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Liver alterations in *Oreochromis niloticus* (Pisces) induced by insecticide imidacloprid: Histopathology and heat shock protein *in situ* localization

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ABSTRACT

Liver is very sensitive to environmental contaminants such as pesticides, it being the first target of toxicity of a substance. The objective of this study was to investigate the possible effects of the insecticide imidacloprid (IMI) on the liver of *Oreochromis niloticus* according concentrations used for growing sugarcane. A semi-quantitative analysis of histopathological alterations of IMI on liver was performed by light microscopy and cellular labeling of heat shock proteins (HSP70) by immunohistochemistry. The most common changes in liver at all concentrations of IMI were hydropic degeneration, pyknotic nuclei, and loss of cell limits. Steatosis and increased levels of HSP70 were detected in hepatocytes with the highest concentration of IMI. In conclusion, the tested concentrations of IMI induced histopathological changes in the liver of *O. niloticus* and active defence mechanisms to maintain the morphophysiological integrity of the liver. This insecticide has a toxicity potential for these fish, which is a non-target organism of its action.

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KEYWORDS
Pesticides; tilapia; histochemistry; HSP70

Introduction

The production of sugarcane has expanded in the last decade and is used for biofuels, such as ethanol, which is one of the most employed fuels today.[1] Now, Brazil is the largest producer of sugarcane in the world and approximately 50% is destined to be used for ethanol.[2] The harvest in 2013–2014 was 653.519 thousand tons of sugar and 27.543 thousand m^3 of ethanol.[3] The increase in sugarcane crops has been accompanied by an increase in the consumption of pesticides, and their indiscriminate application is currently one of the greatest concerns. Among these, the neonicotinoid insecticides are among the most widely used insecticides, with a record use in more than 120 countries.[4] The pesticide with the greatest success is imidacloprid (IMI), the best-selling insecticide for many years and the second most widely used pesticide in the world.[5] In 2010 alone the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA) reported sales of 1.934 tons of IMI.[6] This was used to control pests such as sucking insects in soil, chewers, and termites, and it was used in 140 different cultures such as soybeans, sugarcane, citrus, cotton, coffee, tomatoes, and lettuce.[7]

In soil, the IMI is biotransformed into several metabolites, and degrades completely into carbon dioxide.[8] Its presence in the aquatic environment is increasingly worrying because of the excessive use of commercial products formulated with this substance.[9] IMI can accumulate and generate high risk to the environment and reaches non-target organisms.[10]

Fish are widely used as effective models of water pollution because they accumulate contaminants and show biochemical, cellular, physiological, and histologically differentiated response, making it an excellent biomarker with high application for environmental monitoring.[11,12] Among the most commonly used species of fish is *Oreochromis niloticus*, known as the Nile tilapia, the second most cultivated species of fish in the world.[13] This has been considered optimal in laboratory studies for its many advantages such as a high rate of growth and reproduction, resistance and tolerance in laboratory conditions, and adaptation in commercial food.[14,15]

The effects of contaminants in fish can manifest in organs and tissues. Histological changes could be used as sensitive tools that can detect the direct toxic effects of various compounds, and they act as good markers of environmental stresses.[16,17] Biomarkers in cellular and molecular levels complement histopathological study, offering more information on possible mechanisms of action of pesticides on non-target organisms. Heat shock proteins (HSP) are activated as a primary protection response in organisms against any change induced by environmental stress.[18]

Thus, the aim of this study was to evaluate the toxic potential of IMI in the liver of *O. niloticus* through histological, histochemical, and immunohistochemistry approaches.

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/lesb.

Material and methods

Chemical

Chemical compound IMI (1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine; CAS: N0138261-41-3; molecular formula $C_9H1_0C_lN_5O_2$) was obtained from Bayer Crop Science (Agricultural Experiment Station-SP, Lot EDE 0036241).

Test organism

The test organism used in the experiment was Nile tilapia, *O. niloticus* (Perciformes, Cichlidae). Individuals ($n = 40$) that were two months of age, weighing 30 ± 2 g and12–15 cm in size were used to avoid intraspecific differences associated with size and age. The individuals analyzed were reared on fish culture farms and kept in the Experimental Garden of the Institute of Biosciences, UNESP (São Paulo State University), Rio Claro, São Paulo, Brazil. These animals were acclimated in tanks under controlled conditions, with filtration, aeration systems at an average temperature of 23°C, and fed with commercial food.

