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Studies of Acute and Chronic Toxicity of Commercial Herbicides with Glyphosate against *Danio rerio*

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Abstract

Glyphosate or N-(phosphonomethyl) glycine is a broad spectrum non-selective systemic herbicide, used to kill weeds, mainly in soybean crops. Nowadays there are many controversies about the intensive use of these herbicides due to the potential environmental impact and the effects on human health. The environmental impacts of commercial Glyphosate formulation Roundup were assessed by evaluation of acute and chronic toxicity of Danio rerio fish.

The effects of glyphosate commercial formulations and glyphosate isopropylamine salt solutions were evaluated in different steps. First the lethal doses of Roundup herbicide toward the experimental models were determined. Subsequently the specie was exposed to sublethal concentrations of both, the commercial preparations and pure glyphosate salt in order to evaluate the chronic toxicity.

Acute toxicity was assessed by calculating the mortality indexes and chronic toxicity by measuring several biochemical parameters such as the activity of marker enzymes like aspartate aminotransferase (AST), alanine aminotransferase (ALT) and acetylcholinesterase (AchE). The registers of histological alterations in liver tissue sections were also considered in this study.

Commercial herbicides produce mortality of D. rerio and sublethal doses of these herbicides and the salt of the pure compound produce effects of chronic toxicity at the liver and muscle level; such are enough causes to limit the potential survival of these organisms in the medium.

Keywords: Acute toxicity; Chronic toxicity; Fish; Glyphosate; *Danio rerio*

Introduction

Since 1984 the concept of "sustainable development" has been used worldwide to express the linking development with factors such as human resources, food, species, ecosystems, energy and industry. South American countries (Argentina, Brazil) are producers and exporters of raw materials and agricultural inputs in which soybeans is one of the most important. This oilseed currently presents a great interest because it is a product of great economic value.

In order to increase the yield of crops, transgenic varieties resistant to glyphosate or N-(phosphonomethyl) glycine are used. This is a highly water-soluble substance (10500 mg/L) with a half-life in water of between 3.5 and 90 days [1]. The herbicidal action is due to its ability to inhibit 5-enolpyruvylshikimate-3-phosphatesynthase, an enzyme involved in aromatic amino acids biosynthesis in plants [2].

Glyphosate can be regarded as a compound used extensively in order to control weeds during the soybean crop cycle. This massive and uncontrolled employment has caused concern in urban communities due to the toxicological risks of this pesticide, especially in zones bordering rural areas.

There are multiple commercial formulations of this herbicide, being the most popular and used one known under the trade name Roundup. This herbicide is formulated as the isopropylamine salt of glyphosate with a surfactant called POEA (polyoxyethyleneamine) compound belonging to the family of polyethoxylated alkyl synthesized from fatty acids of animal origin. This last compound is added to the formulation in order to enhance the efficacy of this herbicidal [3,4], however there are a great number of evidences indicating that this compound is the main responsible of the toxic effects of such formulations [5].

A "biomarker" represents a measured response at all levels of biological organization, which may be related to the impact of pollutants [6], it includes responses from organisms in lower organizational levels to the individuals (enzymes, cells, tissues, organs and systems). The term "bio-indicators" refers to responses in the levels of organism, population, community and ecosystem [7].

The fish are used as models to determine the acute toxicity through mortality measure of various species. In addition is evaluated the chronic toxicity by determining possible variations of biochemical parameters such as liver transaminases (AST, ALT) and acetylcholinesterase (AChE) whose altered values may indicate exposure of a few hours or days to several xenobiotics [8]. Histological studies (liver tissue sections or gills) [9] and changes in gene expression are also used.

Our research group has previously used fishes to assess the

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potential toxicity of different novel drugs of natural and/or synthetic source [10-12].

To evaluate the acute toxicity we use mortality and chronic toxicity which were assessed by measuring several biochemical parameters such as the activity of marker enzymes like AST, ALT and acetylcholinesterase as well as registering histological alterations in liver tissue sections.

