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Hematologic, biochemical, genetic, and histological biomarkers for the evaluation of the toxic effects of fipronil for *Rhamdia quelen*

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Abstract: This study aimed to determine the lethal concentration 50% (LC_{50}) and behavioral, hematological, biochemical, histopathological, and genetic disorders of fipronil for *Rhamdia quelen* in acute toxicity tests for 96 h. To determine the LC_{50}, 42 juveniles were distributed in groups of 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.8 mg L^{-1} of fipronil. For other evaluations 36 silver catfish were divided into control, 0.3 mg L^{-1}, and 0.4 mg L^{-1} groups. Blood, kidneys, livers, and gills were collected for evaluation. The LC_{50} calculated with probit-type regression was 0.811 mg L^{-1} at 48 h of study. There was total mortality of 0.8 mg L^{-1}, reduction in hematocrit values, and increase in liver enzymes. Liver samples showed cytoplasmic vacuolization and cellular degeneration, among other changes. Gills presented vascular congestion, complete fusion of secondary lamellae, and epithelial cell hypertrophy. In the kidneys, changes such as Bowman's capsule clearance, tubular degeneration, and glomerular capillary dilatation, among others, were common. Erythrocytes showed morphological alterations without increased micronucleus development. Fipronil induces a clinical condition of anemia, alterations in liver enzyme levels, nuclear erythrocyte changes, and liver, gill and kidney damage in the silver catfish.

Key words: Acute exposure, histopathology, lethal concentration 50%, sublethal concentrations

1. Introduction

The aquatic environment is exposed to various chemicals produced by humans, such as xenobiotics. The accumulation patterns of these substances are different for different organisms and depend on the balance between the rate of assimilation and rates of metabolization and elimination of chemical compounds (1).

Biochemical characteristics are important physiological parameters of fish, changes of which indicate metabolic variations and cellular processes of the organism, evidencing the effects of pollutants and their mechanisms of degradation in the ecosystem (2,3). Hematological parameters are used as indicators of stress resulting from endogenous or exogenous changes in fish (4). Therefore, evaluation of blood parameters can be useful to monitor the physiological status and diagnose fish pathologies and intoxication (5,6).

Toxicity levels can be noted in histological changes in fish, which are sensitive tools for detecting the toxic effects of chemical compounds on target organs considered to be indicators of exposure to environmental pollutants (7).

This study aimed to determine the lethal concentration 50% (LC_{50}) and behavioral, hematological, biochemical, histopathological, and genetic disorders of fipronil for *Rhamdia quelen* in acute toxicity tests for 96 h.

2. Materials and methods

This study was approved by the Committee on Ethics in the Use of Animals of the Pontifical Catholic University of Paraná (PUCPR, Pontifícia Universidade Católica do Paraná) under protocol number 641. The species chosen was a river fish native to southern Brazil, *Rhamdia quelen*, the silver catfish.

2.1. Animals

A total of 78 animals were obtained from the Fisheries Research Laboratory (LAPEP, Laboratório de Pesquisa em Piscicultura) of PUCPR and acclimatized for 7 days in a cement tank with capacity for 3000 L of water with constant aeration, biological filtration, temperature control, dissolved oxygen (DO), pH, and luminosity. The silver catfish were fed daily ad libitum with commercial feed. During the experiments, the animals were kept in plastic buckets with capacity for 20 L of water, with constant aeration. The parameters temperature, pH, and DO were monitored twice daily for the duration of exposure to fipronil.

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2.2. Determination of LC_{50} and behavioral disorders

The silver catfish were submitted to acute toxicity tests for 96 h. The experiment was divided into 7 groups (n = 42), with a control group and concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.8 mg L^{-1} of commercial fipronil. During the experimental period the fish were not fed. At 24, 48, 72, and 96 h the mortality rate in the different groups was observed. Changes in behavior, clinical signs, and postmortem lesions were observed daily.

