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A comparative toxicologic and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate

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Abstract

The herbicide arsenal 250 NA, its technical-grade active ingredient imazapyr, and the surfactant nonylphenol ethoxylate (NP) were evaluated through genotoxicity and toxicity studies in different organisms. A comparative study of these three compounds was carried out to assess how the addition of surfactant components may pose the highest toxicological risk to pesticide formulations. The results showed that arsenal, imazapyr, and NP did not cause chromosome aberration in *Allium cepa* nor increase the frequency of micronuclei in mice. However, toxicological evaluations showed that NP was the most toxic compound to mice, *A. cepa*, *Drosophila melanogaster*, and *Biomphalaria tenagophila.* In this evaluation, it was observed that the adverse effects were produced by the surfactant additive of the pesticide formulation.

Keywords: Arsenal; Imazapyr; Nonylphenol; Toxicity; Genotoxicity

1. Introduction

Many studies with pesticides are showing differences between active ingredients and their formulations with respect to toxicity and genotoxicity. Surfactants and other so-called "inert" components generally increase the toxicity of these formulations (Mann and Bidwell, 1999; Wagner et al., 2003). Oakes and Pollak (2000) demonstrated that inert components such as surfactants contributed approximately 50% of the overall toxicity of the complete pesticide formulation.

Commercial imazapyr products contain nonylphenol ethoxylate, which gives rise to concern for human health and the environment. Nonylphenol (NP) is widely used as a component of detergents, paints, herbicides, insecticides, and other formulated products (Hawrelak et al., 1999; Junk et al., 1974; Kim et al., 2002). Many studies have shown that NP has been implicated in the disruption of endocrine functions in wildlife (Gaido et al., 1997; Soto et al., 1991). The objective of this study was to evaluate the potential adverse effects of inert components of pesticide formulations to understand how these components that cause toxicological concern, when added to the active ingredients, modify their toxicological properties. Genotoxic and toxicological studies were therefore carried out to evaluate the technical-grade active ingredient imazapyr, its formulation arsenal 250 NA, and nonylphenol ethoxylate. These test systems were chosen for their genetic endowment, in addition to their easy availability.

2. Materials and methods

2.1. Test substances employed

The following test substances were used, Imazapyr (technical herbicide), chemical family of imidazolinone, molecular formula $C_{13}H_{15}N_3O_3$, M_w 261.3, CAS No. 081334-34-1, 99.7% purity, Batch 70304. Arsenal 250 NA, Batch 0011-98-7776, formulation of imazapyr with

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components 25 g/L imazapyr, 186 g/L of ammonium hydroxide, 18 g/L nonylphenol ethoxylate, and water. Nonylphenol ethoxylate (RENEX 95%, nonylphenol with nine ethoxylate units), Batch 970919-202, CAS No. 25154-52-3. All three chemicals were obtained from the BASF Company of Brazil.

2.2. Toxicity test

Acute toxicity in mice was tested as follows. The median lethal dose (LD50%) after an intraperitoneal single injection for 7 days was determined. Arsenal, imazapyr, and nonylphenol were administered at six dose levels for each group of five males and five females of 12-week-old Swiss albino mice from the Central Animal Facility of the University of Brasília. The LD50% was calculated using the Trimmed Spearman–Karber method (Hamilton et al., 1977).

Acute toxicity in *Drosophila melanogaster* was tested as follows. Groups of 10 *Drosophila* were randomly isolated from the stock of the Genetics Laboratory of the University of Brasília for each treatment group. Treatments were carried out through diet. Arsenal, imazapyr, and NP were diluted at six multiple-dose levels in 5 mL of Drosophila Instant Medium (Carolina Biological Supply Company). Adults were introduced into the vials, where they were fed for 5 days. The LD50 was also calculated using the Trimmed Spearman–Karber method.

Lethal concentration (LC50) in the snail *Biomphalaria tenagophila* was measured as follows. This test is comparable to the LD50, but is carried out with aquatic organisms by whole-body exposure. Wild type *B. tenagophila* from Southern Brazil have been bred and kept in the Malacology Laboratory of the University of Brasília for more than 8 years. The animals, 8–12 mm in diameter, were 4–6 months old. They were kept isolated in 125-mL vials. Snails were exposed to eight different concentrations of each test-compound for 72 h for the determination of LC50, using the Trimmed Spearman–Karber method.

