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Evaluation of the genotoxic effects of herbicide 2,4-D in *Piaractus mesopotamicus* by micronucleus test

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Abstract

Cowper, C.F.; Jorge, M.J.; Jorge, L.C.: ***Evaluation of the genotoxic effects of herbicide 2,4-D in Piaractus mesopotamicus by micronucleus test.*** *Rev. Vet. 31: 2, 165-170, 2020.* The herbicide 2,4-D is currently one of the most used agrochemicals in agriculture. The fish are target of contamination, these when being in contact with toxics develop later alterations that can be studied, reason why they are used as models in the evaluation of aquatic ecosystems. There is scarce information about the effects of these pesticides in fish. Because of this, the objective of this work was to evaluate the possible impact of a contamination with 2,4-D, in sub-lethal concentrations in *Piaractus mesopotamicus*, through the frequency of micronuclei (MN) and alterations in the shape of the nuclei (NMA) in peripheral blood erythrocytes in chronic conditions (70 days). Two trials were conducted, one with the herbicide 2,4-D pure (P) and another with a commercial formulation (2,4-D bitter amine) (FC). Each experience was composed of five aquariums with two specimens in each, where different concentrations of the pesticide were administered (1 ppm, 1.8 ppm, 3.2 ppm, 5.6 ppm and 10 ppm), and in another with well water (control). A total of 4000 cells per individual were analyzed. Through the test MN and NMA the presence of diverse nuclear alterations was evidenced. The mentioned test for P and dilutions 1 ppm, 1.8 ppm, 3.2 ppm, 5.6 ppm of FC did not show significant differences with the control, while the concentration of 10 ppm of FC differed statistically from its control, this could be due to additional components in FC.

Key words: *Piaractus mesoptamicus*, 2.4-D, mutagenicity, micronucleus and alteration in the shape of nuclei.

Resumen

Cowper, C.F.; Jorge, M.J.; Jorge, L.C.: ***Evaluación de los efectos genotóxicos del herbicida 2,4-D en Piaractus mesopotamicus a través del test de micronúcleos.*** *Rev. Vet. 31: 2, 165-170, 2020.* Los herbicidas están siendo abundantemente utilizados haciendo que los mismos no solo lleguen al organismo específico sino también contaminen el medio ambiente donde son empleados. El herbicida 2,4-D es en la actualidad uno de los agroquímicos más utilizado en la agricultura. Los peces son blanco de la contaminación, éstos al estar en contacto con tóxicos desarrollan posteriormente alteraciones que pueden ser estudiadas, por lo cual son utilizados como modelos en la evaluacion de ecosistemas acuáticos. Existe escasa información acerca de los efectos de estos pesticidas en peces, siendo el actual trabajo el primero en evaluar efectos crónicos. El objetivo de la presente investigación fue evaluar el posible impacto de una contaminación con 2,4-D en concentraciones subletales en *Piaractus mesopotamicus*, a través de la frecuencia de micronúcleos (MN) y de las alteraciones en la forma de los núcleos (NMA) en eritrocitos de sangre periférica en condiciones crónicas (70 días). Se realizaron dos ensayos, uno con el herbicida 2,4-D puro (P) y otro con una formulación comercial (2,4-D amina Sumargo) (FC). Cada experiencia estuvo compuesta por cinco acuarios con dos ejemplares en cada uno, donde se administraron difererentes concentraciones del plaguicida (1 ppm, 1,8 ppm, 3,2 ppm, 5,6 ppm y 10 ppm), y en otra con agua de pozo (control). Se analizó un total de 4000 células por individuo. A través del test MN y NMA se evidenció la presencia de diversas alteraciones nucleares. El mencionado test para P y las diluciones 1 ppm, 1,8 ppm, 3,2 ppm, 5,6 ppm de FC no mostraron diferencias significativas con el control, mientras que la concentración de 10 ppm de FC se diferenció estadísticamente de su control. El presente trabajo aporta luz de los potenciales efectos nocivos del 2,4-D en el medio ambiente y posibilita nuevos estudios prospectivos y retrospectivos.

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Palabras clave: *Piaractus mesoptamicus*, 2.4-D, mutagenicidad, micronucleos y alteraciones morfológicas nucleares.

