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Hepatic effects of the clomazone herbicide in both its free form and associated with chitosan-alginate nanoparticles in bullfrog tadpoles

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HIGHLIGHTS

- The study evaluated the effect of clomazone and nanoparticles in the tadpole livers.
- The exposure to sublethal doses present in the field causes liver damage in tadpoles.
- Exposed groups showed an increase in the frequency of melanomacrophage centres.
- Exposure to clomazone groups caused an increase of eosinophils and hepatic lipidosis.

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ABSTRACT

The use of agrochemicals in agriculture is intense and most of them could be carried out to aquatic environment. Nevertheless, there are only few studies that assess the effects of these xenobiotics on amphibians. Clomazone is an herbicide widely used in rice fields, where amphibian species live. Thus, those species may be threatened by non-target exposure. However, nanoparticles are being developed to be used as a carrier system for the agrochemicals. Such nanoparticles release the herbicide in a modified way, and are considered to be more efficient and less harmful to the environment. The aim of this study was to comparatively evaluate the effect of clomazone in its free form and associated with nanoparticles, in the liver of bullfrog tadpoles (*Lithobates catesbeianus*) when submitted to acute exposure for 96 h. According to semi-quantitative analysis, there was an increase in the frequency of melanomacrophage centres, in the accumulation of eosinophils and in lipidosis in the liver of experimental groups exposed to clomazone – in its free form and associated with nanoparticles – in comparison with the control group, and the nanotoxicity of chitosan-alginate nanoparticles. The increase of melanomacrophage centres in all exposed groups was significant ($P < 0.0001$) in comparison to control group. Therefore, the results of this research have shown that exposure to sublethal doses of the herbicide and nanoparticles triggered hepatic responses. Moreover, these results provided important data about the effect of the clomazone herbicide and organic nanoparticles, which act as carriers of agrochemicals, on the bullfrog tadpole liver.

1. Introduction

Amphibians have been considered to be the most vulnerable targets for environmental changes because they have a biphasic lifecycle and as such could be threatened by disorders in both aquatic and land environments (Becker et al., 2009). In addition,

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amphibians have a permeable, moist and vascularized skin (Stebbins and Cohen, 1995; Wake and Vredenburg, 2008), which facilitates the absorption of xenobiotics that can be present in bodies of water. Besides water contamination, several factors can contribute to the global decline in the number of amphibians, including climate changes, habitat fragmentation, competition with exotic and invasive species and infections, usually caused by fungi and other pathogens (Alford and Richards, 1999; Beebee and Griffiths, 2005; Hayes et al., 2010; Mccallum, 2007; McMenamin et al., 2008; Young et al., 2001). All these mentioned factors, associated or isolated, could cause a decrease in the reproductive and growth rates of amphibians (Hayes et al., 2010), as well as changes in their behavior and performance, which could make them more susceptible to diseases (Alford and Richards, 1999). Among these factors, exposing amphibians to xenobiotics, such as pesticides applied to crops, may negatively affect them. The pesticides can cause death or trigger sub lethal effects (Krishnamurthy and Smith, 2011), which could affect the health of amphibian populations found near to agriculture areas (Mann et al., 2009).

Xenobiotics induce several sub lethal effects on amphibians, such as modification to cardiac functions (Costa et al., 2008; Dal-Medico et al., 2014; Salla et al., 2016; Watson et al., 2014), biochemical changes in several organs (Dornelles and Oliveira, 2014; Güngördü et al., 2016; Maximiliano Attademo et al., 2015; Peltzer et al., 2013; Pereira et al., 2013), as well as morphological changes in the gonads (Abdalla et al., 2013; Li et al., 2015; Medina et al., 2012; Shi et al., 2014), testicles (Hayes et al., 2011), kidney (Çakıcı, 2015; Loumbourdis, 2005; Marques et al., 2009), skin (Van Meter et al., 2014; Walker et al., 1998) and liver (Bernabò et al., 2014; Ganser et al., 2003; Grassi et al., 2007; Lou et al., 2013; Loumbourdis, 2005; Marques et al., 2009). Xenobiotics can affect the reproduction, development and survival (Devi and Gupta, 2013; Finch et al., 2012; Flynn et al., 2015; Hooser et al., 2012; Svartz et al., 2012), among other effects such as endocrine alterations (Falfushynska et al., 2016), genomic damages (Gonçalves et al., 2015) and teratogenicity (Chae et al., 2015).

Brazil is a country with extensive agricultural areas that intensively use pesticides on crops. These pesticides can reach water bodies and lead to damage of non-target species that inhabit regions close to these areas (Botelho et al., 2012; Pateiro-Moure et al., 2011). One of the agrochemicals used in agriculture is the clomazone herbicide, which is widely applied in rice fields located in the south of Brazil (Cattaneo et al., 2012; Marchesan et al., 2007). In fact, studies have confirmed the presence of clomazone residues in water bodies close to Brazilian rice fields (Marchesan et al., 2007; Zanella et al., 2008). The presence of amphibians close to these rice fields and bays have been reported by Pastor et al. (2004) in Spain, Colombo et al. (2008) in Brazil, Hyne et al. (2009) in Australia, Bahaar and Bhat (2011) in India, and Liu et al. (2011) in China. However, there are no studies on the sub lethal effects of clomazone in amphibians. Currently, ecotoxicological studies with the clomazone herbicide are on fish (Menezes et al., 2013; Miron et al., 2008; Pereira et al., 2013). Therefore, it is relevant to evaluate the sub lethal effects induced by clomazone in amphibians.

