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Induction of micronuclei and nuclear abnormalities in tadpoles of the common toad (*Rhinella arenarum*) treated with the herbicides Liberty® and glufosinate-ammonium

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ABSTRACT

The assessment of micronucleated erythrocytes (ME) in blood represents a widely used method for the detection of chromosomal damage by chemical agents, such as herbicides that may occur as water contaminants. We investigated the changes in some circulating blood-cell parameters of tadpoles of the common toad (*Rhinella arenarum*) that were exposed during 48 or 96 h to three sub-lethal concentrations (3.75, 7.5, and 15 mg/L) of a commercial formulation of a glufosinate-ammonium (GLA)-based herbicide (Liberty®, LY®) as well as to the corresponding active ingredient GLA. The frequency of ME and other erythrocyte nuclear abnormalities (ENA, *i.e.*, lobed nuclei, binucleates or segmented nuclei, kidney-shaped nuclei, notched nuclei, and picnotic nuclei) were evaluated and compared with positive (cyclophosphamide, CP, 40 mg/L) and negative (de-chlorinated tap water) controls. The results indicate that the exposure of *R. arenarum* tadpoles to LY® induces a concentration-dependent increase in ME frequency. The ENA frequency at 48 h was also significantly higher than that in the negative control group for all the chemicals assayed (CP, LY® and GLA) whereas at 96 h, increases in ENA over the negative control group were found only for CP and GLA (7.5 mg/L). Our study demonstrates that the commercial formulation of a GLA-based herbicide induces micronucleus formation in *R. arenarum* tadpoles, in contrast to the active ingredient. According to these results, the inert ingredients of the commercial formulation played an important role in the production of genotoxic damage in erythrocytes of amphibian tadpoles.

1. Introduction

Pesticides and related chemicals are used in agricultural farming and often discharged directly or indirectly into water bodies [1]. Much research is currently alerting on the consequences of pesticides in the global decline observed in amphibians [2,3]. These vertebrates are well known to be vulnerable to pesticides that constitute – in view of their genotoxic or mutagenic properties – initial risk factors in the generation of reproductive effects in the long term [4–8]. Latin America has shown a great expansion of genetically modified (GM) soybean cultivations, as well as a simultaneous increase in the application of herbicides [9]. These expansions are driven by crop prices, governmental and agro-industrial support, and demand from importing countries, especially China [10,11]. In particular, Argentina started to experience the biggest expansion in soybean planting in 2005, with GM "Roundup Ready" crops being the most widely used, thus encouraging the increased use of glyphosate-based herbicides [12,13].

The increase in the number of weeds with resistance to glyphosate, and the ever-increasing areas affected by it in the US and South America has led to recommendations that farmers should use other herbicides to control weeds in GM crops tolerant to this herbicide. Glufosinate-ammonium (GLA) is a post-emergent herbicide related to glutamate and it belongs to the organophosphate family [14], which is significantly increasing in worldwide use [15,16].

GLA is highly soluble in water (solubility, about 1370 g/L), it is hydrolytically stable in the range of environmentally relevant pH (5–9) and it is not degraded by photolysis in water [17]. GLA has been classified, in some studies, as a persistent contaminant

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with a reported half-life ranging from 3–42 days [18]. The high risk of GLA contamination of aquatic systems is related to accidental overspray or indirect influx from surface runoff, thus leaching and eroding contaminated soils [19]. According to a review by Schulte-Hermann et al. [20], GLA has been extensively tested for genotoxic properties, with negative results. On the other hand, Watanabe [21] indicated that GLA induced cell death, chromatin condensation, and dissociation of the cytoplasmic structure and cell membrane in the neuroepithelium of mouse embryos, and Kanaya and Tsubokawa [22] reported micronucleus induction (MN) by GLA in gill cells of medaka fish (*Oryzias latipes*).