As a second goal, we have used this model for setting tools allowing us to evaluate gene expression at the mRNA level. As proof of principle, we have chosen the monoamine oxidase gene (MAO), which has been shown to be induced by glyphosate in other species [13].

Methods and Materials

Herbicides and solutions

To perform the different assays, we used formulations of Roundup® with a concentration of the active ingredient of 48% w/v. Besides a solution of the pure active ingredient was used: pure glyphosate STD (high purity) 99.7% provided by Monsanto Argentina. To prepare this solution 3 g of active substance were dissolved in 500 mL of distilled water to form a stock solution, which was diluted for various tests.

Biological experimental models

D. rerio fish were obtained in our laboratory by reproducing adult specimens acquired in commercial shops.

Acute toxicity assay: It was used the technique recommended by the US Fish and Wild life Service [14] which was modified to use a smaller amount of test compounds as was reported by Mascotti *et al*. Specimens of *D. rerio* were purchased in local businesses and were transferred to our laboratory and placed for 21 days in tanks parked 50 L of water to adapt to new conditions. During that period they were fed 1 time per day with a (Tetramin®) specific food and standardized controlled aeration supply; the value of the ambient temperature was a daily average of 23°C and water replenishment undertaken to maintain the volume of the ponds. For the experiences we selected specimens to 2.5 - 3 cm in length, they showed favorable signs of adaptation considering mobility, fins position and overall external morphology.

Ten adult specimens of *D. rerio* were exposed for a period of 96 hours to each concentration of test herbicide solutions using five concentrations in each toxicity test (in the range of 100 µL/L to 6.25 µL/L). Solutions and specimens were placed in a 10 to 20 L vessel (ratio of 1 specimen per 1 or 2 L of water) where they were kept until the end of the evaluations. The numbers of dead specimens in each container were removed every 24 hours. The percentage of mortality was assessed at 96 hs. It was determined the minimum concentration of formulated which produced 100% mortality (MC100%M) and the maximum concentration that did not cause mortality (MC0%M).

Chronic toxicity assays: The specimens were placed in 20 L containers for a period of 30 days. 10 specimens were placed in each recipient (ratio of 1 specimen per 2 L of water) and maintained at average ambient temperature of 23°C with controlled aeration and fed one time per day with a specific food (Tetramin®) and standardized controlled aeration supply. The specimens were exposed to different sublethal doses considering as such the maximum concentration which did not produced mortality (MC0%M) determined in the acute toxicity tests. Besides it was used a solution of pure salt of glyphosate at a concentration of 18 mg/L.

Enzymatic assays: Viscera of fish treated and control were separated and macerated. From these macerated the enzymatic activity of AST and ALT were determined. The protocols followed were those specified by the manufacturer (Wiener Lab) based on the use of a specific enzymatic colorimetric method (transaminases 200, Wiener) and already implemented in other toxicity studies conducted by our research group [15,16]. For the AchE, muscle tissue flow area was used, the method used is indicated in the protocol specified by the manufacturer (Wiener Lab) based on the use of a kinetic method at 405 nm (Cholinesterase, Wiener).

Histologic assays

Liver histological cuts: The animals were sacrificed by rapid decapitation. Then, they were sectioned and the flow area of each fish was dismissed, taking as anatomical reference the anal fin (or the anal orifice). Immediately the trunk was placed in Bouin liquid for fixation during 24 hours. Subsequently the tissue blocks were dehydrated in ethyl alcohol of increasing concentration, clarified in xylene and embedded in paraffin. Sections of 5 microns thick were performed with a sliding microtome Reichert-Jung Hn 40 and were stained with hematoxylin-eosin. The capture of images was performed using an optical microscope Olympus BX-40 with built-in digital camera (Sony SSC-DC50A) and PC connection.

RT-PCR of MAO mRNA

RNA preparation: Total RNA from viscera was purified using TriReagent, using 70 mg/mL, following the manufacturer instructions. Integrity of RNA was confirmed by agarose electrophoresis and quantification was carried out by absorbance at 260 nm. RNA was treated with RNase-free DNase in order to eliminate potential contamination with genomic DNA.