This study also proposes to evaluate whether the association of nanoparticles with clomazone makes it less harmful for non-target amphibians than the active principle of this herbicide in its isolated form, which is usually applied in agricultural fields.

Currently, new technologies are being applied to herbicide formulations in order to release this agrochemical in a modified way, which could decrease their ability to contaminate the environment. Nanospheres, which are a type of nanoparticle, are associated with pesticides for use on agricultural fields (Grillo et al., 2012; Silva et al., 2011, 2012). The chitosan-alginate nanoparticles (AG/QS) associated with the clomazone herbicide release small quantities over time and therefore reduce the amount of the bioavailable chemical compound in the environment (Silva et al., 2010).

Simultaneously with nanotechnology development, it is necessary to assess the safety of nanospheres for different animal species. For this reason, nanotoxicology has recently emerged as a research area with a focus on testing whether or not the presence of nanoparticles in the environment induces toxic effects in the organisms exposed to them. Some nanomaterials can be recognized by animals as foreign substances (Kahru and Dubourguier, 2010; Linhua et al., 2009; Menard et al. 2011) and they could be immunologically harmful to the exposed animals. In this context, the evaluation of the bullfrog tadpole's response to chitosan-alginate nanoparticles (AG/QS) exposure is also important in this research.

This study comparatively evaluated the hepatic response of bullfrog tadpoles (*Lithobates catesbeianus*) of Gosner stage 25, under acute exposure (96 h) to the clomazone herbicide (active ingredient), in its free form and associated with chitosan-alginate nanoparticles, as well as the nanoparticles of chitosan-alginate without the herbicide. The concentration of clomazone used in this study (0.5 mg L^{-1} in Brazil) was similar to the levels of this herbicide found in flooded rice fields (Cattaneo et al., 2012; Miron et al., 2008; Rodrigues and Almeida, 2011).

The liver was the organ selected for this study because it is an important target organ in toxicological, xenobiotic evaluations due to its function in the biotransformation of chemical compounds. Xenobiotics induce molecular, biochemical and cellular responses on liver of frogs (Bernabò et al., 2014; Dornelles and Oliveria, 2015; Li et al., 2014; Regnault et al., 2014). In addition, amphibian livers have melanomacrophage centres (MMCs) that change in quantity and size in stressful conditions, such as exposure to xenobiotics (Agius and Roberts, 2003; Johnson et al., 2004; Ribeiro et al., 2011). Biometric, morphometric and morphological parameters were evaluated in this study in order to detect possible alterations in the liver at organ, tissue and cellular levels.

2. Materials and methods

2.1. Animal care

Newly hatched L. catesbeianus (Shaw, 1802) tadpoles, at Gosner (1960) developmental stage 25 (premetamorphic stage), were acquired from a frog farm located in Santa Bárbara do Oeste, São Paulo State, Southeast Brazil (22°78′S, 47°40′W), in a rural area. During acclimation period (7 days), the 170 (one-hundred seventy) tadpoles were housed in 80 L, glass aquariums that were equipped with a continuous supply (1.2 L/h) of well-aerated and dechlorinated water, at a constant temperature (25 ± 1 °C), under a natural photoperiod (~12 h light/dark cycle). Animals were fed with mashed commercial feed (Alcon Garden Basic Sticks®) once a day during the acclimation period and the supply of food to the animals was stopped 48 h before the toxicological bioassays with the herbicide clomazone and nanoparticles.

The water was monitored daily to ensure that the physical and chemical parameters were kept at acceptable levels (pH 7.1–7.3; hardness of $CaCO_3$ 28–34 mg L^{-1}; dissolved oxygen 6.8–7.5 mg L^{-1}), similar to most Brazilian inland waters (CETESB, 2009; CONAMA, 2005). All physical–chemical parameters were within the acceptable guidelines of American Society for Testing and Materials (ASTM, 2002).

2.2. Ecotoxicological experiment design

One-hundred and twenty tadpoles, at Gosner (1960) developmental stage 25, were submitted to a random distribution in to four experimental groups assayed in triplicate: I) Control (CT); II)

Clomazone herbicide (CL); III) Clomazone associated with chitosan-alginate nanoparticles (CLn); IV) Chitosan-alginate nanoparticles (AQ).