Experimental design

The bioassays were set up in aquariums (40 L) (size: length = 45 cm, height = 25 cm, and width = 20 cm). The fish were exposed to 96 h of treatment, according to other studies with fish,[19–21] with constant aeration at a temperature of 23±2°C, pH = 8.3, and photoperiod of 14-h light/10-h darkness. Eight aquariums were used in the experiment; i.e. two for controls groups (fresh water) and the remaining six were exposed to three IMI concentrations (C1: 250; C2: 125, and C3: 62.5 μg/L). All treatments comprised two replicates with five organisms each. The test concentrations were determined by the concentration used for growing sugarcane, based on previous studies.[22]

Dissection of animals

The study was approved by The Ethics Committee on Animal Use, UNESP, and filed with the number 8937. After 96 h of treatment, fish were removed from aquaria to proceed for dissection. They were anesthetized, euthanized by pithing with surgical scissors, and dissected in saline solution for liver extraction. Liver samples were cleared from adhering tissues, fixed with aqueous Bouin's solution (0.9% picric acid, 9% formaldehyde, and 5% acetic acid) (for histology, total protein and polysaccharides determination), formal calcium (40% formalin, 10% $CaCl_2$, and distilled water) (for lipids determination), and buffered paraformaldehyde (4% paraformaldehyde in 7.4 phosphate buffer saline [PBS]) (for immunolabeling of HSP70). The material remained in fixative for over 24 h.

Histology and histochemistry

Portions of liver were dehydrated through the ascending series of alcohol (70, 80, 90, and 95%) for 15 min. The tissues were embedded in resin (Leica Historesin Embedding Kit, according to the manufacturer's specifications) for 48 h in refrigerator and transferred to plastic moulds with resin. The resin blocks with the material were cut serially using a microtome (6-μm-thick sections). The sections were floated in a tissue flotation bath at 45°C and placed on glass slides. The sections were stained with haematoxylin and eosin (HE) according to histological procedures following the protocol of Junqueira and Junqueira.[23] For the histochemical analysis, the material was subjected to bromophenol blue for the detection of proteins.[24] In addition, periodic acid-Schiff (PAS) and PAS simultaneously with Alcian blue techniques detected the presence of neutral and acid polysaccharides, and Sudan black B and Nile blue were used for lipids – all according to the protocol of Junqueira and Junqueira.[23] Two slides with eight sections of each fish were analyzed under a light microscope, and photographed.

Semi-quantitative analysis of histological results

A total of 80 slides and 640 non-consecutive histological sections were analyzed. The description and evaluation of histological alterations were analyzed in accordance with the Bernet protocol[25] with modifications.[26] Two parameters were established to determine the index of alterations, i.e. the scores (α) and importance factors (w). Histopathological observations were classified in scores on a scale of 0–6 depending on the degree and extension of alteration: 0: no occurrence, 2: mild occurrence, 4: moderate occurrence, and 6: severe occurrence. The control group and each treatment performed were given a score for each alteration and each individual. The importance factor was set for each lesion according to their pathological importance (how it affected organ function and fish survivability). The alterations were classified as one of the three important factors: (1) minimal pathological importance (damage is easily reversed); (2) moderate pathological importance (damage is reversible in most cases); and (3) severe pathological importance (damage is usually irreversible, leading to partial or total loss of organ function). Multiplication of score by the importance factor of each alteration was calculated to establish the index of alterations (I) for each individual using the following equation: $Index_{ind} = \Sigma\ (w \times \alpha)$. From individual indices, the mean values and standard deviations (SD) were calculated for all the groups. The results were compared between the control group and the groups with three concentrations using the non-parametric Mann–Whitney test and the Statistical Package for the Social Sciences for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA).

Histological preparation for HSP70 determination

Liver samples were fixed in buffered 4% paraformaldehyde solution for 24 h, then slowly dehydrated in ascending series of ice-buffered alcohol (pH 7.4) (15, 30, 50, 70, 85, 90, 95, and 100%, xylene, (I) and (II)) for 20 min and embedded in paraplast blocks. Sections of 6 μm were cut serially using a microtome, floated in a tissue floatation bath at 45°C, and placed on glass slides for immunohistochemistry.

Immunohistochemistry for the detection of HSP70

The description and evaluation were analyzed in accordance with the procedure used by Silva-Zacarin et al.[27] with modifications.

