Chemosphere 149 (2016) 304–313

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

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.

ARTICLE INFO

Article history:
Received 7 October 2015
Received in revised form
14 January 2016
Accepted 18 January 2016
Available online 10 February 2016

Handling Editor: David Volz

Keywords:
Frog
Ecotoxicology
Nanotoxicity
Liver
Lipidosis
Melanomacrophage centres

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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http://dx.doi.org/10.1016/j.chemosphere.2016.01.076
0045-6535/

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)