INTRODUCTION

The intensification of agriculture has made crops more vulnerable to pests. Agricultural practices such as multiple crops per growing season, reduced fallow and monocultures have contributed to creating favourable conditions for the emergence of pests and to reducing natural barriers to their spread [10]. Ecosystem will be inevitably affected by the action of pesticides, due to their toxicity, persistence and bioaccumulation.

The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) is one of the oldest synthetic pesticides. It was released in the forties. Being currently one of the most used agrochemicals in agriculture [19]. It is within the group of phenoxy or phenoxyacetic or chlorophenolic herbicides. It is found within the "hormonal herbicides" because it acts similarly to the natural hormone auxin, or indol-3-acetic acid (AIA) [3]. 2,4-D has been classified slightly and moderately dangerous (class II and III) [28].

Fish are particularly targeted for contamination, for this reason, they are used as a model for the assessment of pollution in aquatic ecosystems [21]. Laboratory tests with fish showed that several substances were potentially genotoxic [17], while others proved harmless [4].

Due to the speed, sensitivity and reliability of the technique of *micronucleus and nuclear morphological alterations* (MN and NMA) in peripheral blood of fish, it is intensively used for the evaluation of contaminants in aquatic environment.

For this reason, the aim of the present work was to evaluate the possible impact of a contamination with 2,4-D, in sublethal concentrations in *Piaractus mesopotamicus*, through the frequency of micronuclei and alterations in the shape of the nuclei in erythrocytes of peripheral blood.

MATERIALS AND METHODS

The trials were conducted with juvenile specimens of *P. mesopotamicus* commonly known as "pacu". The fish were acclimatized for 30 days prior to treatment. Two trials were conducted, one with the herbicide 2,4-D pure (P) and another with a commercial formulation, 2,4-D Sumargo amine (FC).

In the experiments 6 aquariums of 20 liters were used with 2 specimens in each one. The duration of the trial was 70 days. Two groups were considered (treated and control). Different concentrations (1 ppm, 1.8 ppm, 3.2 ppm, 5.6 ppm and 10 ppm) of the herbicide were placed in the treated group and only water from artesian wells was added in the control.

After 70 days, the animals were anesthetized with xylocaine to extract blood from the caudal vein. For the analysis of micronuclei in peripheral erythrocytes, a drop of blood was placed on a slide for smears. The smears were fixed in ethanol for 10 minutes and left to air dry at room temperature; they were then stained with 5% Giemsa in Sörenson buffer (pH 6.9) for 20 minutes [26].

The analysis of the smears was carried out by randomly sweeping the smears to count 4000 cells per individual. The MN and NMA were classified with the suggested criteria for fishs [5]. The program Infostat version 2013e [9] was used for statistical analysis. The data were submitted to the variance analysis and Tukey's statistical test.

RESULTS

In the 22 *P. mesopotamicus* specimens studied, an average of 16 erythrocytes per field was observed, with a total of 4000 cells per individual. In order to determine the frequency of micronuclei and nuclear morphological alterations, the shape and structure of the cells were taken into consideration.

The following alterations were identified: MN: micronuclei; BN: binuclei; LOB: nuclear lobulations (lobed nuclei and blebbed nuclei); MUN: nuclear notches (notched nuclei); VAN: vacuoles (vacuolated nuclei) (Figure 1).

In each bioassay, 8,000 cells were counted for the control group and 36,000 for the treated group, evaluating a total of 16,000 erythrocytes (controls) and 72,000