Paraffin wax was removed from slides with xylene and absolute alcohol. Dewaxed sections were then permeabilized with Triton X-100 to ensure free access of antibody to its antigen. These sections were washed in PBS and incubated with primary antibody against HSP70 (anti-HSP70, monoclonal antibody produced in mouse obtained from Sigma, St. Louis, MO, USA). For control sections, the primary antibody was omitted. After overnight incubation at 4°C, sections were washed in PBS and covered with a secondary antibody (Anti-mouse IgG whole molecule, obtained from Sigma) conjugated to alkaline phosphatise and incubated for 1 h at room temperature in a humidified chamber. The EnVision System alkaline phosphate kit (Dako™) was used in accordance with the instructions to obtain a red permanent colored precipitate that indicates the enzyme–substrate reaction product. Quantitative analyses were carried out by the immunostaining of HSP70 stress protein in the liver of the fish through Image J program. For each individual, average values and SD were calculated in all groups. Statistical analysis was performed by *t*-test, $P < 0.05$, accordingly obtained in the normality test of Shapiro–Wilk.

Results

Histological analysis

No fish died during the experiments, and no symptoms of toxicity were observed. The fish in the control group showed typical features of liver tissue (Fig. 1(a)). As described for the species, the liver has exocrine pancreatic acini and hepatocytes of polyhedral shape arranged in rows with well-defined nuclei, some with homogeneously stained cytoplasm and other less homogeneous, interspersed with sinusoidal, in which erythrocytes are observed. In the groups treated with IMI, different alterations were observed in hepatocytes. Table 1 lists the alterations observed with the corresponding importance factors (w). The most frequent and statistically significant alterations ($P < 0.05$) compared with the control group (Table 2) were hydropic degenerations, pyknotic nuclei (Fig. 1(b)), and loss of cell limits (Fig. 1(c)). In some fish (six), vacuolization of cytoplasm was observed in hepatocytes (Fig. 1(d)) but were not statistically significant.

Histochemical analysis

The vacuolization of cytoplasm observed by HE was analyzed for specific histochemical staining. The tests used for histochemical detection of proteins and polysaccharides showed no differences between the treated and the control groups. In the analysis of lipids by Nile blue staining, a difference was observed between the controls (Fig. 2(a)) and the highest concentration of IMI employed. An increased presence of neutral lipids was observed (reddish color) and acids (blue) in the cytoplasm of hepatocyte fish exposed to the highest concentration (Fig. 2(b)).

Detection of HSP70 by immunohistochemistry

Results of detection of HSP70 are shown in (Fig. 3). It was found that the control group had immunostaining of HSP70 protein, indicating that the protein is constitutively expressed in this organ as a molecular chaperone. In the treated groups, HSP70 immunolabeling was higher and statistically significant in the highest concentration of IMI.