2.3. Assessment of hematological, biochemical, histopathological, and genotoxic parameters

To evaluate the hematological, biochemical, histopathological, and genotoxic parameters, the animals were divided into 4 per group, in three groups in triplicate: 0.0, 0.3, and 0.4 mg L^{-1} of fipronil (n = 36).

After 96 h of water intoxication, the animals were anesthetized in 2% benzocaine solution to enable biometry procedures and blood sample collection, 0.8 mL per caudal vessel puncture, in 3% EDTA-washed syringes for hematological analysis and without anticoagulant to obtain serum for biochemical analysis.

Red blood counts (RBCs), white blood counts (WBCs), and total thrombocyte counts (TTCs) were manually determined with a Neubauer chamber under optical microscopy after 1:200 dilution of blood in Natt/Herrick dye. The hematocrit was determined using capillary tubes in a microhematocrit centrifuge (SISLAB/MH) operated at 11,000 rpm for 5 min and the total plasmatic protein (TPP) by means of a refractometer (KERNCO – OS1270). The hemoglobin content, expressed in g dL^{-1}, was determined spectrophotometrically (Fanem – Excelsa Baby II – mod. 206-R) using the cyanmethemoglobin reaction after centrifugation at 3500 rpm for 5 min.

A small aliquot of about 10 µL of blood from each sample was used to make blood extensions stained by Rosenfeld dye to perform differential leukocyte analysis, morphological evaluation, visualization of thrombocytic aggregates, and hemoparasite screening.

For the biochemical tests, a serum sample was obtained by centrifugation (Eppendorf centrifuge 5417 – R), performed for 5 min at 3500 rpm in a refrigerated centrifuge. The biochemical tests performed by spectrophotometry (Drake-mod. Quick Lab/Siel – mod. EPECTROMATIC 710) were aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and albumin, obtained using commercial kits (Labtest, Lagoa Santa, Brazil).

Fish euthanasia was performed by medullary section under deep anesthetic plane in 2% benzocaine solution and samples were collected from the liver, gills (standardized from the second right branchial arch), and caudal kidney for histopathological analysis. The samples were fixed in Alfac solution for 7 days. Subsequently, they were dehydrated in 70%, 80%, 90%, and 100% alcohol solutions, diaphanized in xylol and included in paraffin; the material was included for microtome cutting at a thickness of 5 µm for blade assembly. The slides were stained with hematoxylin and eosin (H&E) for later analysis under light microscopy under an optical microscope adapted for morphological description of the organs sampled (8).

For the analysis of micronuclei (MNs), the technique described by Heddle (9) was used, in which an aliquot of 10 µL of blood from each fish was used to perform a blood smear, which was dried at room temperature for a period of 12 h and then fixed by immersing the slides in absolute alcohol for 20 min. The slides were stained by the 5% Giemsa method, diluted in phosphate buffer at pH 6.8 for 8 min, and then washed gently in tap water and left at room temperature to dry. The cytological analysis of the MN test was performed by the evaluation of 2000 erythrocytes, including in the total composition those that presented MNs and those with nuclear morphological alterations, under light field microscope at a magnification of 100×.

2.4. Statistical analyses

For analysis of the LC_{50} determination data the experimental results were treated using Table Curve 2D software, version 5.01, by probit analysis. For the analysis of hematological, biochemical, histopathological, and genotoxic parameters, ANOVA followed by the Bonferroni test was used to compare the means. The normality test used was Kolmogorov–Smirnov. Data are presented as means and standard deviations. The significance level adopted was 5% ($\alpha < 0.05$) using GraphPad Prism software, version 5.0 (GraphPad Inc., USA).

3. Results

The silver catfish measured 22.30 ± 1.08 cm and weighed 103.47 ± 13.82 g. Monitoring of the water parameters of the pails where the fish were kept accounted for equal and constant temperature at 20 °C, pH 6.8, and DO of 7.65 mg L^{-1}.

