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Original Articles

Histopathological study on gills of *Rhamdia quelen* juveniles submitted to chronic toxicological test with ibuprofen

Estudo histopatológico em brânquias de juvenis de *Rhamdia quelen* submetidos a ensaio toxicológico crônico com ibuprofeno

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A R T I C L E I N F O

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A B S T R A C T

The present study aimed at evaluating histopathological changes in gills of *Rhamdia quelen* juveniles submitted to different concentrations of ibuprofen (0.0; 0.5; 5.0 and 50.0 mg/L); concentrations were determined from LC_{50} acute assays of 5.0 mg/L (120h). For each concentration four replicates were made, with four copies each. The experiment had a total duration of 120 hours in a chronic toxicological trial. After completion, the animals were euthanized, laparotomized, and the gills were localized, isolated and fixed in 10% formaldehyde, preserved in 70% alcohol and subsequently submitted to histological routine technique for paraffin inclusion. Five cuts (6µM) per specimen were made and stained with Hematoxylin and Eosin (H&E). The Average Alteration Score (AAS) and Histopathological Alteration Indices (HAI) were determined according to the degree of the lesion and then classified in stages I, II and III which produced respectively Lamellar derangement, Lamella's epithelium rupture and aneurysm with more expressive alterations. For all concentrations the drug led to histological alterations for *Rhamdia quelen*, putting in evidence damages that go from moderate to severe in the gills of the analysed fishes. Ibuprofen caused branchial lesions in *R. quelen* with a progressive effect in high concentrations, suggesting that the presence of this drug could cause abnormalities and favour mortality in this species.

Palavras-chave:
Lamela
Ibuprofeno
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R E S U M O

O presente estudo teve como objetivo avaliar as alterações histopatológicas, em brânquias de juvenis de *Rhamdia quelen,* submetidos a diferentes concentrações do fármaco ibuprofeno (0,0; 0,5; 5,0 e 50,0 mg/L); as concentrações foram determinadas à partir de ensaio agudo com CL_{50} de 5,0mg/L (120h). Para cada concentração foram feitas quatro replicas, com quatro exemplares cada. O experimento teve uma duração total de 120 horas em um teste toxicológico crônico. Após o término, os animais foram eutanasiados, laparatomizados, sendo as brânquias isoladas e fixadas em formaldeído 10%, preservadas em álcool 70% e posteriormente submetidas a técnica histológica de rotina para inclusão em parafina. Foram realizados cinco cortes (6µM) por exemplar, os quais foram corados com Hematoxilina e Eosina (H&E). Foi determinado os Valores Médios das Alterações e o Índice das Alterações Histológicas, este através do grau das lesões e classificados em estágios I, II e III, os quais tiveram respectivamente, o desarranjo lamelar, a ruptura do epitélio da lamela e o aneurisma, como as alterações mais expressivas. Em todas as concentrações o fármaco ocasionou alterações histológicas para *Rhamdia quelen,* sendo evidenciados danos que vão de moderados a severos nas brânquias dos peixes analisados. O ibuprofeno provocou lesões branquiais em *R. quelen* com efeito progressivo em elevadas concentrações, sugerindo que a presença deste fármaco poderia provocar anormalidades e favorecer a mortalidade nesta espécie.

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INTRODUCTION

Water is seen as an essential and irreplaceable element for the organisms development and most of the times has its quality compromised in many ways, among them, industrial effluents disposal, drugs disposal in general waste, fertilizers drainage, pesticides, heavy metals accidental or not spill, organic and inorganic compounds, among others (GALLI & ABE, 2010).

Population usually have the bad habit of disposing drugs anywhere, contaminating water and soil, which may increase the risks and adverse effects to human, animals, and aquatic organisms health (ZAPPAROLI; CAMARA; BECK, 2011). Various studies and searches have detected drugs, personal care products and cosmetic presence in surface and underground water, in human consuming water, even in soil exposed to sewage sludge application (CARVALHO et al., 2009). Among these compounds, drugs developed to control diseases, increase feed production and people's life expectancy have been highlighted. However, the growing presence of these contaminants in water has become a threat to human and environmental health, mainly because it brings risks to the aquatic ecosystems' biodiversity (TUNDISI, 2005).

