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

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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).

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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.