3.1. Determination of LC_{50} and behavioral changes

Fish treated at different concentrations of fipronil presented mild to severe apathy, proportionally to the concentrations used and time of exposure, in relation to the animals of the control group.

There was total mortality of the group subjected to 0.8 mg L^{-1} on the first day of exposure. The animals had hyperemic areas and/or cutaneous hemorrhagic lesions at concentrations of 0.2 and 0.3 mg L^{-1} in the region of the lateral line of the body. The fish of groups 0.4 and 0.5 mg L^{-1} showed muscle spasms and depigmentation and did not respond to manipulation, whereas the animals of the 0.1 mg L^{-1} group did not show behavioral changes or external lesions.

Table. Values (mean ± standard error) of hematological analyses and the biochemical parameters of silver catfish (*Rhamdia quelen*) exposed to different concentrations of fipronil for 96 h.

Parameters	*Fipronil (mg L^{-1})*		
	0.0	0.3	0.4
Erythrocytes (×10 μL^{-1})	1.75 ± 4.44	1.34 ± 4.60	1.47 ± 3.73
Hematocrit (%)	43.89 ± 15.80^{a}	26.17 ± 15.93^{b}	25.42 ± 14.54^{b}
Hemoglobin (g dL^{-1})	8.13 ± 2.62	6.75 ± 1.38	7.17 ± 1.28
TPP (g dL^{-1})	5.55 ± 1.19	6.95 ± 1.13	6.98 ± 1.86
Lymphocytes (%)	17.00 ± 6.55	17.83 ± 6.74	15.92 ± 4.66
Monocytes (%)	11.33 ± 4.79	12.66 ± 4.59	11.16 ± 4.01
Eosinophils (%)	4.92 ± 2.31	7.50 ± 3.45	7.16 ± 4.78
Neutrophils (%)	67.75 ± 7.39^{a}	68.00 ± 8.03^{a}	77.00 ± 8.83^{a}
Leukocytes (×10^{3} μL^{-1})	136.50 ± 67.97^{a}	122.00 ± 38.24^{ab}	86.83 ± 26.55^{b}
Thrombocytes (×10^{3} μL^{-1})	134.50 ± 67.41^{a}	57.83 ± 45.44^{b}	55.25 ± 43.47^{b}
AST (UI L^{-1})	109.51 ± 21.54^{a}	169.30 ± 41.84^{b}	159.00 ± 20.78^{b}
ALT (UI L^{-1})	41.90 ± 11.80^{a}	78.50 ± 25.43^{b}	85.78 ± 34.49^{b}
ALP (UI L^{-1})	16.58 ± 5.07^{a}	33.50 ± 12.45^{ab}	38.25 ± 29.80^{b}
GGT (UI L^{-1})	3.83 ± 1.65^{a}	12.94 ± 1.99^{b}	14.63 ± 10.64^{b}
Albumin (g dL^{-1})	2.80 ± 2.92^{a}	1.40 ± 1.07^{a}	1.90 ± 1.79^{a}

Different letters in the same line indicate significant difference between the means ($P < 0.05$).
TTP: Total plasmatic protein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: γ-glutamyl transferase.

After 72 h of intoxication, there was mortality in groups 0.3, 0.4, and 0.5 mg L^{-1}, leaving only one individual alive in group 0.3, one in group 0.4, and two in group 0.5 mg L^{-1}. In groups 0.1 and 0.2 mg L^{-1}, all the animals remained alive until the end of 96 h of intoxication but presented neurological signs provoked by fipronil: apathy, erratic swimming, muscle fasciculation, decreased frequency of opercular movements, and fragmentation and necrosis of the fins and barbels.

Through the group of data evaluated by probit-type regression analysis, it was possible to observe an adjusted $R^2 = 1.00$ and a lethal dose to kill 50% of individuals (LC_{50}) of 0.8177 mg L^{-1} at 48 h of study.