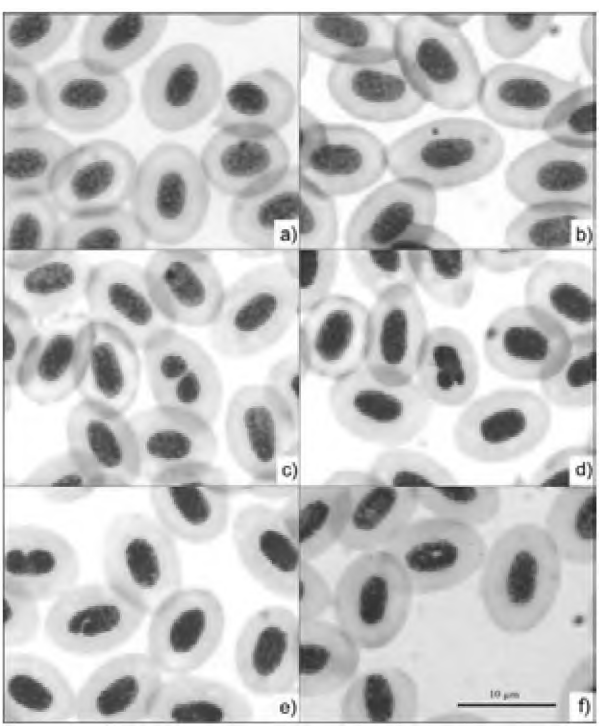

Figure 1. Erythrocytes of *Piaractus mesopotamicus*. a) Normal. b) Micronucleous. c) Binuclei. d) Nuclear lobulations. e) Nuclear notches. f) Vacuolated nuclei.

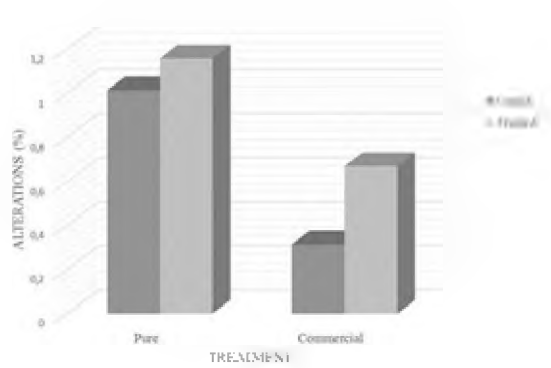

Figure 2. Alterations frequency in erythrocytes of *Piaractus mesopotamicus* in both assays.

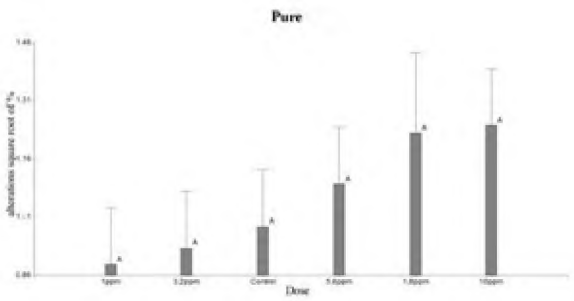

Figure 3. Alteration frequency for pure rehearsal with Tukey comparison.

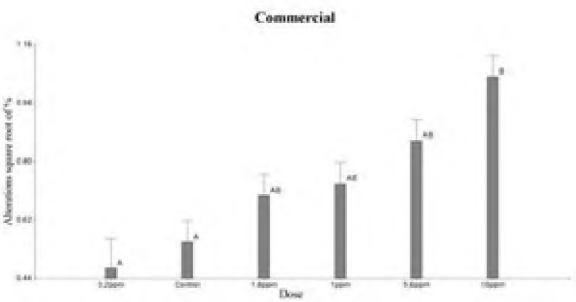

Figure 4. Alteration frequency for commercial rehearsal with Tukey comparison.

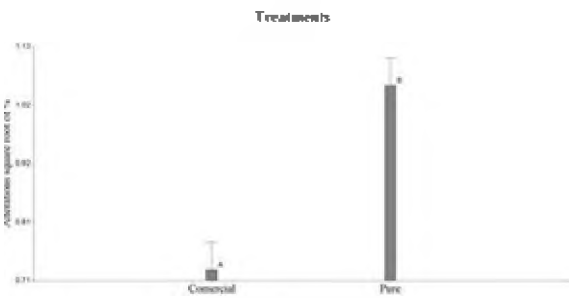

Figure 5. Alteration frequency for pure and commercial test with Tukey comparison.

(treated) for presentation (P) and (FC). In individuals exposed to the pure herbicide, 81 (1%) cells with MN and AMN were evidenced; while in those treated, 416 (1.15%) were identified. On the other hand, *P. mesopotamicus* specimens in contact with the commercial formulation showed 25 (0.31%) cells with MN and NMA, and those treated showed 241 (0.66%) red blood cells with abnormalities (Figure 2).

