Ecotoxicology and Environmental Safety 162 (2018) 201–207

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Toxicity induced by glyphosate and glyphosate-based herbicides in the zebrafish hepatocyte cell line (ZF-L)

Fernanda Moreira Lopes[a], Juliana Zomer Sandrini[a,b], Marta Marques Souza[a,b,*]

[a] Programa de Pós Graduação em Ciências Fisiológicas, Universidade Federal do Rio Grande, Av Itália km 8, 96203-900 Rio Grande, RS, Brazil
[b] Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Av Itália km 8, 96203-900 Rio Grande, RS, Brazil

ARTICLE INFO

Keywords:
Cytotoxicity
Active mitochondria
Metabolic activity
Lysosomal integrity
Apoptosis

ABSTRACT

Glyphosate is the active component of many commonly used herbicides; it can reach bodies of water through irrigated rice plantations. In the present study, we evaluated the effect of glyphosate and Roundup® (a glyphosate-based herbicide) in established culture of the zebrafish hepatocyte cell line ZF-L after 24 and 48 h of exposure to concentrations of 650 and 3250 μg/L. We observed a reduction in metabolic activity and lysosomal integrity, and an increase in cell number after 24 h of Roundup® exposure at the highest concentration. An increase in active mitochondria and apoptotic cells was observed following 24 h exposure to glyphosate and Roundup®, while only exposure to Roundup® induced an increase in necrotic cells. Rhodamine B accumulation decreased after 48 h exposure to 650 μg/L of Roundup®; this reduction is indicative of increased activity of ABC pumps. Overall, the present findings highlighted the hazard of glyphosate exposure not only in the commercial formulation but also glyphosate alone, since both can induce damage in the ZF-L cell line primarily through the induction of apoptosis.

1. Introduction

Glyphosate-based herbicides are widely used. The mechanism of action of glyphosate is to interrupt the synthesis of essential aromatic amino acids in plants (Giesy et al., 2000). In southern Brazil, this herbicide is used in irrigated rice plantations; therefore, the water used in the crop is drained and returns to the water bodies and thus can cause damage to organisms that inhabit this environment (Anvisa, 2010; Costa, 2008; Primel et al., 2005).

Glyphosate concentrations in rivers around the cultivation are relatively high and may be toxic to organisms that inhabit these areas. Silva et al. (2003) detected a glyphosate concentration of 100 μg/L in a river in southern Brazil 60 days after glyphosate application. Peruzzo et al. (2008) evaluated levels of glyphosate in a river in Argentina and determined a range of 100–700 μg/L.

Several aquatic organisms can survive in contaminated environmental conditions because they have a multixenobiotic resistance system (MXR) (Bard, 2000), which consists of ATP-binding cassette (ABC) proteins, which remove substances from inside the cell and are the primary defense against pollutants (Lehman-Mckeeman, 2008). The ABC transporters are a large family of proteins, which includes P-glycoprotein (P-gp), the first identified ABC transporter in teleost fish. P-gp contributes to the efflux of a broad variety of hydrophobic organic chemicals, neutral or cationic (Sturm and Segner, 2005). Multidrug resistance-associated protein performs the efflux of anionic substances and metabolites of phase II biotransformation (Luckenbach et al., 2014). The biotransformation process is considered a second line of defense and includes the enzyme glutathione *S*-transferase (GST), which conjugates glutathione to substrates, like xenobiotics, to increase polarity and facilitate excretion, although GST can also act as an antioxidant by combating reactive oxygen species (ROS) (Van der Oost et al., 2003).