3.2. Evaluations of hematological, biochemical, histopathological, and genotoxic parameters

RBCs, hemoglobin, and PPT did not show a significant difference ($P > 0.05$) between groups. However, there was a significant decrease ($P < 0.05$) in the hematocrit rate in groups 0.3 and 0.4 mg L^{-1} in relation to 0.0 mg L^{-1} (Table).

There was a significant decrease of WBCs ($P < 0.05$) and TTCs ($P < 0.01$) in the samples from the group treated with 0.4 mg L^{-1} fipronil in relation to 0.0 mg L^{-1} (Table). In differential leukocyte counts there was no significant difference ($P > 0.05$) between the groups.

Biochemical analysis showed a significant increase in AST ($P < 0.001$), ALT ($P < 0.01$), and GGT ($P < 0.001$) for dosages of 0.3 and 0.4 mg L^{-1} compared to 0.0 mg L^{-1} (Table). There was no significant difference ($P > 0.05$) between groups in albumin levels. The AF of the 0.4 mg L^{-1} group was increased significantly ($P < 0.05$) when correlated to the 0.0 mg L^{-1} group.

The main lesions observed in the livers of the animals used in the experiment were steatosis, nuclear degeneration, pyknosis, tissue necrosis, hepatic cord disarrangement, cell degeneration, cytoplasmic vacuolation, cholestasis, and loss of cellular contour. In the gill tissues, alterations were observed such as complete melting of secondary lamellae, basal cell hyperplasia, secondary lamella epithelium elevation, secondary lamella rupture, loss of gill tissue integrity and morphology, epithelial hyperplasia, and vascular congestion. In renal tissues, tubular degeneration, tissue necrosis, tubular cell vacuolization, increased Bowman's capsule space, changes in glomerular structure, melanomacrophagous centers, and renal parenchyma and hyaline degeneration were seen.

In the analysis by optical microscopy, blood smears of silver catfish exposed to the insecticide fipronil at concentrations of 0.3 and 0.4 mg L^{-1} showed a significant

increase ($P < 0.05$) in the frequency of occurrence of nuclear morphological alterations in relation to 0.0 mg L^{-1}, where the means and standard deviations were 18.50 ± 5.61, 16.75 ± 9.06, and 5.00 ± 3.11, respectively. However, there was no significant increase ($P > 0.05$) in the appearance of MN type changes.

4. Discussion

Pesticides such as deltamethrin (6), fipronil (10), bifenthrin pyrethroids (10,11), and cypermethrin (12) are related to behavioral changes and/or to the drop in swimming performance in larvae and juveniles of several species of fish exposed to acute intoxications.

The toxicity of fipronil in fish varies according to species with lethal concentrations (LC_{50}) ranging from 0.042 mg L^{-1} in Nile tilapia (*Oreochromis niloticus*) to 0.43 mg L^{-1} in common carp (*Cyprinus carpio*) (13). It is highly toxic for bluegill (*Lepomis macrochirus*) at LC_{50} 96 h = 0.085 mg L^{-1}. For rainbow trout (*Oncorhynchus mykiss*) the LC_{50} of fipronil at 96 h was 0.248 mg L^{-1} and it was 0.13 mg L^{-1} for sheepshead minnow (*Cyprinodon variegatus*), and it affected larval growth in rainbow trout (*Oncorhynchus mykiss*) at concentrations higher than 0.0066 mg L^{-1} (13).

The LC_{50} values presented for these fish are lower than the concentrations used in this study, except for the common carp (*Cyprinus carpio*), for which the LC_{50} is close to that found for silver catfish (*Rhamdia quelen*). Silver catfish (*Rhamdia quelen*) has been shown to be more resistant than other species in acute toxicity tests for LC_{50} determination of several pesticides (6,12).

