139% and 116% of control, respectively) than that of the control. In contrast, Impacto® treatment caused no differences in ROS formation.

3.5. Agrochemicals induces apoptotic cell death involving caspase 3/7 activation

To determine whether apoptosis was involved in cell death, we investigated the contribution of caspase 3/7 dependent pathway on this event. 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 and uniformly stained chromatin. However, when cells were exposed to Atanor®, a 17.7% of the nuclei showed condensed and fragmented chromatin ($p < 0.001$) (Fig. 3), a typical feature of apoptotic cell death.

Nevertheless, we found that caspases 3/7 are significantly activated with LC_{20} concentration of all agrochemicals, what could be indicating that oxidative stress triggers caspase activation. It is observed that with all treatments assayed caspase 3/7 activity is significant higher than control, but cause morphological changes only Atanor® treatment (Figs. 3 and 4).

4. Discussion

In order to provide evidence of influence of spray adjuvant on the glyphosate formulation toxicity, we determined the LC_{50} of Atanor®, Impacto® and the mixture, on HEp-2 cell line.

We have found that all the agrochemicals assayed induce dose and time-dependent cytotoxicity. What is more, adjuvant addition to Atanor® formulation (mixture) showed higher cytoxicity compared to Atanor® alone (Table 2), while Impacto® exhibited lower LC_{50} than Atanor® alone. These results are consistent with the concept that adjuvant by themselves or in commercial formulations, play a role in toxicity attributed to herbicides (Liwarska-Bizukojc et al., 2005; Li, 2008; Jahan et al., 2008; Song et al., 2012a,b).

As with other xenobiotics found in the environment, the effect of an agrochemical mixture on an organism comes into question.

Table 3
Additive indices for HEp-2 cells treated with Atanor®/Impacto® mixture.

Agrochemicals	Time (h)	*S*	Additive index	Index range	Mixture toxicity
Atanor® and Impacto®	24	0.867	0.153	(−0.117)–(0.498)	Additive
	48	0.933	0.072	(−0.128)–(0.330)	Additive
	72	0.898	0.113	(−0.050)–(0.284)	Additive

Table 4
Enzymatic and non-enzymatic antioxidant and detoxification capacity.

	GST (%)	SOD (%)	CAT (%)	GSH (%)
Control	100.0 ± 8.0	100.0 ± 19.4	100.0 ± 15.4	100.0 ± 18.4
Impacto®	87.3 ± 21.0	122.2 ± 29.0	138.0 ± 16.4*	183.6 ± 7.6**
Atanor®	93.8 ± 14.0	108.7 ± 37.1	157.3 ± 24.6**	136.9 ± 12.1**
Mixture	94.3 ± 15.8	94.4 ± 20.2	138.6 ± 21.2**	130.8 ± 13.1*

HEp-2 cells were exposed to LC_{20} of Atanor®, Impacto® or mixture (Athanor® and Impacto®) for 24 h.
Results are reported as mean ± S.D. of 5 independent experiments. Significant differences between treatments and control are indicated by $^{*}p < 0.05$ or $^{**}p < 0.01$.

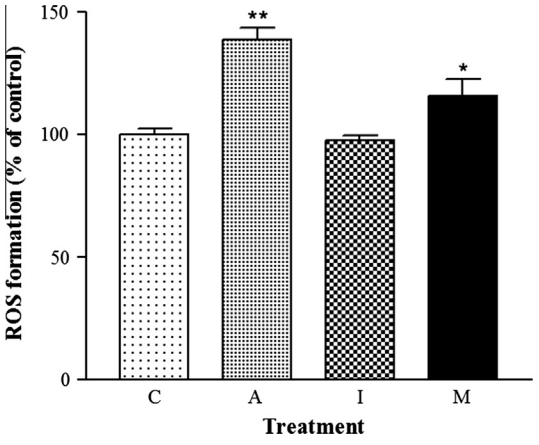

Fig. 2. Reactive oxygen species (ROS) formation by vehicle (C), Atanor® (A), Impacto® (I), or mixture (M) in HEp-2 cells. Cultures were exposed to LC_{50} of each formulation for 24 h. ROS production was presented in percentage comparatively to non-treated cells (C). Results were expressed as mean ± S.D. ($n = 6$), and analyzed as fluorescence units (FU) per milligram of protein. Significant differences between treatments and control are indicated by * $^{**}p < 0.01$ and $^{*}p < 0.05$.

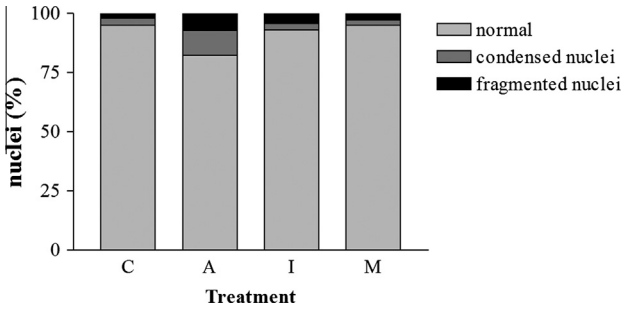

Fig. 3. HEp-2 cells were exposed to vehicle (C) or LC_{20} of Atanor® (A), Impacto® (I), and mixture (M) for 24 h. Percentages of nuclei in each state (normal, condensed, and fragmented) function of all treatments. The results are given as mean of 9 independent experiments ($n = 7$); the standard deviation is evaluated for each cell percentage.