Ibuprofen (isobutylpropanoicophenolic acid), is a drug of great relevance because it is commonly used, it is recommended for fevers, slight or moderate pain, and because of this has antipyretic, analgesic and anti-inflammatory effects. This drug has free sale, it can be acquired without prescription, and it is in the drugs national list of the basic component of pharmaceutical care, which contemplate drugs and inputs available in Unified Health System (hereby SUS) (RENAME, 2010). It presents good oral uptake of which approximately 80% of the dose absorbed in the gastrointestinal tract. The onset in humans occurs between 15 and 30 minutes, with elimination half-life of 1.8 to 2.0 hours. The bioprocessing is hepatic and excretion is almost done in 24 hours after the last dose, being less than 1% excreted in the unchanged form (IBUPROFENO, 2011).

For this purpose, these products have a high contamination capacity, when together with water and soil, which are not completely removed from the sewage system (PINTO et al., 2014). These compounds when in the aquatic environment, may be absorbed by teleost fishes through digestive tract (water and food ingestion), branchial and cutaneous, being the two first the main ones (SENS, 2009). Therefore, fishes have been widely used as experimental models, in aquatic ecosystems health evaluation and toxicological studies (FONTANETTI et al., 2012). Gills are responsible for the osmoregulation process, acid-base balance and nitrogen compounds excretion, therefore, the gas exchanges main place (POUGH et al., 2008). For these functional features, gills are a very used tissue for the water toxicity investigation. In this connection, histopathological alteration evaluations, including the ones affecting gills, are used as environmental biomarkers in fishes, as for laboratory studies as for *in situ* (PAULINO, 2011; REDDY & RAWAT, 2013).

Water toxicology or ecotoxicology, may be an important tool for the pollutants impact in aquatic biota evaluation (LOMBARDI, 2004). The fish species in focus, *Rhamdia quelen* (Silurioformes, Heptapteridae) is a widely distributed specie in South and Central America, in the West of the Andes and between Venezuela and North of Argentina (SILFVERGRIP, 1996). The specie culture in the South of Brazil has increased because of its rapid growth even in the coldest months, which characterise a good management resistance (CARNEIRO, 2002; FRACALOSSI et al., 2002). This is a benthic catfish, substratum speculator, which is fed by ground and water insects, crustaceans, plant remains, in addition to fishes such as lambaris and guarus and it has nocturnal habits (CASATTI & CASTRO 2006, OYAKAWA et al., 2006).

By this context, this paper's purpose was to determine ibuprofen chronic toxicological potential in *Rhamdia quelen* juveniles, and consequently the analysing histomorphological alterations induced by this drug.

MATERIAL AND METHODS

Chronic toxicological tests were made at the Research Institute in Environmental Aquaculture, hereby (InPAA) from the State University of the West of Parana (hereby Unioste), Toledo-PR Campus.

Rhamdia quelen juveniles were acquired in a commercial fish farming and transported to InPAA in aerated plastic bags. After an hour, they were stored in aerated tanks for acclimatisation during 20 days. Fishes were fed once a day with the commercial feed Kowalski (40% protein), corresponding to 0.51g per individual, totaling 3% of biomass. During this period, temperature, pH and oxygen values were watched. At the end of this period fishes were measured using an ichthyometer (Pentair) and weighted in digital scale (YDTECH). Fishes has an average length of 12.50 ± 42cm and weight body weight of 17.80 ± 5.32g.

Chronic toxicological tests were done only at the juvenile phase (04 months). Ibuprofen Lethal Concentration (LC_{50}) was 5.0mg/L, achieved by acute test previously done. From LC_{50} ibuprofen other concentrations for the toxicological test were established, being two concentration ranges selected, 0.5mg/L and 50.0mg/L, which respectively are 10 times shorter and bigger than LC_{50} dose. Ibuprofen curve presented three growing ibuprofen concentrations plus the control (CTL) group (without ibuprofen) according to GHELFI (2014); consequently having, 0.0; 0.5; 5.0 e 50.0mg/L of ibuprofen. For the test, *R. quelen* samples were distributed in an aquarium (8 L) having a density result of 0.5 fishes/L, having each sample considered as an experimental unit.