2.3. Mouse micronucleus test

Swiss mice, from the Central Animal Facility of the University of Brasília, were acclimatized to laboratory conditions for 1 week prior to the study. Males and females (10–12 weeks old), weighing 30 ± 2 g, were fed Purina mouse chow and filtered water ad libitum. The negative control received distilled water. Cyclophosphamide (Enduxan) was injected at 30 mg/kg, as a positive control. Arsenal was tested at 65.5, 131.6, and 196.7 mg/kg body weight, which means 25%, 50%, and 75% of LD50 (262.33 mg/kg), respectively. Imazapyr was tested at 374.5, 749.0, and 1123.5 mg/kg body weight, representing 25%, 50%, and 75% of LD50 (1498 mg/kg), respectively. Nonylphenol was tested only at the maximum tolerated dose of 57.27 mg/kg, previously determined in our laboratory. They were housed at random in groups of 10 and were dosed twice with pesticides at a volume of 0.5 ml within a 24-h interval and sacrificed 24 h after the second injection. The treatment protocol was carried out according to the half-life of these pesticides in rodents. Test substances were administered intraperitoneally. The bone marrow preparations for micronucleus analysis were made according to Schmid (1975). The slides were fixed with methanol and stained with Giemsa. Two thousand cells per animal were counted and classified as polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). The PCEs/NCEs relationship was determined by the first 1000 PCEs or NCEs counted.

2.4. Allium cepa test

This assay was carried out according to the test protocol proposed by Rank and Nielsen (1993) to screen the genotoxicity of complex mixtures. Commercial onion bulbs were obtained from organic growers, without any treatment with growth inhibitors. For each test solution 10 onions were set up. Genotoxicity tests were then carried out with different concentrations of each pesticide. Growth inhibition tests were carried out for each pesticide prior to the genotoxicity test to find the toxicity level of the test chemical. Root tips were exposed to four concentrations of arsenal and imazapyr and three concentrations of NP for 48 h. These were continuously shaken during the period of treatment in a horizontal shaker. Filtered and dechlorinated tap water (pH 7.0) of good quality was used as a negative control and for dilution of pesticides. Methylmethanesulpfonate (MMS) at 10 mg/L was used as positive control mutagen. At the end of exposure, five or six root tips from each bulb were prepared for the microscopic slides. Ten bulbs were used per treatment and 100 metaphase–telophase cells were analyzed, giving 1000 cells per treatment. The root tips were fixed and macerated in a solution of 45% acetic acid (9 parts) and 1 N HCl (1 part) at 50°C for 5 min, followed by squashing them in a 2% orcein stain in 45% acetic acid. Slides were kept in a freezer and examined within 2 weeks. One hundred cells per onion in metaphase or anaphase were examined and classified in the following categories: bridges, fragments, and lagging chromosomes. All slides were coded and examined blind. The mitotic index was determined by counting all stages of mitotic cell out of 1000 cells. Statistical analysis was performed using the Mann–Whitney U-test, $\alpha = 5\%$.

3. Results

As shown in Table 1, there was no statistically significant difference in the means of the frequencies of

micronuclei among groups of mice treated with arsenal, imazapyr, or nonylphenol. Arsenal at 196.7 mg/kg body weight showed cytotoxicity through reduction in the percentage of PCE ($P = 0.0001$). Arsenal, imazapyr, or nonylphenol did not induce chromosome aberrations in the *Allium cepa* assays. Otherwise, a large number of lagging chromosomes were found in treatments with arsenal and nonylphenol, which suggests mitotic spindle disturbance. The mitotic index was changed significantly by arsenal and nonylphenol (Table 2). At the highest treatment, arsenal and nonylphenol were able to induce a drastic reduction in the mitotic index. Even at the lowest treatments, arsenal and nonylphenol showed a high frequency of lagging chromosomes. NP at 100 and 1000 μg/L showed so high a toxicity that it was impossible to find 1000 cells per onion. However, at a low mitotic index we were able to find more than 500 cells. Toxicity assays carried out through lethal dose (LD50) and lethal concentration (LC50), shown in Table 3, indicate that nonyphenol was the chemical most toxic to mice, *D. melanogaster*, and *B. tenagophila*. In contrast, imazapyr showed low toxicity to the three different organisms.