Tadpoles with body mass between 1.37 g and 3.95 g (2.24 ± 0.10 g, mean ± SE) were used in the bioassays. Each experimental group had 30 individuals divided in three glass test aquaria (N = 10 tadpoles per aquarium) comprising a triplicate per group. Each test aquarium was filled with 10 L of well-aerated, dechlorinated water (>6.0 mg O_2/L). During the bioassays, all glass test aquaria with the animals were kept under controlled laboratory temperature (25 ± 1 °C), on a 12:12 h light: dark cycle. Acute exposure to clomazone and nanoparticles was performed in a static system for 96 h.

All procedures followed ASTM (2002) guidelines, and the experiments were previously approved by the University Ethics Committee (Protocols nº 006/2013 – CEUA/UFSCar), which follows Brazilian regulating laws.

2.2.1. Clomazone exposure

The clomazone pestanal® (analytical standard), which was used in the bioassays, was obtained from Sigma–Aldrich Chemical Co. The exposure concentration of the herbicide was selected as 0.5 mg L^{-1} based on field level data (Cattaneo et al., 2012; Miron et al., 2008; Rodrigues and Almeida, 2011).

2.2.2. Nanoparticles

The nanoparticles used in this study were provided by, and prepared at, the Department of Environmental Engineering UNESP (Universidade Estadual Paulista "Julio de Mesquita Filho"), Campus Sorocaba, São Paulo State, Brazil. The chitosan-alginate nanoparticles were developed according to the methodology described by Silva et al. (2010, 2012). The chitosan used in this study was obtained from Sigma Chem. Co. and has a degree of deacetylation of about 75–85%. The developed nanoparticles were previously characterized and showed a size distribution ranging from 200 to 1000 nm, with a polydispersity of about 0.3 (Silva et al., 2010). The chitosan-alginate nanoparticles were cross linked with calcium. The final concentration of clomazone formulation added to aquaria was 0.5 mg L^{-1}, i.e. identical to the concentration applied in aquaria exposed to clomazone in its free form.

2.3. Collection of materials for light microscopy

Four days (96 h) after the beginning of the exposure, 10 tadpoles from each experimental group were randomly collected from test aquaria and were euthanized by cranial concussion. All procedures followed the American Veterinary Medical Association (AVMA, 2001) guidelines, and the experiments were approved by the University Ethics Committee (Protocols nº 006/2013), which follows Brazilian regulatory laws.

Each euthanized tadpole was subjected to a ventral surgical incision in the caudal-cranial direction in order to expose the liver. The liver was removed and weighted in an analytical balance (Bel Engineering, Class I) for the 10 tadpoles of each experimental group (see section 2.4). Thereafter, the five liver samples obtained from of each three exposed groups and control group (N = 5 per group) were fixed overnight in modified Karnovsky (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) and the sections were prepared to be observed under light microscopy.

Subsequently the liver samples was washed in a cacodylate buffer (0.1 M, pH 7.4) for a few minutes and postfixed in osmium tetroxide (0.5% concentration) for 2 h in the dark. After, the material was washed three times in the cacodylate buffer for 30 min (10 min per bath) and processed according the methodology described by Silva-Zacarin et al. (2012) and adapted by Abdalla et al. (2013). Then, the material was submitted to a slow dehydration in increasing series of cold-ethanol for 40 min every bath. After this step, the liver fragments were embedded in resin (Leica®). The histological sections with 1.5 μm of thickness were obtained in the microtome and were then hydrated and submitted to Hematoxylin-Eosin staining (HE) for morphological analysis by light microscopy.

Some slides were stained with Instant Prov Kit (New Prov®) for visualization of different types of leukocytes and others slides were submitted to Sudan Black (Pearse, 1960) for lipid detection in the liver tissue.

For the semi-quantitative analysis of the hepatic parenchyma, five individuals were analyzed per experimental group. Two histological slides per individual for each experimental group were evaluated. Each slide contained 16 liver tissue sections obtained by sectioning at different depths of the livers. The histological features were grouped into three categories (morphological biomarkers): lipidosis in hepatocytes, frequency of eosinophils in capillaries or parenchyma, frequency of melanomacrophage centres. Each histological feature was assessed in a 6-grade score adapted from Bernet et al. (1999), using a score ranging from 0 (zero) to 6 (six). Score was attributed according to alteration degree of each histological parameter and/or its mean prevalence in area of section: 0 – absence; 2 – slight degree in <25% area of section; 4 – moderate degree in 25–50% area of section; 6 – severe degree in >50% area of section. The analyses were done at 400 magnification.

2.4. Biometric and morphometric analysis

The biometric study was conducted on 10 tadpoles collected randomly from each experimental group (CT = control, CL = clomazone herbicide, CLn = clomazone associated with chitosan-alginate nanoparticles, AQ = chitosan-alginate nanoparticles). The total tadpole body mass and the liver mass were used to calculate the hepatosomatic index [HSI= (liver mass/tadpole body mass) × 100] from each experimental group. The result obtained was submitted to statistical analysis by parametric ANOVA test, one way, with Dunnett's Test, *a posteriori*, using the statistical analysis program Graph Pad Prism version 5.0. These tests were performed to compare all exposed groups with the control.