Previously, before the tests, each sample was measured and weighted and had, its feeding interrupted during all

the experiment in order to benefit drug absorption by the organism. The experiment lasted for 120 hours, having the concentrations fixed each 24 hours, when the total volume of each aquarium was replaced with the same initial concentrations (COSTA, 2016).

Aquariums received artificial aeration and water physical and chemical parameters control during this period. These data analysis showed that these measurements did not float significantly along the experiment, the water's temperature is kept at 25°C.

At the end of the experiment, 06 samples from each group were analysed and anaesthetised using benzocaine (100mg/L).

For the gills histological procedures, the right branchial arch was set apart and fixed in 10% formaldehyde for 24 hours and later stored in 70% alcohol for 30 days (ROCHA et al., 2010). After the fixing process, samples were dehydrated in growing ethanol concentrations, from 70% up to pure ethanol, diaphanized in xylol, infiltrated and included in paraffin (DA JANSEN, 2014). Thereafter, 6µm thickness cross sections were obtained with the support of an Olympus cut 4055 microtome, being coloured by hematoxylin-eosin (HE). For the blades reading a microscope was used and the photographs were taken in an Olympus DP71 photomicroscope.

Histological alterations data obtained was quantified in the gills, for each of the 6 animals in each group, 5 cuts were determined, of which 5 random camps per cut were analysed. Lesions incidence and distribution were evaluated according to these criteria: 0=Lesions absence (0 – 10%); 0+=Rarely present (11 – 25%); += Present (26 – 50%); ++= Frequent (51 – 75%) and +++= Highly frequent lesions (76 – 100%) according to Paulino et al. (2014).

Histopathological alterations were semiquantitatively tested in two ways: a) Histopathological Alteration Indices (HAI), based on each lesion severity. For this purpose, alterations were classified in progressive phases according to the organ function impairment: stage I alterations, do not compromise organ's performance; stage II, more severe and damaging to the organ's performance; and stage III, very severe and irreversible (adapted from Poleksic & Mitrovic-Tutundzic, 1994). A HAI value was calculated for each animal through the formula: $HAI = 10^0 \sum I + 10^1 \sum II + 10^2 \sum III$, where I, II and III, correspond to the stages respectively. The average HAI value was divides in 5 categories: 0-10 = normal tissue performance; 11-20 = slight to moderate tissue damage; 21-50 = moderate to severe tissue damage; 51-100 = severe tissue damage; bigger than 100 = irreparable tissue damage; and b) the Average Alteration Score (AAS), based on the lesions incidence, according to Schwaiger *et al.* (1997). For this purpose, a numerical value was assigned for each analysed sample, according to the scale: grade 1 (histopathological alteration absence); grade 2 (small centres); grade 3 (moderate centres); grade 4 (frequent lesions) and grade 5 (widely distributed lesions).

Results (mean ± DP) of HAI and AAS obtained for the animals from each analysed group, were submitted to a variance analysis (ANOVA) with the Tuckey postest for the groups comparison, taken into consideration significant $p<0.05$ values, using SigmaPlot 11.0 software. This evaluation was authorised by Ethics Animal Experiment Committee from the State University of the West of Parana 57/17.

RESULTS AND DISCUSSION

The observed alterations were classified according to the severity and impairment grade of gills functions, the most part being stage I, followed by stage II and III alterations with aneurysm presence (Table 1).

Table 1**.** Histopathology in gills of *Rhamdia quelen* juveniles, submitted to different Ibuprofen concentrations, in a chronic toxicological trial during 120 hours.