The MN test, initially proposed by Heddle [23] and Schmid [24], is a simple assay for the detection of chromosomal damage. Typically, a MN is defined as a small extranuclear chromatinic body originating from an acentric fragment or whole chromosome lost from the metaphase plate. When compared with other DNA-damage detection techniques, the MN test has some advantages: it can be performed rapidly, it is not complex or expensive, and its preparation and analysis are simpler and faster than other tests for chromosomal aberrations [25,26]. The MN test has been widely used in amphibian erythrocytes [27–31] since this cell type is easily handled and cellular dissociation is not required [32]. Also, other erythrocyte nuclear abnormalities (ENA), such as lobed nuclei, binucleated cells, kidney-shaped nuclei and notched nuclei [13,32–36] have been observed in erythrocytes of amphibian tadpoles as a consequence of exposure to environmental and chemical contaminants with cytotoxic, genotoxic or mutagenic activities. Although the mechanism responsible for the formation of all ENA types has not been totally explained, these abnormalities are considered to be indicators of genotoxic damage and therefore may complement the scoring of MN in routine assays for genotoxicity screening [37].

In this study, *Rhinella arenarum* tadpoles were exposed *in vivo* for 48 or 96 hours to three sublethal concentrations (3.75, 7.5, and 15 mg/L) of a commercial formulation of a GLA-based herbicide (Liberty®) as well as to its corresponding active ingredient. Genotoxic effects were investigated in peripheral erythrocytes by use of assays for MN and ENA, whose frequencies were then evaluated in comparison with positive and negative controls.

2. Materials and methods

2.1. Chemicals

We used the commercial formulation of the herbicide Liberty® (LY®, 20% GLA, excipients c.s.) which was obtained from Bayer CropScience®, Argentina. GLA (ammonium-DL-homoalanin-4-yl(methyl) phosphinate CAS 77182-82-2) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, Mo, USA). Cyclophosphamide (CP) (CAS No. 50-18-0, Filaxis, Argentina) was used as a positive control, at a concentration of 40 mg/L [29].

2.2. Tadpoles

Tadpoles of the common South American toad *R. arenarum* were selected as model test organism. This anuran has an extensive neo-tropical distribution [38] and is frequently found in forests, wetlands, agricultural land and urban territories [39]. Its larvae exhibit aggregative behaviour [40] and they have been recently characterized by their sensitivity to end-points for genotoxicity and cytotoxicity [41]. Larvae were collected during November 2012 from temporary ponds in natural floodplains of the Paraná River (31°11′31″S, 60°9′29″W, Argentina) where no pesticides were used. The average size (snout-tail tip) was 15 ± 0.5 mm and weight was 0.045 ± 0.007 g, Gosner stages (GS): 29–31 [42]. Tadpoles were acclimated for 48 h to a 12-h light/dark cycle with dechlorinated tap water (DTW, pH: 7.4 ± 0.05, conductivity: 165 ± 12.5 μmhos/cm, dissolved oxygen concentration: 6.5 ± 1.5 mg/L, hardness: 50.6 mg/L of $CaCO_3$) at 22 ± 2 °C, and fed on boiled lettuce (*Lactuca sativa*) from the beginning of the experiment.

2.3. Experimental design

Preliminary experiments were conducted in order to determine the concentrations at which tadpoles did not show mortality or signs of reduction in food uptake. The no-observed adverse-effect level (NOAEL) was 20 mg/L for both, LY® and GLA.

Thereafter, 96-h sub-lethal tests were conducted according to US-EPA Standard Methods [43], with 10 larvae per concentration per time of exposure. In the assay, LY® and GLA formed an emulsion with DTW and it was applied as such in three different concentrations: 3.75, 7.5, and 15 mg/L. The nominal test concentrations of LY® are given as the nominal concentration of the active ingredient (GLA). Negative controls were conducted in DTW during the same period of exposure. CP was used as a positive control, at a concentration of 40 mg/L. All test solutions were prepared in triplicate immediately before each experiment. Each solution was replaced every two days with freshly prepared solution of the same concentrations (LY® and GLA, respectively) and food. The MN and ENA frequencies in each group were measured after 48 and 96 h.