Primer design and RT-PCR experiments: The following oligonucleotides were designed in order to selectively amplify a 150 bp fragment from the MAO mRNA (GenBank: AY185211.3): Fw_MAO: ACAATGGATAAGATGGGAATGGAG and Rev_MAO: GTTTACGAACAGAGTGGCAAAG. In order to use as control, the following primers were designed amplifying a 133 bp fragment of the actin mRNA (GenBank: AY222742): Fw_Actin: AACGTCCCAGCCATGTATG and Rev_Actin: GGCAGAGCATAACCCTCATAG. RT-PCR reactions were performed using 0.6 µg of total RNA in a final volume of 4 µL, using the ProtoScript kit (New England Biolabs), following the manufacturer instructions. PCR conditions were as follows; 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 sec, 60°C for 45 sec, 72°C for 30 sec; followed by a final elongation step at 72°C for 10 min. Amplicons were visualized on 1.5% agarose electrophoresis.

Statistical analyzes

The frequency of mortality of acute toxicity tests were compared using Chi Square to analyze and compare the frequencies between mortality rates between different concentrations for the same species studied.

The results of the enzymes activity were analyzed by analysis of variance one-way, after square root transformation of data, in order to homogenize the variances. The subsequent comparisons were performed using t test Tuckey.

In all cases it worked for a significance level of 95% using statistical soft Graph Pad Instat.

Results and Disscusion

Acute toxicity

In the first stage of the work, it was studied the acute toxicity of the commercial herbicide Roundup and an aqueous solution of pure salt of glyphosate (without excipients) against *D. rerio* fish.

The parameter used was mortality evaluating in each case the concentration values of commercial formulation producing 100% mortality (MC100%M) and 0% of mortality (MC0% M), respectively, These results are shown in Table 1.

Roundup herbicide produces 100% mortality of the specimens at a concentration of 50 µL/L (equivalent to 24 µg/L of glyphosate salt) and does not produces mortality at 25 µL/L equivalent to 12 µg/L of pure salt). The pure salt solution of glyphosate was evaluated at a concentration of up to 18 mg/L and it did not produce mortality at such concentration. These results are consistent with multiple studies which have shown that the excipients which are part of the formulation would be responsible for potential toxicity [5,17].

Statistically we observed an effect of all or nothing, with no differences between mortality rates of values higher than 50 µL/L or lower than 25 µL/L ($p \geq 0.95$) resulting in the total mortality of fish at values 50 µL/L ($p \leq 0.000001$).

Chronic toxicity

Taking the values MC0%M in µL/L (maximum concentration values that produces 0% of mortality) as reference (25 µL/L); we carried out different chronic toxicity studies according to the previously described methodology.

Enzymatic assays: The liver is an organ used by fish to perform various functions related to the metabolism of xenobiotics [18]. There is a group of liver enzymes that indicate this activity among which stand out Glutathione-S-transferases (GST); ALT and AST which catalyzes the conjugation of glutathione (GSH) with a variety of electrophilic metabolites involved in the detoxification process [19]. These transaminases (AST and ALT) are enzymes widely distributed in the body and their normal activity in serum is very low or zero. It has been shown that the activity of these enzymes vary in fish exposed to various contaminants as herbicides [15], besides an organic low level contamination can lead to increased GST hepatic activity in fish [20] or a significant reduction [21].

Acetylcholinesterase (AChE) belongs to a family of enzymes defined as a group of serine esterases capable of hydrolyzing choline esters such as acetylcholine. Cholinesterase has a very wide distribution, from unicellular organisms, plants, invertebrates and vertebrates in which appears in very early stages of embryonic development before the synaptogenesis, and is predominant in muscle and nervous system.

The inhibition of AChE in brain tissue or other organs: such as muscle is considered as the most specific and sensible biomarker for insecticides possessing carbamate and organophosphate in freshwater and marine water fish [22-24].