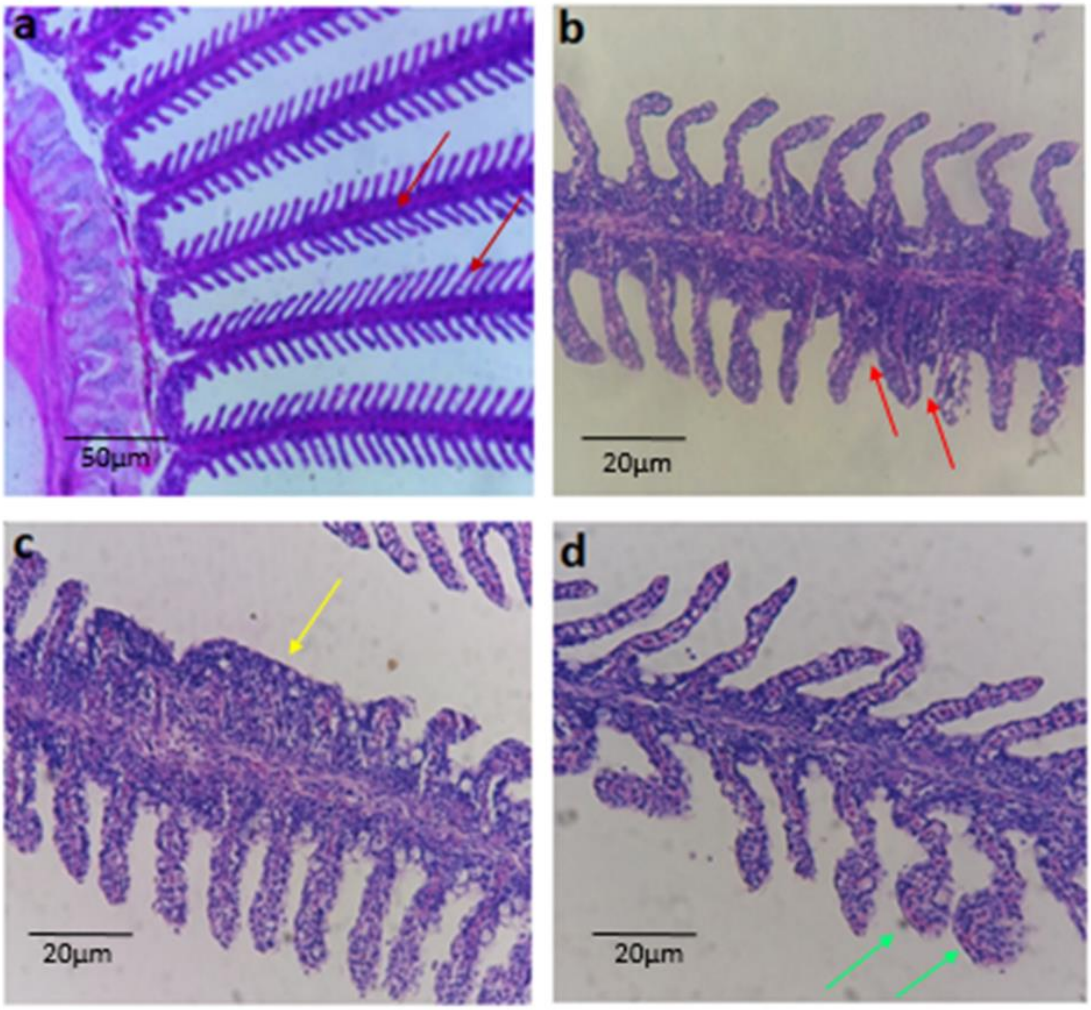
Histopathology	Stages	Control	0.5mg/L	5mg/L	50mg/L
Lamellar derangement	I	0	+++	+++	+++
Lamellas incomplete fusion	I	0	++	+++	+++
Lamellas complete fusion	I	0	+	++	+++
Lamellar epithelium gathering	I	0	++	+++	+++
Lamellar epithelium hyperplasia	I	0	++	+	+++
Parasite presence	I	0	0	0	0
Lamellas epithelium rupture	II	0	++	++	+++
Proliferative tissue uncontrolled thickening	II	0	+	++	+++
Aneurysm	III	0	++	+++	+++

Stage classification according to the severity and lesions occurrence. 0 = absent (0-10%); 0+ = rarely present (11-25%); + = present (26-50%); ++ = frequent (50-75%) and +++ = highly frequent (76-100%).