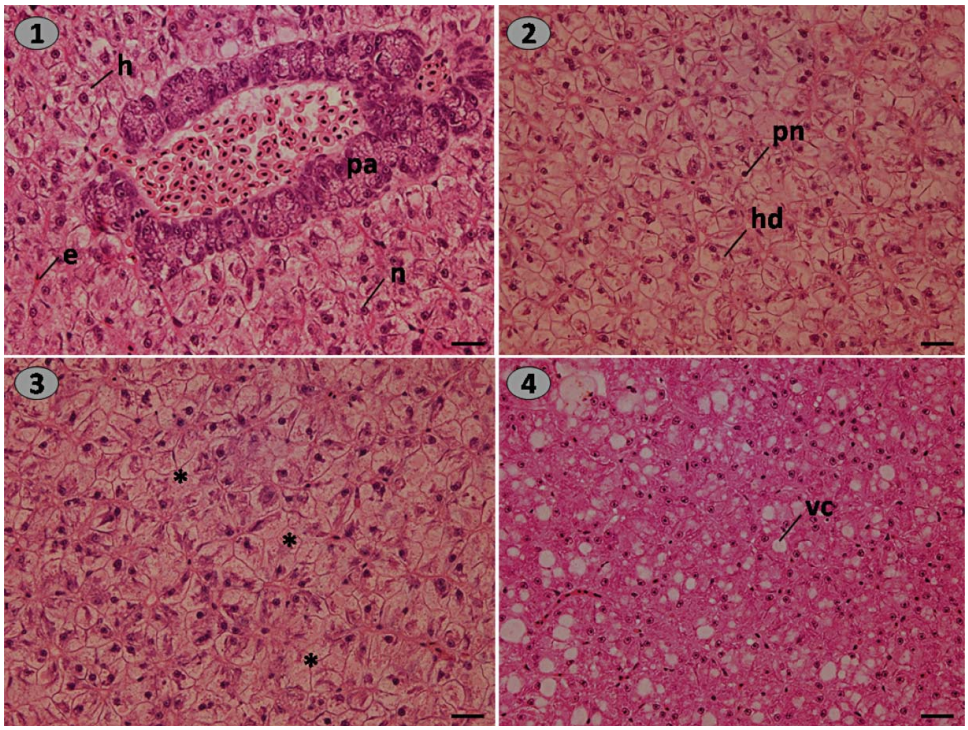

Figure 1. Liver sections of *O. niloticus* stained with hematoxylin-eosin. (a) Control group, and (b), (c), and (d) treatment groups. Bars: 20 μm; pa: pancreatic acini; h: hepatocytes; n: nucleus; e: erythrocytes; hd: hydropic degeneration; pn: pyknotic nucleus; vc: vacuolated cytoplasm; *loss of cell limit.

Table 1. Alterations observed in *O. niloticus* livers exposed to IMI, and their corresponding factor of importance.

	Characteristics examined	Factor of importance (w)
Hepatocytes	Hydropic degeneration	2
	Pyknotic nucleus	3
	Loss of cell limit	3
	Vacuolated cytoplasm	1
	Nuclear alterations	2

Discussion

Some time ago, environmentalists and government officials increased actions for water resource protection worldwide. Several decisions were based on the scientific literature, whose main objective was to assess the action of several potentially toxic products on water bodies. In addition to the contamination of water, the poisoning of aquatic animals is of concern. In this context, the presence of products that are not designed specifically for use in bodies of water is highly disturbing. However, they enter the aquatic environment through spraying practices, draining, or leaching, and reach non-target organisms, such as fish.

The neonicotinoid insecticides have a potential for leaching, and several studies have characterized their presence in water.[28] A wide variety of these insecticides have been found in water bodies such as tanks, lakes, groundwater, and streams.[6,29] The concentrations detected in water vary depending on the study; for example, surface waters in the United States of America showed that 50% of the collected water samples had a presence of IMI, and neonicotinoids low biodegradation in water.[30] Another study in California showed that 89% of the water samples contained IMI and 19% exceeded the reference index established by the US Environmental Protection Agency (USEPA) Aquatic Life Benchmark.[29] In Sydney, Australia, a concentration of 4.56 μg/L of IMI was reported in rivers.[31]

For these reasons, studies conducted on aquatic organisms are of great importance to predict the possible environmental effects of IMI. Fish are organisms widely employed in studies to assess pesticide formulations.[32–34] Pathological changes in fish are the result of biochemical and physiological changes unfavourable to the body.[35,36] Liver histology is a useful tool for the evaluation of the relationship between damage in fish and environmental pollution.[27,37–39] Liver is a key organ for the determination of pollutant actions as it is very sensitive to environmental contaminants, as well as detoxification of endogenous and exogenous products such as pesticides. The hepatocytes are considered prime targets for toxicity of a substance.[40] Multiple histological studies have been carried out on fish exposed to different insecticides. For example, studies in *Rhamdia quelen* exposed to Folidol 600® (active ingredient is methyl parathion) showed that there were several cellular changes in the liver, simulating what happens in the natural environment when water bodies are contaminated with this product.[41] Banaee et al.[42] performed chronic treatment of diazinon in *Oncorhynchus mykiss*, which caused morphological alterations in liver. Another example is acute and chronic exposure to endosulfan, which caused significant impact on the *Channa punctatus* liver tissue, showing that the fish are very sensitive to the presence of this insecticide.[43]

Various histological alterations were observed in the groups exposed to IMI. Hydropic degeneration, pyknoticnuclei, and loss of cell limits showed statistically significant differences when compared with the control group. The hydropic degeneration is characterized by an increase in cell volume due to the accumulation of water and electrolytes inside the cell.[44] The pyknotic nucleus with reduced diameter is a change that indicates condensation of chromatin, suggesting the onset of the cell death process. The loss of cell limits can result in drastic changes in organ function.[45]