Reduction of RBCs of fish was associated by Borges et al. (2) and Galeb et al. (6) to acute toxicity tests of the pesticides cypermethrin and deltamethrin, respectively, using the species *Rhamdia quelen*. Differently from that observed in the present study (without alterations), Montanha et al. (3) reported increased RBC values of silver *Rhamdia quelen* exposed to cypermethrin.

In the present study there was a decrease in hematocrit rates in groups exposed to fipronil, las Borges et al. (2) found in silver catfish (*Rhamdia quelen*) exposed to cypermethrin. However, Montanha et al. (3) reported a significant increase in the rate of hemoglobin and hematocrit in this species when exposed to the same pesticide. According to Pimpão et al. (5), changes in hematocrit, hemoglobin, and erythrocyte rates can be attributed to the mechanism of reactivation of erythropoiesis induced by the spleen and liver in response to cerebral hypoxia and stress.

As well as the hemoglobin level, total plasma protein levels of *Rhamdia quelen* obtained in this study did not show a significant difference between treatments. In contrast, a significant increase of this parameter in fish exposed to pesticides in trials was described by Pimpão et al. (5) and Velisek et al. (14) as being associated with immunological and hepatic responses to the stimulation of erythropoiesis in situations of hypoxia under intoxication conditions. According to the results of this study, Galeb et al. (6) do not report significant differences in these parameters evaluated in *Rhamdia quelen* intoxicated with deltamethrin. Plasma proteins are synthesized mainly in the liver and are constituted of amino acids obtained after intestinal breakage and absorption (15).

The WBCs revealed a significant reduction in the group 0.4 mg L^{-1} fipronil, so leukopenia may be associated with reduced neutrophil production and survival due to viral diseases, severe bacterial infections, drugs, pesticides, and neoplasms (5). Results are similar to those observed by Galeb et al. (6) in a study with *Rhamdia quelen* exposed to deltamethrin at doses of 1.0 and 1.5 mg L^{-1}. Montanha et al. (3), however, reported leukocytosis in *Rhamdia quelen* exposed to acute intoxication by cypermethrin, relating it to the immunogenic stimulus due to pesticide exposure.

There was a significant decrease in TTC values of silver catfish (*Rhamdia quelen*) exposed to fipronil, corroborating the findings of Pereira et al. (16), who exposed curimbatá (*Prochilodus lineatus*) to pollutants, and those of Galeb et al. (6), correlating with the stress levels of the fish at the moment of management for blood collection, taking into account that the cortisol released during stress reactions is an important cause of decrease in the quantity and quality of thrombocytes.

The ALT results showed a significant increase in *Rhamdia quelen* exposed to fipronil. Borges et al. (2) and Galeb et al. (6) demonstrated a decrease in ALT levels in this species when exposed to cypermethrin and deltamethrin, respectively. Diseases that cause hepatocyte lesions, such as exposure to chemical agents, increase the amount of ALT extravasation into the bloodstream (3,6).

In the present study significant increases of AST, ALP, and GGT were observed, but Borges et al. (2) and Galeb et al. (6) observed a decrease in AST and ALP in *Rhamdia quelen* exposed to cypermethrin and deltamethrin, respectively. However, Galeb et al. (6) did not observe a change in GGT.

In the present study, no significant difference was found in the serum albumin levels of the fish exposed to different treatments. Albumin is synthesized in the liver, and its decrease is usually observed in liver disease, when the liver lose 60%–80% of its function. However, there seem to be some differences between species (15,17).

The silver catfish (*Rhamdia quelen*) of the present study presented fin degeneration that can be associated with fipronil's irritative effect in contact with the animal. They also presented histopathological lesions in the analyzed gills, caudal kidney, and liver samples, unlike Ghisi et al. (18), who, in a chronic toxicity test with the same species and pesticides, did not observe a significant difference in the appearance of branchial lesions between groups.