The morphometric studies were made from counts on histological slides using pre-established parameters: I) Count melanomacrophage centres to confirm or refute the hypothesis that these cells increase in frequency in the exposed groups; II) Ratio hepatocytes by melanomacrophage centres to verify if the increase (hyperplasia) or the decrease (degeneration) of the number of hepatocytes in response to the exposure to clomazone and/or nanoparticles (adapted from Crunkhorn et al., 2004).

For counting melanomacrophage centres, liver fragments of each tadpole (N = 5 animals per group) were sectioned into 2 μm thick sections, which were collected at intervals of 12 μm and deposited on the slide, in order to obtain different depths of the liver in the same slide, totaling 20 non-overlapping sections per slide and ten slides per animal. To determine the frequency of the melanomacrophage centres, 20 non-overlapping sections in non-consecutive slides were digitized for each tadpole (N = 5 animals per group), so that one-hundred fields for the experimental group were photo-documented under a 4x objective (final magnification = 40×) in order to visualize the larger area for counting. All quantitative analyses were counted using the IMC-50 Leica Software.

To determine the ratio of hepatocytes by melanomacrophage centres, five non-overlapping sections in non-consecutive slides were digitized for each tadpole (N = 5 animals per group), so that fifty random fields for experimental group were photo-

documented at 400× magnification, in order to quantify the number of melanomacrophage centres and hepatocytes. All quantitative analyses were counted using the IMC-50 Leica Software. Mean numbers of MMCs and hepatocytes and the mean ration of hepatocyte number to MMCs number per field for each animal were calculated.

The morphometric data obtained from counting melanomacrophage centres and ratio hepatocytes by melanomacrophage centres was statistically analyzed using the ANOVA test, one way, with Tukey's Multiple Comparison Test, *a posteriori*. These tests were applied in a paired way, thus the data of the experimental groups were analyzed by comparison to the controls for each exposed group. Subsequently, the results were plotted together in the graphs. The program used for statistical analysis was the Graph Pad Prism version 5.0.

3. Results

3.1. Biometric analysis

The HSI of the exposed animals showed no significant difference from the control group value (ANOVA One-Way test, with Dunnett's test, *a posteriori*, $P > 0.05$) (Fig. 1). The HSI for each exposed group was CL = 2.47 ± 0.165; AQ = 2.29 ± 0.173; CL_n = 2.35 ± 0.181 (mean ± SE) and for the control group (CT = 2.19 ± 0.118).

3.2. Morphometric analysis

The quantity of melanomacrophage centres of the exposed animals showed significant difference from the control group value (ANOVA One-Way test, with Tukey's Multiple Comparison test, *a posteriori*, $P < 0.0001$) (Fig. 2).

Although the number of MMCs is similar in all exposed groups (Fig. 2), the number of hepatocytes per area unit independent of the other components varied (mean ± SE): Control = 45.4 ± 1.16; Herbicide Clomazone = 43.7 ± 1.30; Chitosan-Alginate nanoparticle = 50.7 ± 1.37; Clomazone associated with Chitosan-Alginate nanoparticle = 38.9 ± 0.85. The number of hepatocytes per area unit (12,739.5 μm^2) significantly decreased in the group exposed to Clomazone associated with Chitosan-Alginate nanoparticle in relation to the control and others exposed groups (ANOVA One-Way test, with Tukey's Multiple Comparison test, *a posteriori*, $P < 0.0001$).

The ratio of hepatocytes by melanomacrophage centres showed no significant difference among the experimental groups (ANOVA One-Way test, with Tukey's Multiple Comparison test, *a posteriori*, $P > 0.05$) (Fig. 3).

3.3. Histological analysis

The morphological data on the liver of bullfrog tadpoles showed the typical cytoarchitecture of the hepatic parenchyma of amphibians: hepatocytes forming acini, the occurrence of leukocytes, bile ducts, blood vessels of different sizes and sinusoidal capillaries (Fig. 4A–D) in the control and exposed groups. There are no modifications in the liver stroma. Vascularization was similar in all experimental groups, without rupture in blood vessels and absence of dilatation of sinusoids. In the groups exposed to nanoparticles (Fig. 4C, D), hepatic sinusoid was completely full of erythrocytes. In all experimental groups (CT – Fig. 4A; CL – Fig. 4B; AG – Fig. 4C; CL_n – Fig. 4D), there were melanomacrophage cells diffusely dispersed throughout the liver, which presented a black–brown pigmentation.

The lipidosis in the cytoplasm of hepatocytes, as well as the presence of granulocytes and melanomacrophage centres in the hepatic parenchyma, were considered morphological biomarkers in the evaluation process of the sublethal effects on tadpoles (Table 1). The lipidosis was visualized as small vacuoles not stained by hematoxylin-eosin in the cytoplasm of the hepatocytes. The lipidosis in the hepatic tissue was observed in individuals exposed to clomazone in its free form (Fig. 4B) and associated with nanoparticles (Fig. 4D).