Numerous studies have demonstrated the effects of glyphosate-based herbicides related to an alteration in redox balance such as changes in the activity of antioxidant enzymes, lipid damage, genotoxic effects, histological damage, altered acetylcholinesterase activity, and reproductive damage (Cai et al., 2017; Ferreira et al., 2010; Lushchak et al., 2009; Modesto and Martinez, 2010; Sinhorin et al., 2014; Topal et al., 2015; Webster et al., 2014). Reproductive damage has been observed following exposure of both commercial formulations and pure glyphosate in fishes (Harayashiki et al., 2013; Lopes et al., 2014). Guilherme et al. (2014) and Marques et al. (2014) showed a genotoxic effect on *Anguilla anguilla* following exposure to 116 μg/L of Roundup® Ultra. This damage remained 2 weeks after exposure. In vitro experiments also showed a DNA damage after exposure to 42.5 and 85 mg/L pure glyphosate in peripheral blood mononuclear cells and 1.7–4.25 g/

* Corresponding author at: Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Av Itália km 8, 96203-900 Rio Grande, RS, Brazil.
E-mail addresses: nanilopes_@hotmail.com (F.M. Lopes), juzomer@hotmail.com (J.Z. Sandrini), martasouza@furg.br (M.M. Souza).

https://doi.org/10.1016/j.ecoenv.2018.07.005
Received 29 March 2018; Received in revised form 20 June 2018; Accepted 1 July 2018

L in human lymphoma cells (Kwiatkowska et al., 2017; Townsend et al., 2017). Peixoto (2005), using isolated mitochondria demonstrated that both glyphosate and Roundup® were able to perturb the bioenergetic functionality.

When the toxicity of Roundup® and pure glyphosate is compared in aquatic environments, the high toxicity has been attributed to the surfactant polyoxyethylene amine (POEA) (Giesy et al., 2000; Tsui and Chu, 2003). In fact, some studies have demonstrated effects of POEA on the antioxidant defense system, resulting in lipid damage, as Navarro and Martinez (2014) showed in the fish *Prochilodus lineatus* exposed to 0.15–1.5 mg/L of POEA for 24 h. However, the majority of studies performed with pure glyphosate use much higher concentrations than those used in commercial formulations, making comparison difficult.

Studies with comparable concentrations of pure glyphosate and its commercial formulation are necessary to relate the effects of the active component to its commercial formulation. Therefore, the present study aimed to evaluate the toxicity and cellular defense capability in an established culture of zebrafish hepatocytes (ZF-L) following glyphosate exposure and to compare the effects of the active component (glyphosate) with the commercial formulation (Roundup Original®). Several liver cells cultures have been established and applied to toxicity tests of pesticides in an in vitro system, since the liver is a major target organ for most toxins (Huang and Huang, 2012). Also, in vitro studies could characterize the mechanisms by which compounds cause adverse effects besides avoid the animal use, providing results with smaller residues generation, and for this reason ZF-L cell line was chosen to evaluate possible cell targets of this herbicide.

2. Materials and methods

2.1. Cell culture

The zebrafish hepatocyte cell line (ZF-L) was maintained in the Cell Culture Laboratory at the Federal University of Rio Grande at 28 °C in cell culture flasks with 50% L-15 medium and 40% RPMI 1640 medium, supplemented with sodium bicarbonate (0.2 g/L), L-glutamine (0.3 g/L), HEPES (25 mM), 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotic and antimycotic (penicillin [100 U/mL], streptomycin [100 mg/mL], and amphotericin B [0.25 mg/mL]; Sigma-Aldrich). Phosphate-buffered saline (PBS) enriched with calcium and magnesium (136.9 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.9 mM of $CaCl_2$, and pH 7.0) was used to wash cells and photograph cell culture plates.

2.2. Cell exposure

Cells were transferred to 96- or 24-well culture plates (depending on the assay) and incubated for 48 h for complete cell adhesion. Following this, the cells were exposed to glyphosate (analytical standard, Sigma-Aldrich) or Roundup® Original (Monsanto) and analysis was performed after 24 and 48 h.