Chronic tests are important, since in aquatic environments chronic effects are more frequent, due to the dilution of contaminants, which grant a longer exposure time, to aquatic organisms. Therefore, this test allowed us to verify that histopathological's analyses made in the individuals gills submitted to different ibuprofen concentrations indicated different morphological alterations presence. Contrasting with a branchial strand image with no histological changes (Picture 1a) is the lamellas fusion, which is another important lesion that arises from the lamellas epithelial cells and brachial strands hyperplasia, so that the fusion grade depends on the hyperplasia intensity and location, this way, fusion will be partial if hyperplasia narrows to the strand basis or only to part of the lamellas. According to Melo (2012) this kind of alterations together with epithelium gathering, occur as a defence mechanism when exposed to a pollutant agent. Resulting in the block of water passing through lamellas, which makes the organ respiratory function difficult (Pictures 1b and 1c).

Aneurisms occurrence (Picture 1d) for *Rhamdia quelen* may have been stimulated by ibuprofen presence, leading to the secondary lamellas modelling loss, and consequently lamellar strand disruption. However, pillar cells rupture occurs because of the lamellas vascular axis vasodilation that lose their bearing capacity, shaping lamellar aneurysms (SANTOS, 2007).

Figure 1. Gills of *Rhamdia quelen* juveniles representative photomicrographs submitted to different ibuprofen concentrations, branchial strand with no histological alterations, highlighting primary and secondary lamellas (a). 10x magnification; lamellas incomplete fusion (b); lamellas complete fusion (c); aneurysm (d). 40X magnification. HE Staining.



According to Da Jansen (2014), lesions observed in *Centropomus undecimalis* brachial tissue may prejudice blood flow, reducing gas exchanges, which compromise gills primordial function, as well as prejudicing animal acid-base and osmotic balance maintenance. In the current study, lesions found in fishes compromise organ respiratory function partially or even completely.

Lamella epithelium gathering (red arrow) (picture 2a), probably occurred because of oedematous alterations which was frequently observed in branchial lamellas, these lesions were also observed by GUERRA-SANTOS et al. (2012). Epithelial gathering is one of the first alterations that occur when the animal is under a specific kind of stress (WINKALER et al., 2008). When it comes to lamellar epithelium hyperplasia (green arrow) (picture 2a) it happens the same way, although it does not compromise organ's function, it is possible by its evolution to cause oedematous alterations leading to the fish asphyxia death. That kind of alteration is extremely

