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Effects of anthropogenic metallic contamination on cholinesterases of *Gambusia holbrooki*

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ABSTRACT

Metal contamination causes multiple biological dysfunctions, including impairment of key physiological functions by targeting enzymes. This feature is a matter of concern, since it may imply significant disturbances in energy allocation, behaviour, reproduction, and survival. Inhibition of the cholinesterase (ChE) activity of aquatic organisms by metals has been described, and systematically used in biomonitoring studies as effect criterion of environmental exposure to these compounds. The present paper addresses the feasibility of using ChE inhibition to quantify the adverse acute and chronic effects of metals (copper, zinc, lead, and cadmium) on nervous tissue of *Gambusia holbrooki*. With the exception of acute exposure to copper, ChE activity was not significantly impaired. The meanings of the reported findings are further discussed, aiming at a more comprehensive use of this biomarker in environmental assessment. Based on the obtained results, the role of ChE inhibition in environmental metal contamination scenarios should be questioned or even discarded.

1. Introduction

Metals are widely dispersed in the aquatic environment, as a consequence of anthropogenic activities, such as surface or deep-sea mining (Oebius et al., 2001), or dissolution of metallic particles in the atmosphere resulting from the burning of fossil fuels (Abdollahi et al., 2013), and input from natural sources (e.g. submarine volcanoes; Rubin, 1997). This dispersion favours the establishment of toxic interactions with biota. Metallic species can thus exert multiple biologic effects, such as neurotoxicity, genotoxicity, carcinogenicity (Florea and Büsselberg, 2006), and also enzyme inhibition (El Khalil et al., 2008; Garceau et al., 2010).

Cholinesterases (ChE) are esterases, with capability of hydrolysing carboxylic esters. ChE can be distinguished from other esterases since they exhibit preference for the hydrolysis of choline esters rather than other carboxylic esters and are inhibited by physostigmine (eserine) at concentrations of 10^{-5} M (Eto, 1974). ChE can be grouped in two major distinct subdivisions: true cholinesterases (also designated as acetylcholinesterase, AChE) and pseudocholinesterases (represented by enzymes such as butyrilcholinesterases, BChE). These two different forms can be differentially classified according to their preference for specific inhibitors and substrates. The biologic importance of ChE is indisputable. AChE, for instance, plays a central role in the mechanism of neurotransmission, since it cleaves the neurotransmitter acetylcholine after its release at the nervous cleft of cholinergic synapses. Inhibition of AChE often takes place following environmental exposure to several chemical agents, known as anticholinesterasic compounds (e.g. organophosphorous pesticides, OPs); as a consequence of this inhibition, the cleavage of acetylcholine is impaired and it accumulates in the nervous cleft, causing overstimulation of post-synaptic membranes. This set of effects culminates in acute toxicity, frequently resulting in death of the exposed organisms (Nunes et al., 2005). The diagnostic of environmental contamination by anticholinesterasic agents has long been based on the quantification of cholinesterases in exposed organisms. To assess exposure to OPs, one can evaluate the hydrolytic activity of the cholinesterases of exposed organisms. However, the role of cholinesterase inhibition was somewhat broadened, when several studies pinpointed the feasibility of using this toxicity criterion to study effects caused by other chemical agents, such as metals (Guilhermino et al., 1996; Labrot et al., 1996; Payne et al., 1996). Nevertheless, the number of such studies is still limited, and other contradictory theories have been postulated suggesting that cholinesterase inhibition by metals can be a matter of experimental artefacts being erroneously misinterpreted as a true inhibition phenomenon (Nunes, 2011).

Gambusia holbrooki is an euryhaline, globally distributed Poeciliidae, found mainly under temperate climate conditions (Nunes

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et al., 2005). It was selected as the test organism for the present study since it presents particular characteristics, such as natural abundance, simple capture, easy laboratorial rearing, large biological representativity (secondary consumers in the habitats in which they were introduced) and adaptability to brackish water (Nunes et al., 2005). Furthermore, the cholinesterasic forms present in this organisms have been thoroughly characterised, and it is possible to infer that AChE is the most abundant form (Nunes et al., 2005).

The present work intended to characterise the anticholinesterasic response elicited by metals (zinc, cadmium, lead and copper) on AChE of *G. holbrooki* total head homogenates, following both acute and chronic exposures.

2. Materials and methods

2.1. Chemicals and test organisms

Fish were collected at Pateira de Fermentelos, located in the central region of Portugal, using a hand dip net. This lagoon is characterised by low levels of anthropogenic pollution (Ferreira et al., 2003). Fish were temporarily (4–5 days) kept in 30 l plastic tanks, filled with water from the sampling site. A first phase of laboratory acclimation involved a period of quarantine (15–30 days), in which the animals were kept in dechlorinated tap water, continuous aeration with a temperature of 20 ± 1 °C. Inspections were conducted twice a day in order to discard wounded, diseased and dead individuals. After this period of quarantine, males and females were separately maintained in dechlorinated tap water. The photoperiod was 16 h light/8 h darkness, and the temperature was 20 ± 1 °C. Continuous aeration was provided. Fish were fed *ad libitum* with commercially available fish food (Sera Vipan® flakes). Partial medium changes occurred once a week.