When AChE is inhibited, acetylcholine hydrolysis it is not allowed, resulting in continuous transmission of impulses that causes an over stimulation of nerve cells. This may result in muscle contraction and a respiratory failure [25].

Enzymatic activity studies were performed on fish for Roundup and the solution of pure salt of glyphosate for *D. rerio*. Thus, we determined the liver enzyme levels (AST and ALT) and acetylcholinesterase (AChE) of the different specimens and the results obtained are shown in Table 2.

According to the data of Table 2 it can be seen that for the liver transaminases the values obtained for the specimens are significantly increased with respect to the values of the control group. The mean of the average value of the control group for AST was 56 U/L and 57.33 U/L for ALT; while the average value of the group exposed to herbicide was 122 U/L for AST and 130 U/L for ALT.

Regarding the activity of AchE in fish muscle, we observed a decrease in enzyme activity according to values of the control group. This enzyme is considered as one of the most significant environmental pollution biomarkers in fish, therefore the results demonstrate effects of chronic toxicity of the solutions tested at sublethal doses.

It is interesting to note that the values of transaminases and AchE for the set exposed to the solution of pure salt of glyphosate did not show mortality in acute toxicity assay; however it produces significant increase in the enzymes evaluated. Due to the levels of these enzymes might be considered as biomarkers of toxicity, it is clear that exposure for a period of 30 days at doses MC0%M of the commercial herbicide and the solution of pure salt of glyphosate at 18 mg/L might produce chronic toxicity effects on *D. rerio* fish.

The analysis of variance for both transaminases and AchE shows very significant differences ($p \leq 0.0001$). Comparisons between pairs of measurements (samples and control) were performed using Tukey-Kramer test.

Histological tests

Hepatic histological sections: Histological sections were performed looking for cellular morphological changes indicating toxicity. It was observed that the liver parenchyma of *D. rerio* of the control group (Figure 1A) exhibits a characteristic histoarchitecture. Hepatocytes show round or oval nucleus with smooth or slightly granular chromatin and eosinophilic cytoplasm. Sinusoids displayed typical and bile ducts subtly detectable by the presence of erythrocytes.

The sample which was exposed to the pure salt of glyphosate (Figure 1B) shows a hepatic parenchyma with moderate signs of alterations of its histoarchitecture. Vacuolated cells with irregular nucleus and cytoplasm were observed at different stages of degeneration. The dilated sinusoids are a suggestive evidence of hyperemia.

Finally the liver tissue exposed to Roundup MC0%M (Figure 1C)

Species	Roundup µL/L		Solution of pure salt mg/L	
	A	B	A	B
Danio rerio	50	25	-	≥ 18

A: **MC100%M in µL/L (**minimum concentration values that produces 100% mortality)

B: **MC0%M in µL/L** (maximum concentration values that produces 0% of mortality)

Table 1: Acute toxicity values of the commercial herbicide Roundup and an aqueous solution of pure salt of glyphosate.

Sample	AST (U/L) X ± SD	ALT (U/L) X ± SD	AChE (U/L) X ± SD
Control	56 ± 4.89	57.33 ± 2.49	22.36 ± 1.51
Roundup 25 µL/L	122 ± 6.16	130 ± 8.52	6.35 ± 0.55
solution of pure salt 18 mg/L	100 ± 1.63	106 ± 2.94	10.85 ± 0.25

Table 2: Enzymatic activity values for Roundup and the solution of pure salt of glyphosate for *D. rerio*.