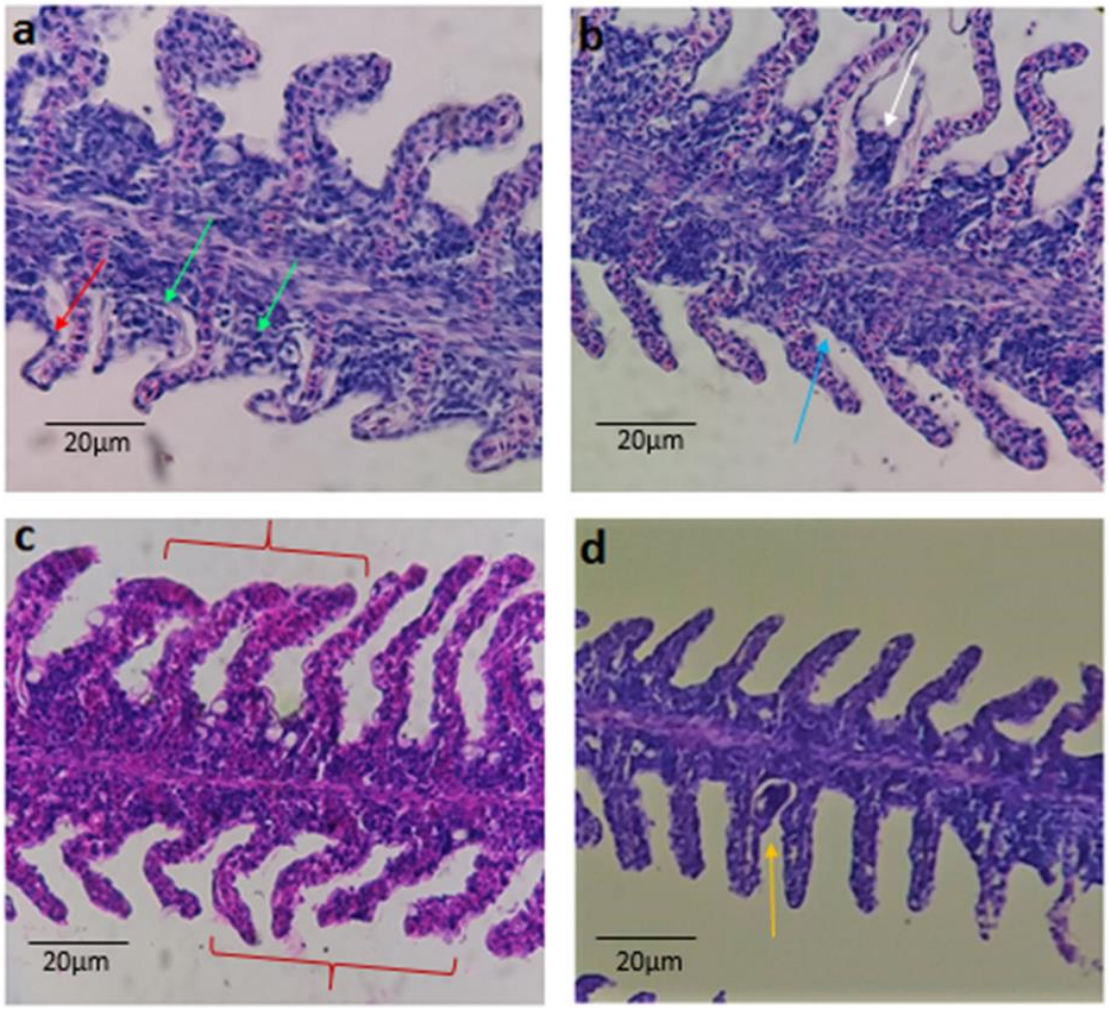
damaging to fishes gills and physiological activity. In this study it is suggested that when occurred together with lamellas complete or incomplete fusion, it was in response to the drugs permanent action, frequently registered in histological cuts in widespread focal form. Therefore, the study demonstrates the importance of chronic tests, as acute toxicity tests are not always sufficient to characterize a toxic effect of a substance or its harmfulness potential to species (MAGALHÃES & FERRÃO FILHO, 2008).

Uncontrolled thickening of the proliferative tissue (blue arrow) and lamellas epithelium rupture (white arrow) (picture 2b) are stage II alterations, these changes are more severe and compromise gills function; in case of water quality improvement , these lesions may be repairable, but, if the pollution level increases it can progress to the third stage (POLEKSIC & MITROVIC – TUTUNDZIC, 1994). For *Rhamdia quelen* specie these alterations were highly frequent for the group tested with higher drug concentration (50.0mg/L).

Lamellar derangement (Picture 4c) is the modelling loss that leads to filamentary apoptosis, these alterations were constant in gills exposed to organophosphate in studies carried with *Orechromis niloticus* (VIRGENS et al., 2015). Also evidenced in the current study, because of the exposure to the drug, this alteration difficult water flow, compromising gas exchanges.

Parasite presence was observed in two analysed fishes (Picture 2d) between secondary lamellas, taking into consideration that the samples were acquired in a fishing farm which may cause serious structural damage, such as cysts between lamellas, inflammation and a possible proliferative tissue uncontrolled thickening increase (CAMPOS et al., 2011). Most of the ectoparasites outbreaks lead to mucous cells in the branchial tissue hyperplasia (HINES & SPIRA, 1974).

Figure 2. Gills of *Rhamdia quelen* juveniles representative photomicrographs submitted to different ibuprofen concentrations, lamella epithelium gathering and lamellar epithelium hyperplasia (a); proliferative tissue uncontrolled thickening and lamella's epithelium rupture (b); lamellar derangement (c); parasite presence (d). 40X magnification. HE Staining.