In the exposure to the active ingredient in the herbicide group (Table 1, Fig. 4B), there was an increase in the frequency of melanomacrophage centres compared with the control groups (Table 1, Fig. 4A). High frequency of melanomacrophage centres was observed in the groups exposed to the nanoparticles (Table 1, Fig. 4C) and to the nanoparticles associated with clomazone (Table 1, Fig. 4D).

The Sudan Black histochemical test was performed to confirm the presence of lipid granules in vacuoles not stained by hematoxylin-eosin (Fig. 5A), which allowed for the detection of lipidosis in liver tissues. Additionally, a kit for the characterization of leukocytes (Instant Prov Kit) was used to confirm the occurrence of eosinophils in the liver tissue (Fig. 5C), since the hematoxylin-eosin showed granulocytes intensely stained by eosin (Fig. 5B).

4. Discussion

This study was the first to evaluate the adverse effects of the clomazone herbicide on amphibian tadpoles. Clomazone is an herbicide widely used in rice fields and specimens of the order Anura are usually found in these areas (Bahaar and Bhat, 2011; Colombo et al., 2008). Clomazone is water soluble and its half-life in water solution is greater than 30 days (CDPR, 2003). This herbicide does not suffer photolysis in water (CDPR, 2003; USEPA, 2007). In the present study, in order to investigate the clomazone herbicide stability, its concentration in water have been determined by HPLC and during the experimental time (4 days) there was not observed changes in the herbicide concentration (data not shown). Clomazone can be biotransformed in the organism (Pereira et al. 2013) and even bioconcentrate in the animal body (Lazartigues et al., 2013). In this scenario, probably the tadpoles absorbed the clomazone and the nanoparticles.

In order to minimize the effects of pesticides on non-target organisms, new technology in agriculture is emerging with the development of nanoparticles as carriers of agrochemicals (Grillo et al. 2016). However, there is need to evaluate the nanotoxicity of these nanoparticles on non-target organisms, which was the focus of this study. Studies about the adverse effects of nanoparticles in amphibians are scarce (Bonfanti et al., 2015; Bour et al., 2015; Bacchetta et al., 2012; Mouchet et al., 2008; Nations et al., 2015).

The results of this study indicated the effects caused by the clomazone herbicide, in its free form or are associated with the nanoparticles, as well as by the nanoparticles without herbicide. These data about nanotoxicity of an organic nanoparticle (chitosan-alginate) associated to an herbicide (clomazone) are pioneer in bullfrog tadpoles and anuran amphibians. Studies about the environmental health impacts of nanomaterials in aquatic ecosystem, especially in frogs, are in progress. Carbon nanoparticles in the frog *Xenopus laevis* induced abnormalities in the movements of swimming and decreased growth of tadpoles, and histological changes in the kidney (Mouchet et al., 2008). Copper nanoparticles of titanium dioxide and zinc oxide induced malformations in *X. laevis* tadpoles and, additionally, histopathological lesions were caused in the intestine (Bacchetta et al., 2012). In addition, zinc oxide nanoparticles induced a high incidence of malformations, in particular misfolded gut and abdominal edema in *X. laevis* tadpoles (Bonfanti et al., 2015).

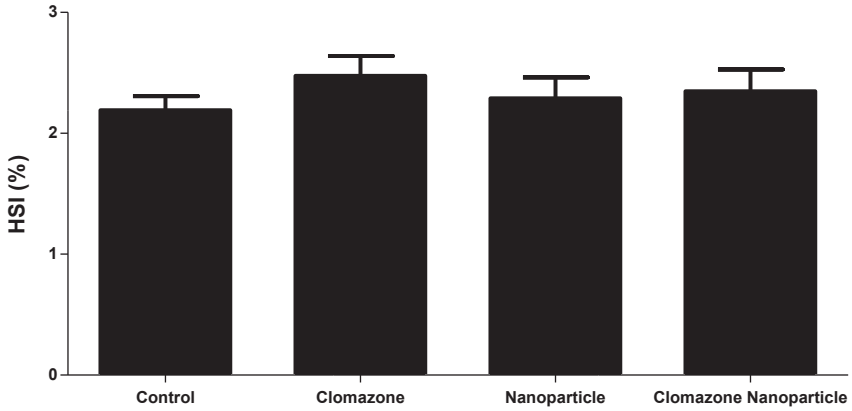

Fig. 1. Hepatosomatic index (HSI) of bullfrog tadpoles (*L. catesbeianus*) between the control group, exposed to the clomazone group (active principle), exposed to nanoparticles and exposed to nanoparticles associated to the active principle (clomazone). ANOVA One Way Test, with Dunnett's Test, *a posteriori*. The bar observed indicate the standard error. The exposed and control groups showing no significant difference with two-tailed P value > 0.05.