2.4. MN assay

Approximately 50 μl blood was taken from each tadpole by cardiac puncture [29] and blood smears were prepared on clean slides, fixed, and stained by means of the May-Grünwald/Giemsa method [44,45]. The MN frequency and mitotic index (MI) were determined in 1000 erythrocytes from each tadpole, with a microscope under 100× magnification [46]. It is important to note that red blood cells in amphibians are nucleated and undergo cell division in the circulation, particularly during the developmental stages [47]. Coded and randomized slides were scored blind by a single observer. The criteria for distinguishing a micronucleus are: (a) the intensity of a stained MN should be similar to that of the principal nucleus but with an inferior diameter, (b) it should be round with a nuclear membrane and not connected to the principal nucleus, (c) it should not overlap with the principal nucleus and has to be located within the cytoplasm [48,49].

2.5. Classification of other ENA

The presence of other ENA was assessed according to the procedures of Guilherme et al. [50] in mature erythrocytes, by determining the frequency of the following nuclear lesions: lobed nuclei (L), binucleate or segmented nuclei (S), kidney-shaped nuclei (K), notched nuclei (NN), and picnotic nuclei (PN). The results were expressed as ENA frequency, the mean value (‰) of the sum (L + S + K + NN + PN) of all the lesions observed. Coded and randomized slides were scored blind by a single observer.

2.6. Data analyses

The data from the assays were analyzed with a binomial proportion test [51]. Statistical analyses were performed with the BioEstat software 5.0 [52]. A p-value <0.05 was considered to correspond with statistical significance.

3. Results

The mature erythrocytes of *R. arenarum* tadpoles are oblong/oval-shaped with a central nucleus (see erythrocytes