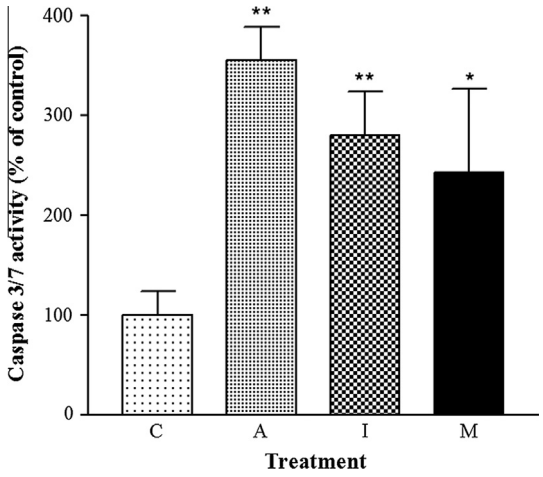

Fig. 4. Caspase 3/7 activity. HEp-2 cells were exposed to vehicle (C) or LC_{20} of Atanor® (A), Impacto® (I), and mixture (M) for 24 h. The effect was evaluated by the substrate Ac-DEVD-pNA for caspase 3/7, and the results are presented in percent compared to untreated cells (% of control). Results were expressed as mean + SD ($n = 5$). Significant differences between treatments and control are indicated by $^{**}p < 0.01$ and $^{*}p < 0.05$.

This is especially true with glyphosate-based herbicides which are used intensively in Argentina. Herbicide combinations with spray adjuvants (like Atanor® and Impacto®) are considered potentially useful in controlling weeds (Romero et al., 2011). The additive relationship between the formulation and adjuvant was at least in part due to the surfactant's ability to facilitate adsorption of herbicide into cells. In this regard, we found that toxicity of Impacto® with Atanor® (mixture) was additive on cell culture (Table 2). Moreover, Deneer (2000) reported that the effects of most herbicide mixtures on aquatic organisms were additive in nature. Moreover, Lajmanovich et al. (2013) described synergism for toxicity when exposed Rinella arenarum tadpoles to herbicides mixture. In our study we used a glyphosate-formulation combined with adjuvant, while Lajmanovich's study combined two types of herbicide formulations. There are very few studies in which the joint toxicity of glyphosate with the addition of adjuvants are analyzed (Romero et al., 2011; Song et al., 2012a,b; Mesnage et al., 2013; Bernal et al., 2009).

It has been reported that many pesticides (including herbicides) generate intracellular ROS. We evaluated the involvement of oxidative stress in cytotoxicity, and 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 (Chaufan et al., 2006, 2014; Sabatini et al., 2009). Free radical scavengers with antioxidant properties in animal cells compensate for damaging effects caused by reactive free radicals. We also demonstrated that antioxidant enzymes such as SOD and CAT constitute the major defensive system against ROS formation (Sabatini et al., 2009; Chaufan et al., 2014) and that glutathione-S-transferase is also recognized as an important catalyst in xenobiotic biotransformation, including drugs, environmental pollutants, and by-products of oxidative stress (Hayes et al., 2005; Chaufan et al., 2014).

A significant increase in CAT activity and GSH levels for all agrochemicals was observed. No differences between control and treatment groups were observed for SOD and GST activities in HEp-2 cells. This result agrees with our previous findings that observed an increase in GSH levels when cells were treated with other glyphosate formulation (Chaufan et al., 2014).

Other studies have reported that increases or decreases in enzymatic activities, induced by oxidative stress, not only depended on toxic source but also of cell culture used (Chaufan et al., 2014; Gasnier et al., 2010; Gehin et al., 2006; Halliwell and Whiteman, 2004). We evaluated whether the addition of adjuvant to the mixture increases ROS formation. We found an important increase in ROS production (139% and 116% of control, $p < 0.05$) in cells treated with Atanor® and mixture respectively. These results agree with our previous findings and others (Chaufan et al., 2014; Heu et al., 2012) where ROS formation in different type of cells was measured. All together these findings demonstrated that Impacto® and Atanor® cause changes to redox state and ROS production as shown by studies (Heu et al., 2012; Gehin et al., 2006; Li, 2008; Okai et al., 2004; Gong and Han, 2006).

In addition to the increase in ROS, we observed high levels in caspase 3/7 activity with all the agrochemicals used. In this area we demonstrated that cell death induced by glyphosate formulation is predominantly apoptotic, as determined by DAPI staining and caspase-3/7 activation (Chaufan et al., 2014). However, the addition of adjuvant to the glyphosate formulation did not increase morphological changes (condensed and fragmented nuclei) consistent with apoptotic cell death.

In conclusion, our results demonstrated that the addition of adjuvant to glyphosate formulation increase the toxicity of the mixture in cell culture. Furthermore, cell culture exposed to mixtures, frequently used in Argentina, showed an increase of ROS production and antioxidant defenses.

Taking into account these results more investigation on this area should be necessary to understand the possible additive or synergistic effect of agrochemical mixture with spray adjuvants.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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