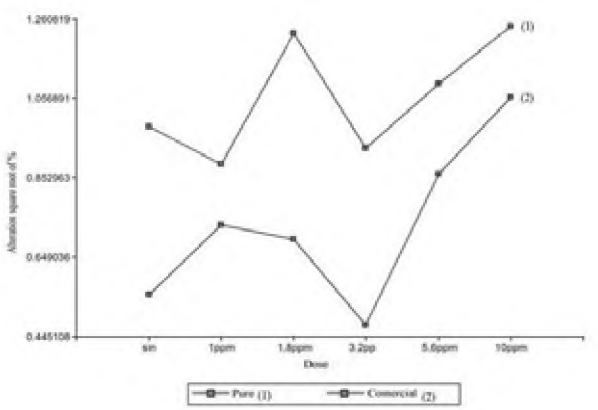

Figure 6. Dose comparison of the frequency of alterations for the pure and commercial trial.

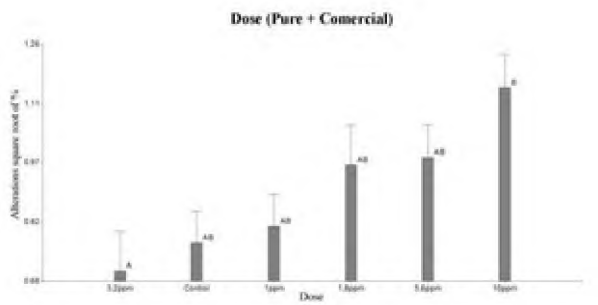

Figure 7. Tukey comparison of dose (pure + commercial) trials.

The study of the data obtained revealed that the control individuals treated with pure 2.4-D presented higher amounts of MN and NMA compared to the specimens exposed to the commercial formulation. When contrasting the control and treated group within each experience the differences are 0.15% (2.4-D pure) and 0.35% (2.4-D amine Sumagro).

Figure 3 shows that the doses used of pure 2.4-D were not statistically significant with respect to control. Whereas, in Figure 4 the 10 ppm dose of the commercial formulation was significant with the control. When comparing the 2.4-D pure and FC treatments, Tukey's test showed significant differences in the behavior of both trials (Figure 5 and Figure 6). On the other hand, comparisons according to the dose of the herbicide, did not show differences between controls and treated (Figure 7).

DISCUSSION

When comparing the data obtained in both trials, pure 2.4-D showed a higher amount of MN and AMN in the control and treated groups. This difference was probably due to the fact that both experiences were not carried out simultaneously in time. This led to the assumption that in the trial with pure 2.4-D other factors inherent to the treatment itself, such as temperature,

oxygen level, etc, could be interacting. The impact of external factors such as temperature were demonstrated and evaluated in genotoxicity tests in fish [18].

The situation in this work where only *P. mesopotamicus* specimens subjected to the 10 ppm concentration of the herbicide 2.4-D amina Sumagro showed a significant difference with the control could be due to the fact that besides the active principle (2.4-D), these may have other compounds as byproducts of their industrial synthesis such as polychlorinated dibenzodioxins [15], nitrosamines [13] and other chlorinated phenols that may express clastogenic activity [13].

On the other hand, manufactured products contain adjuvants (solvents, thinners, dispersants, emulsifiers, enhancers) in unknown proportions [30]. There are works in which it is evidenced that commercial formulations both *in vivo* and *in vitro* induce a greater genotoxic activity than pure drugs [29].

On *Oncorhynchus mykiss* exposed to the commercial formulation of 2.4-D 480 for 24 and 96 hours revealed significant differences in the frequency of micronucleated erythrocytes in control and treated individuals exposed to 10 ppm of the herbicide. No discrepancies were observed in concentrations of 2 ppm and 20 ppm regarding the control; although dilutions of 0.8 ppm and 5 ppm showed differences at 96 h and 24 h, respectively.

According to the author, this may be due to factors such as inhibition of hematopoiesis or the action of DNA repair enzymes [20]. In the same way, specimens of *Cnesterodon decemaculatus* contacted at doses 252 mg/l, 504 mg/l and 756 mg/l of the commercial formulation DMA (58.4% 2,4D) showed at 48 h and 96 h an increase in the frequency of MN and NMA, evidencing significant effects and for the 3 doses used [25].

In relation to the pure 2.4-D trial, no statistically significant differences were observed between the control and treated individuals. This differs from what was found in specimens of *Clarias batrachus* with the pure herbicide 2,4-D at doses of 25 ppm, 50 ppm and 75 ppm during 48, 72 and 96 hours [2]. On the other hand, similar toxicity test was performed in *Channa punctatus* which evaluated the genotoxicity of pure 2,4-D with the same concentrations and time [11].