The alterations observed here have been detected in other studies with other insecticides, such as fenvalerate in *Cirrhinusm rigala*,[46] endosulfan in *Cichlasoma dimerus*,[47] deltamethrin in *Oreochromis niloticus*,[48] and cypermethrin in *Oreochromis mossambicus*.[49] Effects of the mix of benzo-a-pyrene (BaP), dichlorodiphenyltrichloroethane (DDT), and tributyltin (TBT) were evaluated in *Rhamdia quelen*,[38] suggesting that the presence of these compounds may affect metabolic processes and produce pathological lesions in liver, which is the central organ of detoxification.

Many of these studies related liver damage to increased biochemical biomarkers related to oxidative stress.[38,50,51] Free radicals are important components in the toxic effects of pesticides and other environmental chemicals that induce oxidative stress by interacting with biomolecules.[52]

In addition, some cells were found with corresponding lipid storage vacuolization (Fig. 2(b)) and signs of degradation. This is steatosis (accumulation of lipid droplets).

This may be a failure of lipid metabolism due to exposure to IMI. Liver and muscle and adipose tissues are the main organs for the deposition of lipids, and their accumulation is the result of the balance between lipogenesis and β oxidation, with many enzymes involved in these metabolic processes.[53] Increased synthesis and uptake of fatty acids bring with it increased accumulation of lipids in these organs.[54,55] In most species of fish, lipids are predominant sources for obtaining energy, but excessive deposition of lipids can cause problems for the animal's health.[56] Several studies have indicated that exposure of fish to environmental stress can disrupt lipid metabolism, and they observed vacuole accumulation in liver.[53–57]

The determination of biomarker at molecular level complements the histopathology study. The first response of an organism to any change-induced environmental stress happens at sub-cellular level and could be detected by means of immunohistochemistry methods. These responses activate antioxidant defense systems and HSP, which are a primary protective

Table 2. Frequency of occurrence of significant liver alterations found in *O. niloticus* exposed to IMI.

Liver alterations	Control group	IMI [C1]	IMI [C2]	IMI [C3]
Hydropic degeneration	1.6 ± 0.6	8.0 ± 1.0*	8.4 ± 0.9*	6.0 ± 0.9*
Pyknotic nucleus	0 ± 0	6.6 ± 0.6*	8.4 ± 1.0*	5.4 ± 0.6*
Loss of cell limit	0.6 ± 0.6	9.6 ± 1.3*	11.4 ± 1.4*	9.0 ± 1.0*
Vacuolated cytoplasm	1.8 ± 0.7	2.2 ± 0.7	2.4 ± 0.7	1.6 ± 0.5
Nuclear alterations	0.4 ± 0.4	1.6 ± 0.6	3.2 ± 0.5	1.6 ± 0.6

Results presented as mean ± SD. IMI [C1]: imidacloprid (250 μg/L); IMI [C2]: imidacloprid (125 μg/L); IMI [C3]: imidacloprid (62.5 μg/L). *$P < 0.05$. Values statistically significant, compared with the control group with the Mann–Whitney test.

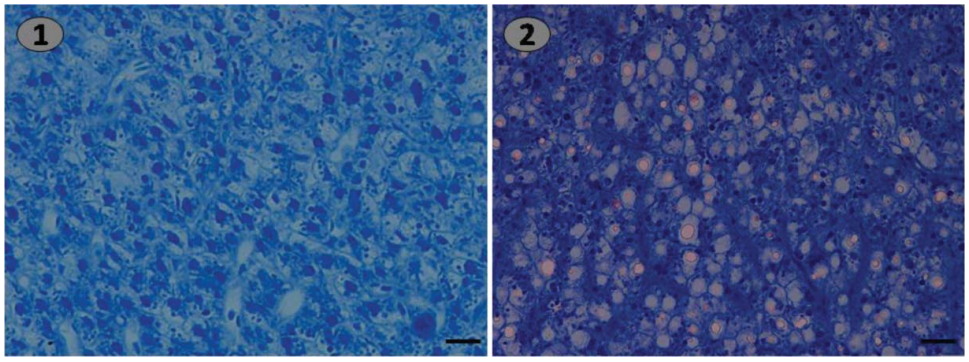

Figure 2. Liver sections of *O. niloticus* stained with Nile blue. Bars: 20 μm. (a) Control group, and (b) IMI treatment group shows vacuoles reddish color.