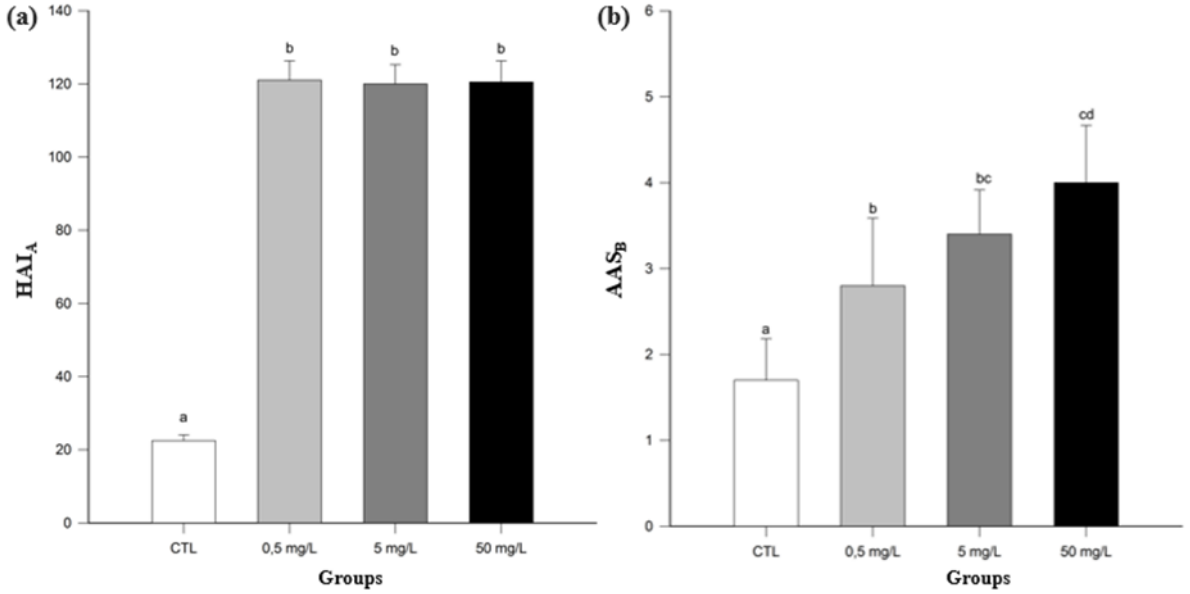


Histopathological Alteration Indices (HAI), allowed branchial lesions severity comparison in the fishes submitted to different ibuprofen concentrations, indicating great difference among the control group (0.0mg/L) and the other groups; groups (0.5; 5.0 and 50.0mg/L) indicated the same lesion indices (Picture

3a). HAI results have allowed the observation of the lesions that caused severe gills damage. These alterations, according to Poleksic & Mitrovic-Tutundzic (1994), do not deplete organs function capacity, but may revert it in case the aggressor agent is eliminated, in ibuprofen case, however, if persistence occurs, these lesions may lead to the organ's function loss.

Drugs can have a long reach in water bodies, depending on the physical-chemical properties of the compound and the characteristics of the receiving environment, either in the unchanged form or in metabolites (EBELE; ABOU-ELWAFA ABDALLAH; HARRAD, 2017). Therefore, chronic tests are very important to assess damage to aquatic species, exposed to these substances for a prolonged period. The study allowed to evaluate the Average Alteration Score (AAS) calculated for the fishes in the concentrations 0.0; 5.0 and 50.0mg/L, indicated lesions incidence in the gills of analysed samples, however, lesions observed in the control group (0.0mg/L) were only small centres, which may be directly related to the environment switch between fish farming and ambiance tanks. However, there was a high difference in lesions frequency among the other groups (0.0; 5.0 and 50.0mg/L) and even more significant in the control group (Picture 3b).

Figure 3. Histopathological Alteration Indices (HAI) and Average Alteration Score (AAS) ± for gills. CTL group (0.0mg/L); N = 06 animals/group. Different letters indicate significant statistics differences.



CONCLUSIONS

The analyses result allows the affirmation that even in low or higher concentrations, and in the tested exposure time, ibuprofen caused reversible and irreversible damage to fishes, but, lesions in higher doses classified in stages I, II and III, were mostly of highly frequent occurrence, which may result in the fishes' death. Therefore, our results prove, through the chronic toxicological test done, ibuprofen drug toxic potential for *Rhamdia quelen* specie.

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