In both studies, significant differences were observed in the amount of micronuclei and nuclear morphological alterations in the erythrocytes of the treated individuals with respect to the controls. The data reveal the existence of a positive correlation between the amount of micronuclei and nuclear deformations depending on the time and dose used. The results of these authors showed significant differences even in the lowest dose 25 ppm, which exceeds the concentration of 10 ppm of the present work. This difference probably led to not finding significant values in our dilutions.

It should be noted that despite the similarity in concentrations and exposure times cited in *Clarias batrachus* [2] and *Channa punctata* [11], it was observed that the alterations differ in number, this could be due to the sensitivity of the bioindicator (species). Such an assumption leads to the consideration that the response of *P. mesopotamicus* to the herbicide may have been due to its rusticity. The way species react to different xenobiotics seems to play a fundamental role in the evaluation of contaminants.

Comparative studies of the frequency of micronuclei and nuclear abnormalities in erythrocytes in three fish species *C. carpio, Astyanax eigenmanniorum* and *Cheirodon interruptus,* from Villa Dalcar lagoon (Río Cuarto, Córdoba, Argentina) showed presence of MN and NMA, being more frequent in *A. eigenmanniorum* and less in *C. carpio* [23]. The sensitivity in the species could have certain relation with the rusticity of each one of them [16].

Thus, it was also shown that *Salmo trutta* (brown trout), *Salmo trutta* (european eel) and *Phoxinus phoxinus* (european minnow) species exposed to cyclophosphamide, cadmium and colchicine for 72 hours show differences in response to genotoxic agents. In specimens of *S. trutta* all three chemical compounds induced MN. *Phoxinus phoxinus* evidenced micronuclei only at cadmium exposure. However, *Anguilla anguilla* did not present significantly MN in any of the treatments [24].

It is known that MN formation occurs during cell division as a result of two events: chromosomal breakage and/or mitotic spindle dysfunction, which can lead to incomplete distribution of chromosomes in daughter cells during mitosis [1]. The frequency of MN in peripheral erythrocytes is then the result of the dynamic balance between the formation of micronucleated cells and their elimination, where a modification of the basal MN frequency would reveal an alteration in one or both processes [22].

Entry of erythrocytes into circulating blood may be caused either by new cell production by the cephalic kidney, or under stressful conditions, by the release of cells stored in the spleen. Splenic contraction has been shown in fish in response to exercise and hypoxia [14].

Elimination may be by apoptosis [7] or splenic removal which has been demonstrated in mammals [6], although the latter would not affect to a large extent, as the elimination mechanism involves spleen sinusoids and fish have a non-sinusal structure [27]. The balance between the processes that are promoting MN frequency may vary over time [27]. Previous studies have suggested that exposure to contaminants inhibits erythropoiesis [8].

Consequently, fewer erythroid cells are subjected to differentiation into potentially micronucleated and altered erythrocytes. Then the formation of MN and NMA decreases, leading to a change in balance. As a result, the peak of micronucleated erythrocytes is observed between 1 and 5 days after exposure to contaminants [1, 27]. This duration is short compared to the average life span of circulating erythrocytes estimated at 51 days for *Carassius auratus langsdorfii* [12].

Since in this work the fish remained under the exposure of the herbicide for 70 days with herbicide renewal every two days, this time allowed all circulat-

ing erythrocytes to be under the influence of the agrochemical. Therefore, it would be expected that the presence of MN and NMA should be greater in both bioassays, however this increase was only evident in fish treated with FC at a dose of 10 ppm.

Therefore, the data from this experience allow us to conclude that only the juveniles of *Piaractus mesopotamicus* put in contact with the 10 ppm concentration of the herbicide 2,4-D amine Sumagro showed a significant increase in the frequency of erythrocytes with micronuclei and nuclear morphological alterations with respect to the controls. The results differ from those existing in the literature, possibly due to different causes: *1)* the concentrations used were higher than those of the present work; *2)* the sensitivity expressed by the different species exposed to 2,4-D; and *3)* exposure time to the contaminant agent and the variability in the response in acute and chronic trials.

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