The solutions of glyphosate and Roundup® was prepared in injection water at a concentration of 200 times higher than final exposure and diluted in culture medium before the distribution to the plate wells. Exposure concentration was 650 and 3250 μg/L for both glyphosate and Roundup® Original (based on the glyphosate present in the formulation). Previous cytotoxicity tests (MTT and NR) were performed in a range of 65–6500 μg/L (data not show) and a reduction in viability began from the 3250 μg/L concentrations in the commercial formulation, chosen as the sublethal concentration in those studies in order to study the pathways of toxicity. The concentration of 650 μg/L was chosen because reflects a concentration which can be found environmentally.

Cell exposure was performed in 96-well plates at a concentration of 1×10^5 cells/mL for the trypan blue, Mitotracker Green, and apoptosis assays, 2.5×10^5 cells/mL for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR), sulforhodamine B (SRB), and Rhodamine B assays, and 5×10^5 cells/mL in 24-well plates for the GST activity assay.

2.3. Cytotoxicity of ZF-L cells

The cytotoxicity of glyphosate in ZF-L cells was analyzed with several different assays. Metabolism was measured according to Freshney (2005), with the MTT assay. This method consists of the reduction of MTT to formazan crystals through the action of dehydrogenases. These crystals are dissolved in dimethyl sulfoxide and the absorbance is measured at 550 nm with a microplate reader (ELx808, Biotek, Winooski, VT, USA).

Lysosomal integrity was measured with the NR retention assay, which is based on the incorporation and retention of NR by intact lysosomes. If the lysosomes are not intact, the incorporation and retention is diminished, indicating damaged cells (Freshney, 2005). Exposure medium was discarded, and cells were washed twice with PBS, incubated with NR (40 μg/mL) for 3 h, fixed for 5 min in formaldehyde (0.5%) in $CaCl_2$ solution (1%), and disrupted with acetic acid (1%) in ethyl alcohol (50%). The absorbance was measured at 550 nm with a microplate reader (ELx808, Biotek).

Analyses were performed independently five times with five replicates. Data are expressed as a percentage relative to the control, which was considered 100%.

2.4. Cell quantification following exposure

The number of viable cells was determined by trypan blue exclusion assay, which is based on cell membrane integrity. Viable cells do not retain the trypan blue dye, while non-viable cells exhibit blue staining. Following exposure, the glyphosate-containing medium was discarded, cells were washed twice with PBS, 100 μL of trypan blue (0.04%) was added, and cells were incubated for 10 min at room temperature. The cells were washed twice with PBS and imaged with an epifluorescence inverted microscope (Olympus IX 81). Analysis was performed independently three times with five replicates, with two images taken of each well and counted with ImageJ software. The mean number of cells was used for data analysis.

Cell quantification, based on protein content, was performed as described by Skehan et al. (1990) with modifications described by Gerhardt et al. (2009). After 24 and 48 h of glyphosate exposure, the medium was removed, cells were washed twice with PBS, and fixed for 40 min with 4% formaldehyde (in PBS). Following this, cells were incubated for 1 h with sulforhodamine B (SRB), which binds to basic amino acid residues within proteins, and washed with deionized water to remove unbound stain. The plates were dried at room temperature and proteins were solubilized in 1% sodium dodecyl sulfate. Absorbance was measured with a plate reader at 490 nm. Data are expressed as a percentage relative to the control.

2.5. Active mitochondria

Active mitochondria were measured with the Mitotracker® Green (MTG) assay, according to the manufacturer's instructions (Molecular Probes, Invitrogen, Carlsbad, CA, USA). The MTG probe contains a mildly thiol-reactive chloromethyl that covalently binds mitochondrial proteins, resulting in accumulation in active mitochondria. Analysis was performed independently six times with three replicates. Following exposure, the medium was discarded, cells were washed twice with PBS, and incubated for 45 min with 200 nM of MTG in PBS. This solution was replaced with PBS and images were captured with an epifluorescence inverted microscope (Olympus IX 81). The fluorescence intensity was measured with ImageJ Software and expressed relative to the total number of cells in the area. Data are expressed as a percentage relative to the control group (100%).