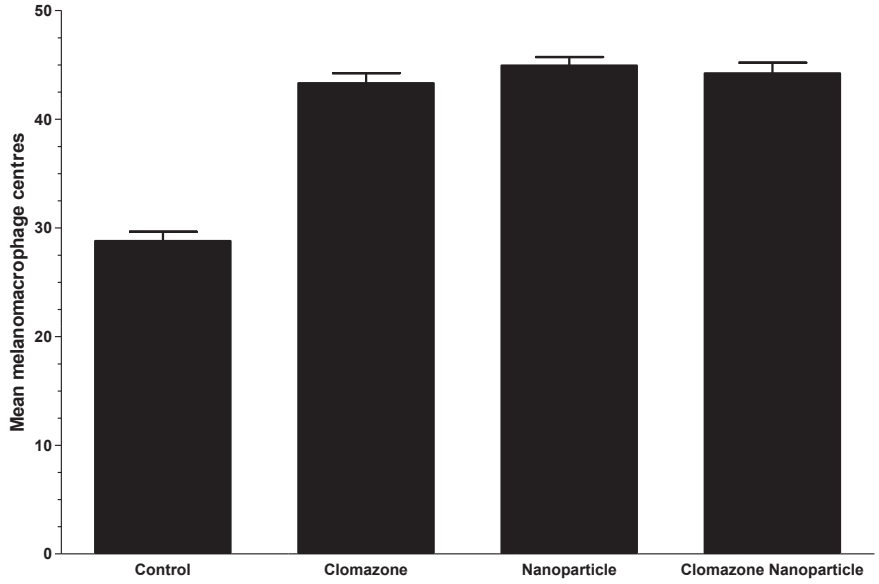

Fig. 2. Count of melanomacrophage centres obtained by analysis of liver section by IMC-50 Leica Software, showing that the mean number of melanomacrophage centres in tadpoles' liver increased in all groups exposed in comparison to the control group. Anova One Way test, with Tukey's Multiple Comparison Test, *a posteriori*. The exposed compared with control groups indicates significant difference with two-tailed P value is <0.0001.

The absence of significant difference in the hepatosomatic index (HSI) and the ratio of hepatocytes by melanomacrophage centres does not necessarily mean that there was no change among the components of liver tissue (hepatocytes and MMCs) of the exposed groups, in comparison to the control group. The comparison of the biometric and morphometric data suggests a compensatory response of liver from exposed animals. The significant decreased in number of hepatocytes in the group exposed to clomazone associated with nanoparticles possibly was compensated by the increase of number of MMCs, as well as their high development degree, which is evidenced by the increase of area. There was high frequency of MMCs with smaller area (100–2000 μ^2) in control group and MMCs with larger area in exposed groups, mainly in the group exposed to Chitosan-Alginate nanoparticle (12,001–20,000 μ^2) [data not shown]. In this scenario, the ratio hepatocytes by melanomacrophage centres did not changed in exposed groups in comparison to the control group because the compensatory response.

The increase in the number of melanomacrophage centres in relation to the control might be the first response from the defense system by exposure to the xenobiotics (Agius and Roberts, 2003) and, later, the defense system acts in the inflammation due to their phagocytic properties (Loumbourdis and Vogiatzis, 2002). This increase in the melanomacrophage centres to the xenobiotics or others environmental stresses was described by several authors (Agius and Roberts, 2003; Johnson et al., 2004; Ribeiro et al., 2011).

The abundance of melanomacrophage centres in the groups exposed to the nanoparticles associated, or not with the herbicide, reflects that the tadpole organism might recognize the chitosan-alginate nanoparticle as a toxin. However, the exact mechanism for immune modulation involving the melanomacrophage centres and the organic nanoparticle used in this study remain unknown, as well as the congestion of erythrocytes in hepatic sinusoids in these nanoparticle-exposed tadpoles. Hepatic confinement of erythrocytes was described in liver of *X. laevis* exposed to a thermic stressful condition (Maekawa et al., 2012).

Among the morphological alterations identified in the hepatic parenchyma of the tadpoles in the groups exposed to clomazone,