response.[18] The HSP70 proteins play an integral role in the cellular response pathways to stress in most organisms, including fish.[58,59] Alterations in the fish HSP70 transcription levels in liver may serve as a rapid, reproducible, sensitive, and simple ecotoxicological biomarker.[60] In liver, the HSP70 gene expressions are related with the development of antioxidant and detoxification responses.[61] The highest concentration of HSP70 in liver occurs in hepatocyte cytosol, but it can be translocated to nucleus and mitochondrial membrane in response to a toxic exposure.[62] In fish, HSP70 can be induced by exposure to pesticides.[63] Oxidative stress can induce HSP70 mRNA expression, indicating that oxygen radicals contribute to the expression of HSP70.[64,65] Probably, the biggest concentration of IMI activates the expression of HSP70 in response to oxidative stress generated by this pesticide, strengthening tolerance to environmental stress.[66]

Many studies have indicated that environmental stressors, such as pesticides, modulate HSP70 expression in fish.[63,67,68] Some authors relate the induction of the expression of this protein with stress conditions caused by pesticides.[69,70]

In this study, increased *in situ* localization of HSP70 in liver, mainly in the highest concentration of IMI, may be related to proteotoxicity in hepatocytes, presumably induced by oxidative stress that occurred by insecticide exposure, which shows correlation with histopathological results observed in liver. It can be concluded that the stress-inducible HSP70 could emerge by inducing HSP70 gene expression and, consequently, increase of immunolabeling of HSP70 in tissue to refold damage proteins as a protective mechanism in response to liver microscopic lesions induced by IMI.

The Convention on Biological Diversity [71] showed loss of diversity as one reason for the loss and degradation of habitats rich in species in many developing countries. The increased use of neonicotinoids may play a role in the decline of populations. The accumulated concentrations in soil, water, plants, nectar, and pollen are enough to subject large groups of organisms to environmental risk. We should evaluate possible strategies for achieving a balance between the use of neonicotinoids to meet the demands of production of food and fuel and the need for managing world's biodiversity to ensure the health of ecosystems.

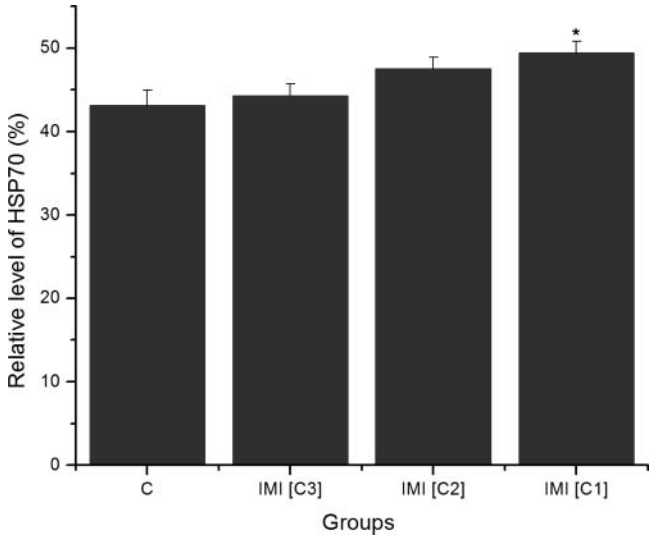

Figure 3. Relative level of HSP70 (mean ± SD) in *O. niloticus* (Pisces) livers. C: Control group (fresh water); IMI [C1]: imidacloprid (250 μg/L); IMI [C2]: imidacloprid (125 μg/L); IMI [C3]: imidacloprid (62.5 μg/L). $^{*}P < 0.05$, significant differences compared with the control group, as shown by *t*-test.

Conclusions

The tested concentrations of IMI induced histopathological changes in the liver of *O. niloticus*, and the semi-quantitative analysis used in this study proved to be an important tool for assessment of adverse effects in liver induced by toxic processes. In addition, the highest concentration of IMI produces steatosis and activated cytoprotective mechanisms mediated by HSP70 to maintain the morphophysiological integrity of liver. Determination of HSP70 levels in the field of ecotoxicology can be used as a biomarker of effect in the fish liver exposed to IMI insecticide that could be applied for environmental risk assessment of pesticides for aquatic animals (i.e. fish).

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