The effects of atrazine on *Rhamdia quelen* during an acute toxicity test for 96 h were assessed by histopathology by Mela et al. (19), wherein leukocyte infiltration, vacuolization of hepatocytes, increase in the number of free melanomacrophages, and areas of steatosis and necrosis at all concentrations (2, 10, and 100 μg L^{-1}) were observed.

The decrease in the number of melanomacrophages may be associated with immunosuppression, whereas the increase of leukocyte infiltrate in the liver may be associated with the appearance of necrosis. According to Mela et al. (19), the presence of many areas of hepatic necrosis in *Rhamdia quelen* is a morphological indication of the toxic effects of the tested pesticides, even in low concentrations, indicating the occurrence of inflammatory processes.

Hepatic tissue vacuolation is common in fish exposed to toxic substances (16,19) and may be related to disorders of lipid or cell skeletal metabolism, even as a defense mechanism, since fat droplets can sequester the lipid-soluble pesticide, minimizing its toxic effects on liver tissue.

The morphological alterations of the affected gills result from the collapse of the pillar cells and the breakdown of vascular integrity, which generates a large extravasation of blood responsible for the thickening of the lamellar epithelium, in an attempt to remove the xenobiotic from the bloodstream. In addition to altering the gill barrier function, pesticides may affect respiration, osmoregulation, acid–base balance, and excretion of nitrogenous products from the fish, leading to acute intoxication and, afterwards, parasitic or microbial disease (20).

Mela et al. (19) evaluated the gills of *Rhamdia quelen* and observed loss of microcells of the squamous epithelium at the highest concentrations (10 and 100 μg L^{-1} of atrazine) under electron microscopy. Fusion of secondary lamellae was evidenced by Pereira et al. (16) in a hematological study of curimbatá (*Prochilodus lineatus*) exposed to waters naturally contaminated with toxic substances from domestic sewage.

As a large volume of blood flows through the kidney, lesions found in this organ may be related to intoxication, acting as indicators of environmental contamination (21). Annabi et al. (20) and Pamplona et al. (22) report these characteristic changes of exposure to xenobiotics in acute toxicity studies with fish, in addition to clusters and cell vacuolization in proximal and distal tubules, distortion and increase of Bowman's capsule space, and necrosis.

No micronucleus damage was observed in the present study. In contrast, Ghisi et al. (18), in a silver catfish (*Rhamdia quelen*) chronic exposure toxicity test to fipronil, observed a significant increase in MN formation in fish samples, suggesting damage to erythrocyte DNA.

The application of the MN test from peripheral blood samples is particularly indicated for chronic exposure conditions. Often prolonged exposure is required to accumulate the toxic substance in the body to a concentration capable of causing changes (23). These results show that exposure to pesticides associated with a longer duration of action can lead to DNA damage.

With the many reports of the ability of pesticides to damage and change the genetic material of animals, including humans (24), this study made it possible to demonstrate the mutagenic potential of the insecticide fipronil to erythrocytes of fish of the species *Rhamdia quelen* (silver catfish) in an acute toxicity test.

According to Bolognese and Hayashi (25), the response of fish to toxic agents is similar to that of mammals, which may allow the comparison of the evaluation of potentially dangerous substances to humans, despite the smaller amount of DNA per cell and the low mitotic activity of fish cells.

At sublethal concentrations fipronil is toxic to *Rhamdia quelen* and poses a risk to the aquatic environment. *Rhamdia quelen* is more resistant than other fish species when exposed to fipronil. At sublethal concentrations, fipronil affects the hematological parameters of *Rhamdia quelen*. The values found in the serum biochemistry indicated hepatic tissue damage associated with the hepatotoxic effect of fipronil in water at sublethal concentrations.

The results of this study demonstrate the toxic effect of fipronil on renal, hepatic, and gill tissues as well as nuclear morphological changes in erythrocytes, which characterize genotoxic damage in silver catfish (*Rhamdia quelen*) when exposed to sublethal concentrations of the pesticide by acute toxicity test for 96 h.

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