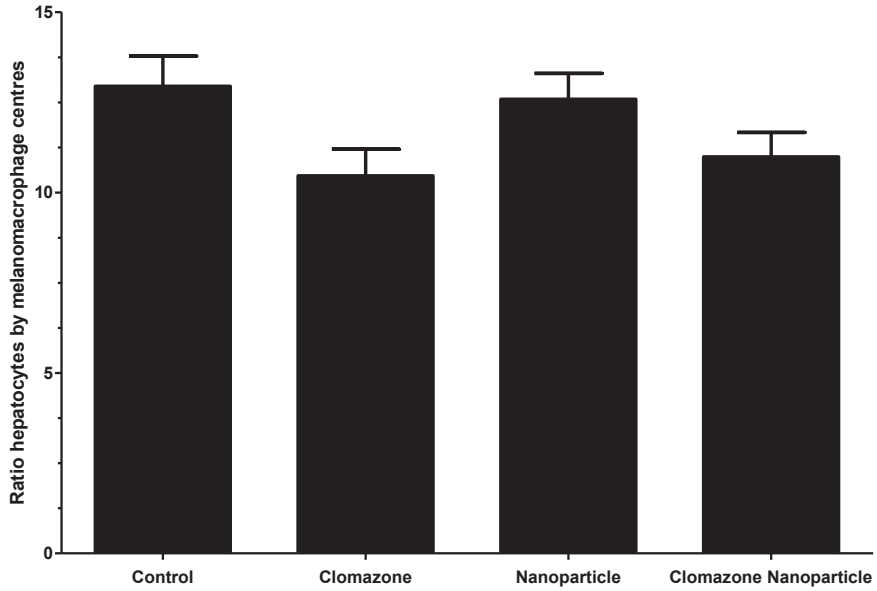

Fig. 3. Ratio hepatocytes by melanomacrophage centres obtained by analysis of liver section by IMC-50 Leica Software, showing the ratio of the number of hepatocytes by melanomacrophage centres number in tadpoles' liver not increased among the experimental groups. Anova One Way test, with Tukey's Multiple Comparison Test, *a posteriori*. The exposed and control groups showing no significant difference with two-tailed P value >0.05.

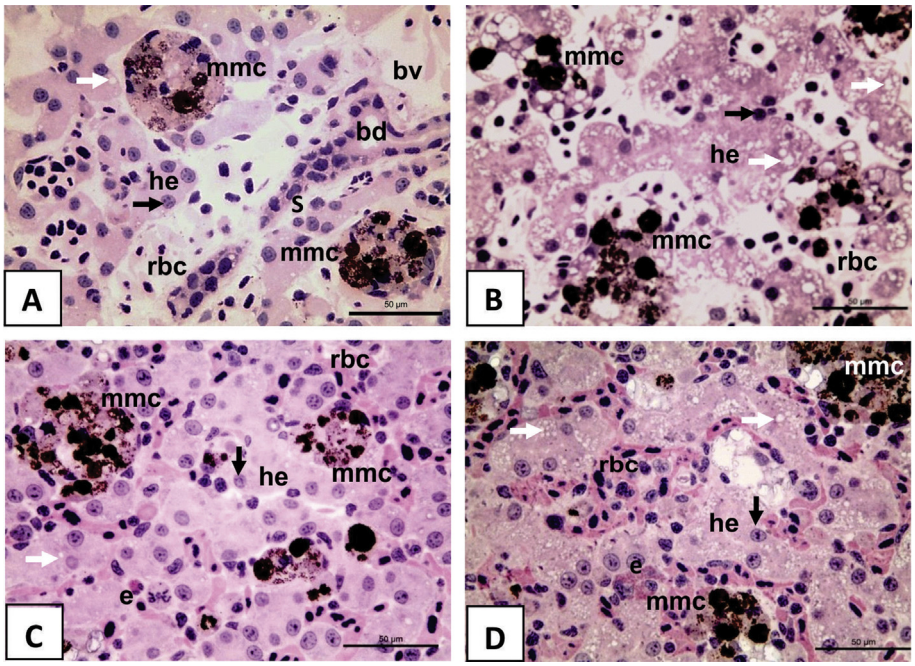

Fig. 4. Bullfrog tadpole livers (*L. catesbeianus*) at stage 25 of Gosner. Histological sections stained with hematoxylin-eosin (HE). A) Control group; B) Exposed to the active ingredient of the herbicide group; C) Exposed to nanoparticle group and D) Exposed to nanoparticle associated with the active ingredient group. Note the structure of liver tissue, such as hepatocytes (he), and their nuclei (shown by black arrows), and bile ducts (bd), blood vessels (bv) and sinusoids (S) containing red blood cells (rbc) in its interior, defense cells such as eosinophil (e) Note the presence of melanomacrophage centres (MMC) in all experimental groups. The lipidosis is more frequent in the exposed groups (shown by the white arrow). A – D) Bar = 100 μm.

both in its free form and associated with nanoparticles, one of the most important is lipidosis, characterized by lipid accumulation in the vacuoles of different sizes in the cytoplasm of the hepatocytes (Brum et al., 2014). The lipidosis observed in the liver of the animals from the exposed groups reflects the standard hepatic response to clomazone in the initial phase, when only small vacuoles were observed in the cytoplasm. Other feature that reinforce that the lipidosis is the initial phase is the absence of the diminishing sinusoidal space due to the expansion in the cytoplasmic volume of the hepatocytes with a high degree of lipidosis (Shaw and Handy, 2006).

Micro and macro lipidosis were described in the hepatocytes of catfish (*Rhamdia quelen*) exposed to the commercial formula of clomazone (Brum et al., 2014) and in the hepatocytes of carp (*Cyprinus carpio*) exposed to different concentrations of TiO_2 nanoparticles (Federeci et al., 2007; Linhua et al., 2009). Other

Table 1
Semi-quantitative analysis in tadpoles' liver in the groups exposed to clomazone herbicide (active ingredient), in its free form and associated to nanoparticles, and chitosan-alginate nanoparticle without the herbicide, as well as the control group. The dates were presented as score values, ranging from 0 to 6, depending on the degree and extent of the alteration.