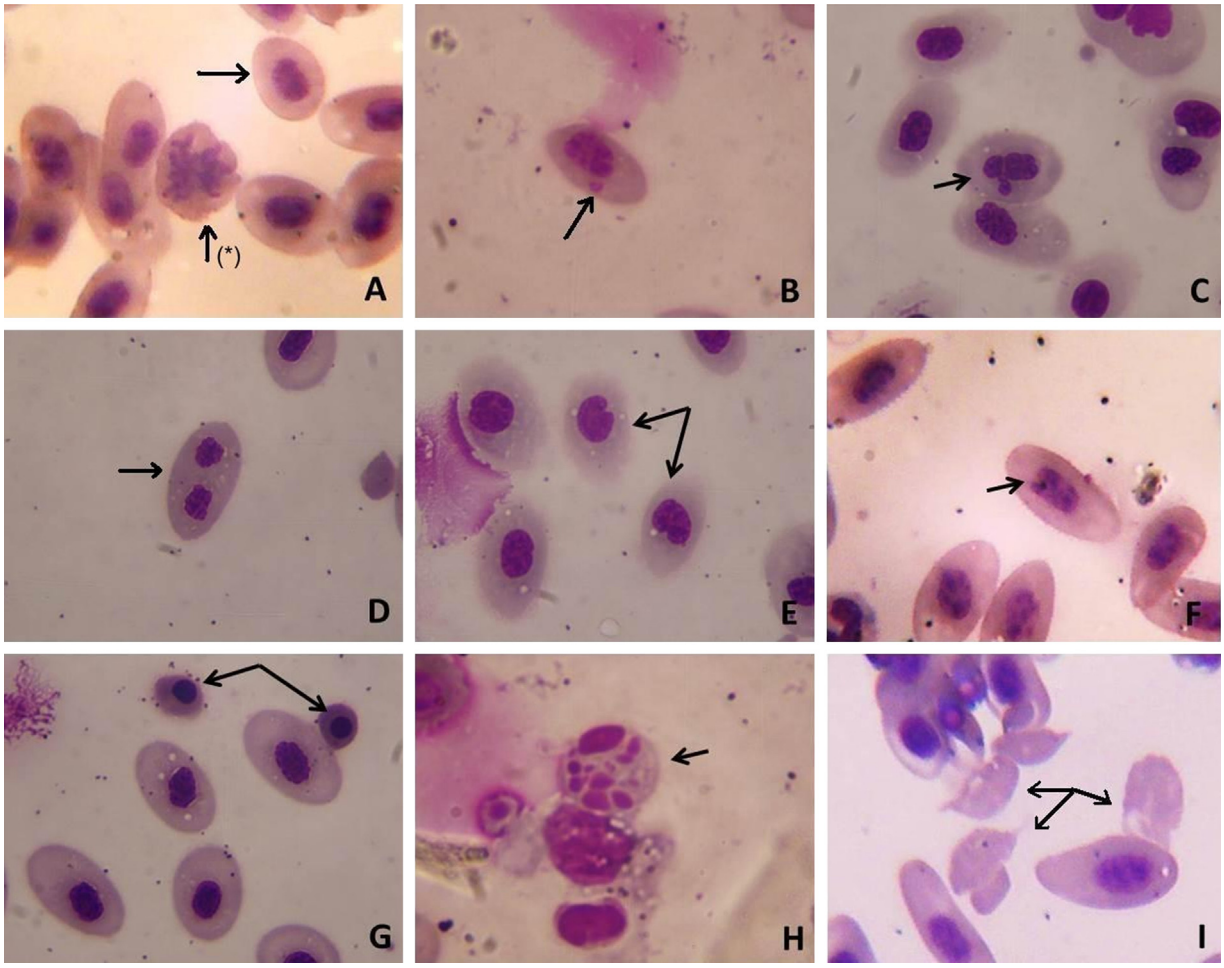

Fig. 1. Detail of red blood cells observed in tadpoles exposed to LY® and GLA. (A) Normal and mitotic (*) erythrocytes; (B) micronuclei (MN); (C) lobed nuclei (L); (D) binucleated cell (S); (E) kidney-shaped nuclei (K); (F) notched nuclei (NN); (G) pyknotic nuclei (PN); (H) apoptotic cell (AP); (I) erythroplastid (EP). May Grünwald-Giemsa, 100×.

in cell division, Fig. 1A). The nucleus was visibly structured and had a well-defined boundary, which facilitated the recognition of fragments in their cytoplasm. The MN observed were spherical nuclear fragments separated from the parent nucleus. In the erythrocytes analyzed, single MN were predominant (Fig. 1B). However, some erythrocytes clearly presented other morphological alterations, such as apoptotic cells, induced by exposures to the commercial formulation and the active principle (Fig. 1C–I).

The data obtained on the MN frequency in erythrocytes of *R. arenarum* tadpoles showed a concentration-dependent increase for the different LY® and GLA concentrations: the MN frequency generally decreased between 48 and 96 h for LY® and GLA. Even though the increase in MN frequency correlated with the increasing concentration, only concentrations of 7 and 15 mg/L from the commercial formulation (LY®) showed statistically significant differences with the negative control group at 48 and 96 h. In addition, significant differences were observed in the frequency of micronucleated erythrocytes at 96 h between tadpoles exposed to CP and the negative control group. These data are shown in Fig. 2. It is noted that the positive control (CP) is not positive at 48 h.

The MI was used to determine the rate of cell division. The mean MI/1000 at 48 h was 1.46 (±0.26), decreasing to 0.57 (±0.4) at 96 h. Despite the fact that the MI decreased with exposure time, no statistically significant differences from the negative control group were found for any of the test compounds (Fig. 3).

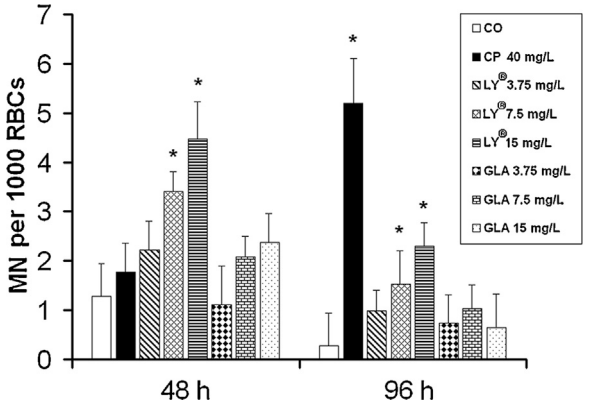

Fig. 2. Frequency of micronuclei (MN) (per 1000 cells) in *R. arenarum* larvae treated with different concentrations of test compounds. Significantly different from negative control: $^{*}p < 0.05$; binomial proportion's test. CO: negative controls; CP: cyclophosphamide, positive control; LY®: Liberty®; GLA: glufosinate-ammonium.