The metals (lead, cadmium, copper and zinc) to which fish were exposed were used in the forms of lead chloride, cadmium chloride, copper (II) sulfate pentahydrate and zinc sulfate, in degrees of purity of 99.9%, 99.99%, >98% and 99.0%, respectively. Chemicals were purchased from Sigma–Aldrich.

2.2. Exposures

The experimental design generally followed recommendations of OECD guidelines (OECD, 1993). Fish (total length between 2 and 2.5 cm) were used in acute tests. Fish were individually exposed in food grade plastic containers (previously rinsed with distilled water). The containers were filled with 200 ml of dechlorinated tap water. Ten specimens were used per concentration, and an additional treatment (control) was added. Stock solutions of every metal were prepared by dissolving the pure compounds in dechlorinated tap water. The ranges of concentrations for each metal, for both acute and chronic exposure, were calculated according to previously published data: concentrations of metals tested were based on a combination of previous studies (LC_{50} values – for acute exposures) and levels already found and described in real conditions of Portuguese estuaries (Mucha et al., 2003; Fernandes et al., 2007), to simulate a low level of exposure that is normally available in the environment (for chronic exposures).

Temperature, photoperiod and aeration were as previously described. No food was provided during the acute tests; the duration of exposure was 96 h. Chronic exposures were conducted for 28 days. Feeding was performed once every other day. Renewal of test medium was also performed once every other day. Conductivity, pH, O_2 concentration and temperature were monitored everyday, for test validation purposes. After the end of the exposure periods, animals were sacrificed by decapitation, and whole heads were homogenised in ice-cold phosphate buffer (0.1 M, pH = 7.2) using an YSTRAL D-79282 homogeniser. After homogenisation, samples were stored at −80 °C until enzymatic determinations were performed. Before the biomarker determinations, samples were centrifuged using a refrigerated centrifuge.

2.3. Enzymatic determinations

AChE activity was determined in head homogenates following the method of Ellman et al. (1961) adapted to microplate. Protein quantification was performed using the Bradford method (Bradford, 1976), adapted to microplate, in order to express enzymatic activities per mg of protein. AChE determinations were performed using a microplate reader Labsystems, model Multiskan Ex.

2.4. Statistical analysis

Data were compared by one-way ANOVA, followed by a Dunnet multi-comparison test to discriminate differences in relation to the control treatments. The adopted level of significance was 0.05.

3. Results and discussion

Acute cadmium exposure was not responsible for any significant change in AChE activity in *G. holbrooki* ($F = 0{,}53$; d.f. = 5,54; $p > 0.05$; Fig. 1). Similarly, chronic exposure to cadmium did not cause any significant modification in AChE activity ($F = 0{,}11$; d.f. = 3,35; $p > 0.05$; Fig. 2).

Lead, following short-term exposure, did not elicit any measurable change in AChE activity ($F = 1.84$; d.f. = 4,45; $p > 0.05$; Fig. 3). A similar tendency was observed after chronic exposure of *G. holbrooki* to the same metal ($F = 1.26$; d.f. = 5,54; $p > 0.05$; Fig. 4).

Acute exposure of *G. holbrooki* to the metal zinc caused a significant increase in AChE activity ($F = 3.39$; d.f. = 3,36; $p < 0.05$; Fig. 5) for the intermediate tested concentration; however, the higher test concentrations elicited a rise of this enzyme's activity, albeit not significant. In contrast, chronic exposure to the same compound was not responsible for any significant alteration in activity ($F = 1.90$; d.f. = 5,54; $p > 0.05$; Fig. 6).

Acute exposure to copper caused a significant inhibition of AChE ($F = 5{,}48$; d.f. = 5,54; $p < 0.05$; Fig. 7). However, chronic exposure to this metal did not cause any measurable modification in activity ($F = 1.80$; d.f. = 5,54; $p > 0.05$; Fig. 8).

The major point that is inherently connected to the assessment of toxicological effects caused by metals on cholinesterases is the large variability of results, when considering different metals and distinct test species. The major drawback about the use of cholinesterase inhibition for the assessment of metals was made clear in the review by Nunes et al. (2011), and is directly linked with the enzymatic assay used to quantify cholinesterase activity. According to the work by Frasco et al. (2005), AChE inhibition by

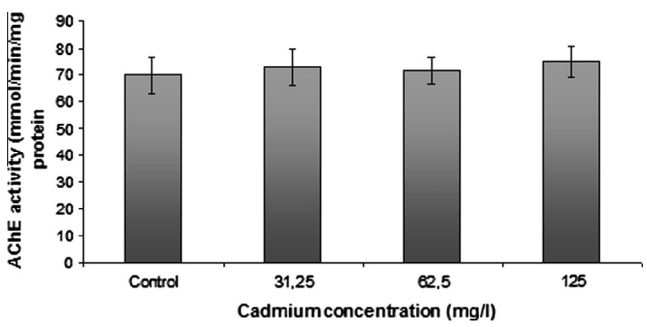

Fig. 1. Effects of acute exposure of *G. holbrooki* to cadmium; values are the mean of ten replicate assays and corresponding standard error bars.