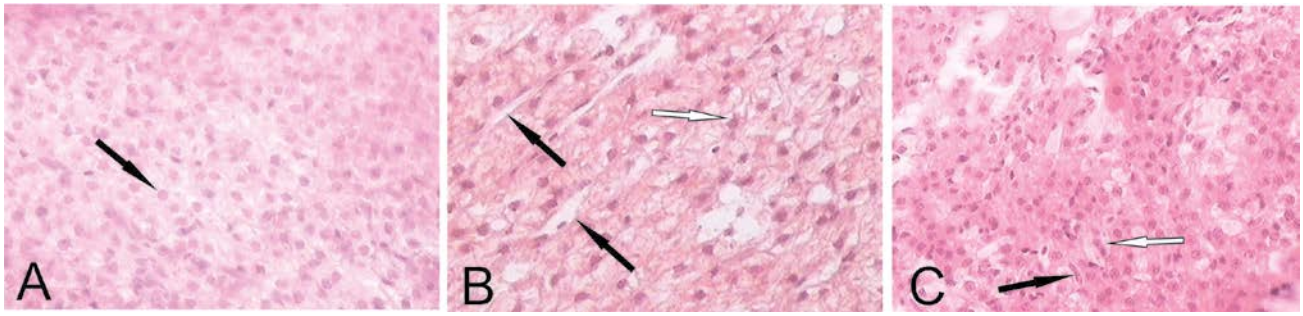

Figure 1: Photography of hepatic histological section of *D. rerio.* A: liver parenchyma of *D. rerio* in control group. B: sample exposed to pure glyphosate salt solution and C: liver tissue exposed to Roundup MC0%M.

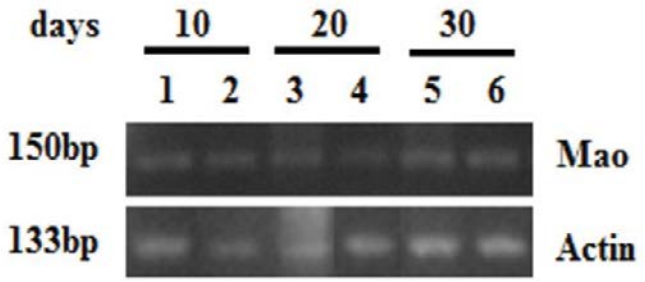

Figure 2: RT-PCR assay for determining relative mRNA expression level of MAO gene. Expression was determined in samples from control (lanes 1, 3 and 5) and treated fish (lanes 2, 4 and 6) with Roundup for 10, 20 or 30 days. The housekeeping gene β-actin was amplified as constitutive control.

showed a marked loss of parenchymal histoarchitecture; showing irregular nucleus with clumped chromatin, hypertrophic and vacuolated nucleus. A great quantity of cytoplasm exhibited many cytoplasmic vacuoles and a marked degeneration. Damaged and dilated blood vessels were frequently observed, probably due to increased blood flow known as hyperemia.

It must be considered that sublethal doses of these herbicides and the salt of the pure compound produce effects of chronic toxicity at the liver and muscle; such are enough causes to limit the potential survival of these organisms in the medium. Although it is only possible to assess these effects for the specie studied here; these results adding with the extensive literature reporting similar effects on other species are worrying considering the wide use with these herbicides in countries of South America.

MAO mRNA expression analysis

Monoamine Oxidases (MAO) are enzymes located in the outer mitochondrial membrane. This enzyme is part of a diverse family of enzymes involved in metabolizing various monoamines, diamines and polyamines produced endogenously or absorbed in the diet, as well as toxic xenobiotics [26,27]. It has been reported that xenobiotics induce the expression of the genes encoding for these enzymes. Mammals, including humans, possess two forms of this enzyme, which are transcribed from separate genes, named as MAO A and MAO B. In contrast, it has been reported that zebrafish, a popular teleost organism suitable for various pharmacological applications, harbors a single MAO gene.

In order to determine the effect of glyphosate-based formulations on expression of MAO, groups of two fish were exposed to Roundup or water, as a control. Total RNA was extracted from the whole visceral homogenates of control and treated fish after 10, 20 and 30 days of exposure. RNA was submitted to reverse transcription and the cDNAs corresponding to each sample was used as template to amplify MAO and Actin sequences. In contrast to other analysis, we have found basal expression of MAO, and no induction by glyphosate could be detected (Figure 2). The described protocol would be used as basis for analyzing the expression of other potential target genes employing both end point as well as real time PCR in future studies using this experimental model.

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