In addition to MN, other nuclear anomalies were noted in tadpoles exposed to LY® and GLA (Fig. 1B–G). After a 48-h exposure, all test concentrations of CP, LY® and GLA showed a significant increase in ENA frequency compared with the negative control group. At 96 h, only the results with CP and GLA (at 7.5 mg/L) were significantly different from the values for the negative control group (Fig. 4).

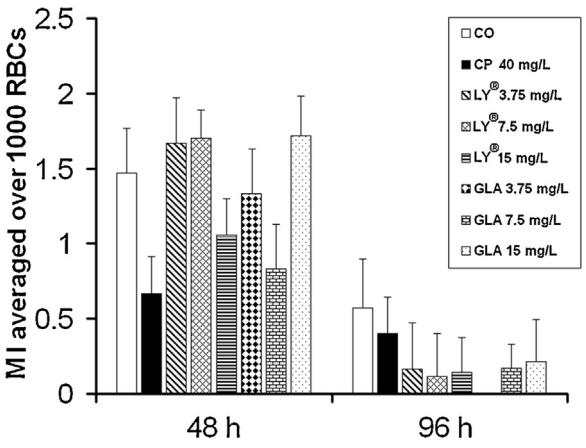

Fig. 3. Mitotic index (MI) (per 1000 cells) in *R. arenarum* larvae treated with different concentrations of test compounds. Significantly different from negative control: * $p < 0.05$; binomial proportion's test. CO: negative control; CP: cyclophosphamide, positive control; LY®: Liberty®; GLA: glufosinate-ammonium.

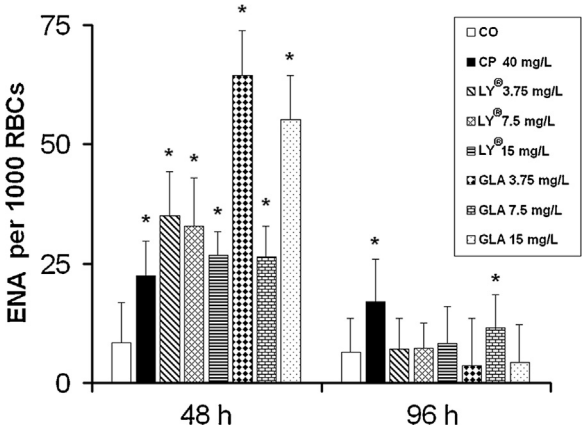

Fig. 4. Induction of erythrocyte nuclear abnormalities (ENA) (per 1000 cells) in *R. arenarum* larvae treated with different concentrations of test compounds. Significantly different from negative control: * $p < 0.05$; binomial proportion's test. CO: negative controls; CP: cyclophosphamide, positive control; LY®: Liberty®; GLA: glufosinate-ammonium.

4. Discussion

Amphibians have been considered as bio-indicators of aquatic and agricultural ecosystems [39,53,54]. Also, several studies demonstrated that amphibians are sensitive organisms, suitable for detection of genotoxic agents [55–58]. Among the methods to detect genetic and genotoxic effects, the MN test is often used since it allows convenient and easy application, particularly in amphibian larvae [25,59].

The literature on the genotoxicity of GLA is controversial, depending on the genetic system or the assay used. In a review to evaluate the safety of GLA formulated at 200 g/L the authors postulated that the compound posed no genotoxic risk to humans [20]. The conclusion was based on the prior research of Ebert et al. [60] including numerous mutagenicity tests. Moreover, as affirmed by Bayer CropScience® (Safety Data Sheet according to Regulation (EC) No. 1907/2006; Version 2/EU 102000012341, Revision Date: 08.11.2010) GLA was not mutagenic or genotoxic in a battery of *in vitro* and *in vivo* tests. However, GLA or their commercial formulations can have genotoxic effects at low concentrations that are not predicted by effects at higher concentrations (*i.e.*, 2.5–5 μM) [22]. In this sense, studies with herbicides show differences between active ingredients and their formulations with respect to genotoxic and cytotoxic effects [61,62]. For this reason, the first step taken in this assessment was an evaluation of clastogenic properties of commercial formulations (*i.e.*, LY®) that are actually used on the fields.