Morphological biomarkers	Control	Clomazone	Nanoparticle	Clomazone Nanoparticle
Lipidosis in hepatocytes	2	4	2	4
Eosinophils	2	4	2	6
Melanomacrophage centres	4	6	6	6

Ranging: Score were attributed according to alteration degree of the histological parameter and its mean prevalence in area of section: 0 – absence; 2 – slight degree in <25% area of section; 4 – moderate degree in 25–50% area of section; 6 – severe degree in >50% area of section.

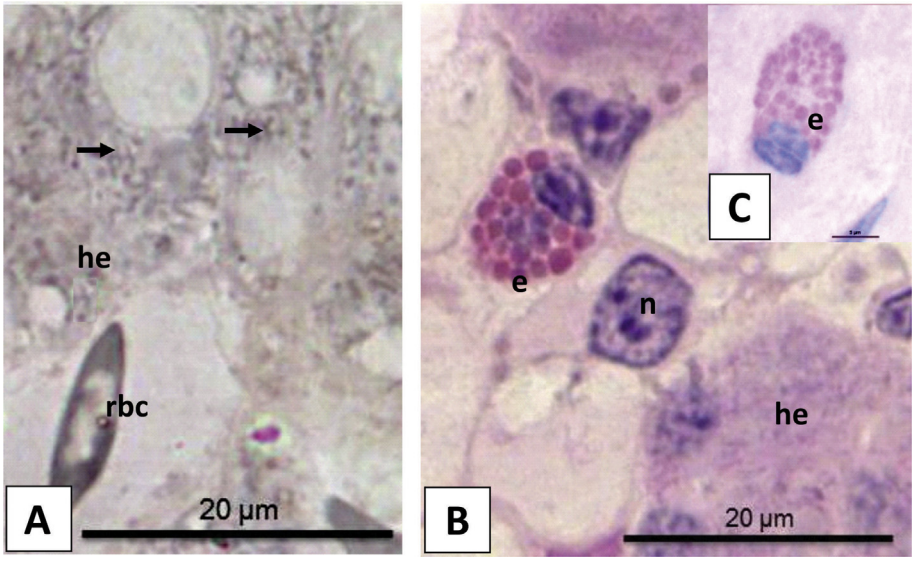

Fig. 5. Bullfrog tadpole livers (*L. catesbeianus*) at stage 25 of Gosner. Histological sections stained with Sudan Black (A), hematoxylin-eosin (B) and Instant Prov (C). A–C) Exposed to the active ingredient of the herbicide group. In (A), note the positive staining of Sudan Black for lipids in the liver parenchyma (shown by black arrow); B and C) Eosinophils (e) are observed. Caption: hepatocytes (he) and their nuclei (n), red blood cells (rbc).

studies have also observed the presence of lipidosis in the hepatocytes of animals exposed to different xenobiotics (Greenfield et al., 2008; Peebua et al., 2008; Ribeiro et al., 2005; Samanta et al., 2015; Smith et al. 2007). However, the mechanisms can be different; generally, the accumulation of lipids in the liver is accompanied by biochemical alterations in blood (Hodgson and Levi, 2004).

The mechanism by which the occurrence of lipidosis in all of the groups exposed to clomazone remains unknown. The abnormal accumulation of lipids in the liver was also described by Glover et al. (2007) in salmon (*Salmo salar*) exposed to endosulfan, and by Costa et al. (2013) in Senegalese sole fish (*Solea senegalensis*) exposed to cadmium. An injury or inflammation induced by xenobiotics could prejudice lipid oxidation and protein synthesis, causing an accumulation of triglycerides in the hepatocytes (Greenfield et al., 2008; Melvin et al., 2013). In this context, the increase in eosinophil frequency in the groups exposed to clomazone (both in its free form, and associated with the nanoparticles) could at least partially explain the lipidosis seen in this study. Leucocytes increased in number during toxicosis in fishes affected by pesticides, including the herbicide paraquat (Rojik et al., 1983).

According to Agius and Roberts (2003), the increase of environmental stress in the derived vertebrate leads to changes in the circulation of leukocytes and, consequently, the number of granulocytes increases in the liver. In the studies by Costa et al. (2013) performed on *S. senegalensis* exposed to cadmium, the authors observed highly elevated concentrations of eosinophil in the liver and noted a correlation between this data and liver damage.

In this way, although this study has indicated that the exposure time induced hepatotoxicity in tadpoles, a hepatic compensatory response was also observed, thus we cannot infer how much these changes could interfere with the health and development of the adult animal. However, the results of the present study represent an alert with respect to the sub lethal effects in low doses of the clomazone herbicide found in agricultural fields as well as showing the importance of evaluating the effect of biodegradable nanoparticles in non-targeted organisms. Studies about nanotoxicity are important and should be encouraged to assess the level of the ecotoxicological risk of nanoparticles by means of careful analysis of the data, which will contribute to the development of new systems that cause less impact to the environment, so that the association of pesticides with nanocarriers achieve the desired benefits in pest control in agriculture. However, it is necessary further studies to investigate the potential effects of agrochemicals and nanoparticles present in the environment in low concentrations on the amphibians populations since they are currently endangered.

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