Table 1
Micronuclei in PCE and NCE of mice treated with arsenal, imazapyr, and nonylphenol

mg/kg	MN-PCE	MN-NCE	PCE%
Control	1.5±0.9	0.5±0.1	48.9
Cyclophosphamide			
30.0	12.6±3.0	4.5±1.0	42.4
Arsenal			
65.5	0.8±0.6	0.4±0.2	33.7
131.6	2.1±1.0	1.4±1.0	36.1
196.7	2.5±1.3	0.9±0.4	21.6[a]
Imazapyr			
374.5	2.0±0.2	0.8±0.2	38.3
749.0	1.5±0.1	0.7±0.02	49.8
1123.5	0.8±0.01	0.3±0.01	56.3
Nonylphenol			
57.27 (MTD)	0.8±0.4	0.3±0.2	35.8

Note: PCE = Polychromatic erythrocyte; NCE = Normochromatic erythrocyte. MN frequencies given per 1000 cells. MTD = maximum tolerated dose.
[a] $P = 0.0001$, Mann–whitney *U*-test.

4. Discussion

Nonylphenol ethoxylate has been found in aquatic environments, particularly in sediments and in drinking water (Clark et al., 1992; Kvestak and Ahel, 1994). A

Table 3
Evaluation of toxicity of arsenal, imazapyr, and nonylphenol through LD50 mice and in *D. melanogaster* and LC50 in *B. tenagophila*

Mice	LD50 (i.p.)
Arsenal	262.33 mg/kg
Imazapyr	1498.00 mg/kg
Nonylphenol	75.63 ml/kg
D. melanogaster	LD50 (oral)
Arsenal	0.174 mg/L
Imazapyr	>2000 mg/L
Nonylphenol	0.043 ml/L
B. tenagophila	LC50
Arsenal	20.10 mg/L
Imazapyr	45.970 mg/L
Nonylphenol	12.56 ml/L

Table 2
Results of *A. cepa* anaphase–telophase aberration study carried out with different concentrations of arsenal, imazapyr, and nonylphenol

Treatment (μL/L)	Cells analysed	Bridges	Fragments	Chromosome lagging	Total of aberrant cells	Mitotic index (%)
Water	1000	1	3	—	4	9.5±2.2
MMS						
10 mg/L	1000	26	14	17	57	4.2±1.6[a]
Arsenal						
0.25	1000	—	2	13[b]	15	8.1±2.7
0.50	902	—	2	10[a]	12	4.1±2.8[a]
1.0	950	—	4	9[a]	13	3.9±2.7[a]
100	601	—	—	—	—	2.2±1.3[b]
Imazapyr						
500	1000	—	2	5	7	9.3±2.2
1000	1000	—	6	4	10	9.4±1.7
2000	1000	—	3	8[a]	11	12.2±3.8
6000	1000	—	2	5	7	11.0±2.1
Nonylphenol						
10	1000	—	2	12[b]	14	10.0±3.2
100	878	—	1	6[a]	7	5.0±3.8[a]
1000	572	—	—	—	—	1.9±0.6[a]

[a] $P < 0.05$.
[b] $P < 0.01$; Mann–Whitney *U*-test.

literature search did not list any results that suggest, that NP is genotoxic in bacterial or mammalian cell test systems. In the present study, NP did not increase the frequency of micronuclei in mice nor the frequency of chromosome aberrations in *A. cepa*, which means that it was not clastogenic. *A. cepa* was a very sensitive test system because a herbicide was tested against a plant system, where high cytotoxicity really should be expected. Generally, a mitotic poison causes disturbance of the spindle apparatus, resulting in c-mitosis effects, which means complete absence of a spindle. A weak c-mitotic effect produces lagging chromosomes that do not attach to the spindle apparatus. Chromosome lagging means evidence of genotoxic effects, such as aneuploidy (Babich et al., 1977). The results of our studies are presented in Table 2 and demonstrate that NP act as a weak spindle poison in the roots of *A. cepa*. Strong inhibition of mitosis by NP, at the highest concentration, showed evident cytotoxicity. Arsenal 250 NA is a formulation of imazapyr, which have 18 g/L of NP in their composition. So the observed effects of arsenal on *A. cepa* could be attributed to NP, rather than to imazapyr. On the other hand, with imazapyr being the technical-grade active ingredient of arsenal without NP in the composition, the frequency of lagging chromosomes did not increase, and nor was mitotic index inhibition observed. A comparative toxicological study carried out in three highly distinct organisms showed that NP is responsible for the toxicity of arsenal to mice, *D. melanogaster*, and *B. tenagophila*, while imazapyr presented very low toxicity.

5. Conclusions

This study could demonstrate that surfactant nonylphenol increased the toxicity of herbicide formulation Arsenal 250 NA, comparing with its active ingredient Imazapyr. Many studies report the importance of evaluating the potential toxicities of complete formulations, rather than just evaluating the toxicities of the active components, because it is the commercial formulation that really goes to the field. These compounds were more toxic than mutagenic.

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