CP is an anticancer drug that is widely used as positive control in a variety of biological systems [63]. For example, in *Xenopus laevis*, 5 mg/L of CP during 96 h induced 3.5 ± 0.4 MN/1000 cells [64] and in *Lithobates catesbeianus*, 5 mg/L of CP during 96 h induced 3.25 ± 0.66 MN/1000 cells [28]. Concentrations and MN frequencies in our study (40 mg/L of CP during 96 h, 5.2 ± 0.6 MN/1000 cells) were similar to those reported by Ossana et al. [65] for *L. catesbeianus* (40 mg/L of CP during 96 h, 10.50 ± 0.65 MN/2000 cells).

MN and ENA frequencies in peripheral blood erythrocytes of *R. arenarum* tadpoles exposed to LY® and GLA at sub-lethal concentrations were significantly higher than those in the negative control group. LY® at nominal concentrations of 7.5 and 15 mg/L (48–96 h) caused increased MN frequency, whereas GLA at the same concentrations did not. Our preliminary results indicate that GLA, when mixed with inerts and surfactants in the commercial formulations, is potentially more genotoxic than as active ingredient alone. Usually, GLA-based commercial formulations (*i.e.* Liberty®) contain a sodium polyoxyethylene-alkylether sulfate as surfactant [66].

Another parameter used in the present study to determine the cytogenetic and toxic potential of LY® and GLA was the MI. Cell division is an essential condition for MN formation [67]. Hence, the MI is critical in determining the rate of cell division [68]. Little is known about the effects of experimental stress on rates of cell division or on mitotic activity in tadpoles. However, it is clear that the rate of cell division decreased throughout the present experiments, including in the negative controls. Consequently, ENA and MN values may have diminished at 96 h in response to experimental stress. Remarkably, the MN frequency in the CP group increased at 96 h.

Some authors [44,69,70] have suggested that variations in the shape of the red blood cell could provide a complementary approach for detecting genotoxicity. The increased frequency of these nuclear abnormalities is indicative of an adverse cellular reaction and/or a surveillance mechanism to eliminate cells with genetic damage [71]. Unusual forms of erythrocytes (*e.g.*, bilobed, anucleated) have been reported to increase in situations of stress, *e.g.*, diet alterations, pathology, metabolic damage, *etc.* [72]. On the other hand, pyknosis and condensed chromatin occur at elevated levels in response to cellular injury [71]. Pyknotic nuclei are associated with apoptosis and DNA damage [73]. The occurrence of erythrocytes with bilobed nuclei and segmented cytoplasm is considered to be an expression of direct cell division (amitosis) which, in relation with mitotic cell proliferation, could represent a short-term means for increasing the oxygen-carrying capacity of the blood in amphibian species [44,74,75]. Moreover, the finding of apoptotic-like cells is in line with published results [21] showing that GLA induced apoptosis in the neuroepithelium of developing mouse embryos. In the same sense, the presence of erythroplastids, *i.e.*, anucleated forms of circulating red blood cells of some urodeles [76], may represent a particular device for increasing oxygen transport efficiency, particularly in conditions of water pollution (*i.e.*, with pesticide residues) by improving the cell surface/volume ratio. Erythroplastids containing a micronucleus further support the hypothesis of a possible formation of normal anucleated erythrocytes through cytoplasmic segmentation of cells with eccentric nuclei [44]. The sporadic appearance of anucleated erythrocytes in adult frogs in contrast to tadpoles may be attributed to the independence of breathing amphibians from the aquatic environment [44].

In conclusion, if we consider ENA as indicators of cytotoxicity and MN as indicators of genotoxicity, as proposed by Çavas et al. [77], our results demonstrate that the damages LY® and GLA caused in erythrocytes of tadpoles are cytotoxic and genotoxic. Finally,

these results reveal potential adverse effects of GLA formulations on the erythrocytes of amphibians in aquatic ecosystems.

Conflict of interests statement

None declared.

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