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Metabolic and histopathological alterations in the marine bivalve *Mytilus galloprovincialis* induced by chronic exposure to acrylamide

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ABSTRACT

Although the neurotoxic and genotoxic potential of acrylamide has been established in freshwater fish, the full breadth of the toxicological consequences induced by this xenobiotic has not yet been disclosed, particularly in aquatic invertebrates. To assess the effects of acrylamide on a bivalve model, the Mediterranean mussel (*Mytilus galloprovincialis*), two different setups were accomplished: 1) acute exposure to several concentrations of waterborne acrylamide to determine lethality thresholds of the substance and 2) chronic exposure to more reduced acrylamide concentrations to survey phases I and II metabolic endpoints and to perform a whole-body screening for histopathological alterations. Acute toxicity was low ($LC_{50} \approx 400$ mg/L). However, mussels were responsive to prolonged exposure to chronic concentrations of waterborne acrylamide (1–10 mg/L), yielding a significant increase in lipid peroxidation plus EROD and GST activities. Still, total anti-oxidant capacity was not exceeded. In addition, no neurotoxic effects could be determined through acetylcholine esterase (AChE) activity. The findings suggest aryl-hydrocarbon receptor (Ahr)-dependent responses in mussels exposed to acrylamide, although reduced comparatively to vertebrates. No significant histological damage was found in digestive gland or gills but female gonads endured severe necrosis and oocyte atresia. Altogether, the results indicate that acrylamide may induce gonadotoxicity in mussels, although the subject should benefit from further research. Altogether, the findings suggest that the risk of acrylamide to aquatic animals, especially molluscs, may be underestimated.

1. Introduction

Acrylamide is an unsaturated amide with diverse applications, mainly the production of polymers used as additives in water treatment, agricultural processes, textile manufacturing and life science research (e.g. Myagchenkov and Kurenkov, 1991; Bajdur, 2008). Acrylamide has been classified a "probable carcinogen for humans" by IARC (International Agency for Research on Cancer) in 2002, following reports of its formation during food processing (Stadler et al., 2002; Tareke et al., 2002). Besides concerns towards human health, under the toxicological point-of-view, mostly related to the presence of acrylamide in foodstuff (Vikström et al., 2011; Watzek et al., 2012; Dobrowolski et al., 2012), there has been some work about the environmental fate of acrylamide monomers after degradation of polyacrylamide (co)polymers and subsequent consequences to the biota (e.g. Brown et al., 1980a, 1980b; Friedman, 2003; Larguinho et al., 2013). Polyacrylamide (co)polymers eventually undergo degradation to acrylamide molecules and these monomers may partition to aquatic niches, so water contamination with acrylamide and its effects to aquatic wildlife remain a critical concern (NICNAS, 2002; Bajdur, 2008; Weston et al., 2009). In fact, waterbodies are acknowledged to be the main reservoirs of the substance, in large part owing to its high solubility in water, low vapour pressure and ability to become adsorbed to particulates (NICNAS, 2002).

Mostly through in vitro and in vivo studies with human cells lines and murine models, exposure to acrylamide has been related to genotoxicity, neurotoxicity (Seale et al., 2012; Prasad and Muralidhara 2012), oxidative stress (Yousef and El-Demerdash, 2006; Sen et al., 2012) and even reproductive impairment (Sakamoto and Hashimoto 1986; Zenick et al., 1986; Chapin et al., 1995; Tyl and

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Friedman, 2003; Wang et al., 2010; Camacho et al., 2012). Acrylamide or its metabolites is known to interact with a wide range of biomolecules, which aids in explaining the potential broad scope of its toxicological effects. Particularly, acrylamide is metabolised to glycidamide, an epoxide which is highly reactive towards DNA (Besaratinia and Pfeifer, 2004; Carere, 2006; Watzek et al., 2012). In fact, the formation of DNA adducts is acknowledged to be one of the main factors involved in acrylamide carcinogenicity (see Besaratinia and Pfeifer (2007) for a review). Acrylamide may also interact with peptide side chains and other cellular nucleophiles through Michael's addition (Mather et al., 2006; Zamora et al., 2010).

The concerns of acrylamide monomers to aquatic wildlife led chiefly to studies focusing on acute toxicity to fish and crustaceans, revealing moderate to low effects (Bridié et al., 1979; Krautter et al., 1986). Still, chronic effects of acrylamide monomers to aquatic organisms have seldom been addressed, perhaps as a consequence of reduced acute toxicity. Nonetheless, there is evidence in freshwater fish at several levels of biological organisation, from genotoxicity to biochemical defences (such as the deployment of oxidative stress-related enzymes) and tissue-level alterations to the hepatopancreas (Larguinho et al., 2013), that account for potential chronic toxicity associated with acrylamide. These findings, together with the studies in vivo or in vitro with model subjects, indicate that acrylamide monomers may indeed cause multi-level adverse effects to wildlife that will only be revealed through more prolonged, therefore more ecologically-realistic, exposures to the xenobiotic. It must be stressed that most of the studies concerning acrylamide toxicity to aquatic biota targeted fish models (Woodiwiss and Fretwell, 1974; Bridié et al., 1979; Krautter et al., 1986; Waddell et al., 1990). Only a few studies have focused on invertebrates, mostly model organisms like *Daphnia magna* (ABC Labs, 1983a); the midge *Parathanyarsus parthenogenetica* (ABC Labs, 1983b); the rotifer *Adineta vaga* (Örstan, 1992), and the saltwater mysid shrimp *Mysidopsis bahia* (Springborn Bionomics, 1985; EG&G Bionomics, 1986). Overall, invertebrates show very different sensitivities to acrylamide, as opposed to what happens in fish models, where effect concentrations typically yield little interspecific variation (see for instance Krautter et al. (1986), Walker (1991), Weston et al. (2009) and Larguinho et al. (2013)), which further endorses the need or understanding the effects of this xenobiotic to aquatic invertebrates.

Bivalve molluscs, in particular mussels (*Mytilus spp.*), are widely surveyed within the field of environmental toxicology, whether to test the hazards of pollutants ex situ or in biomonitoring (Fernandéz et al., 2010). Their ecological and economic importance, sensitivity to toxicants, relative simplicity of the mollusc body plan and easy handling render these organisms as key targets to address the effects of traditional and novel, so-called "emerging" pollutants, from endocrine disruptors to nanotoxicants (see for instance Aarab et al. (2006), Tedesco et al. (2010) and Canesi et al. (2012)).

In face of the limited knowledge on the effects of acrylamide to marine molluscs, the present work aims at determining the chronic and acute effects of waterborne acrylamide in the mussel *Mytilus galloprovincialis*. Specifically, it is intended to: i) determining lethality thresholds; ii) determining acrylamide-induced biochemical responses in whole-soft body homogenates through a multi-level biomarker approach focusing on phases I and II responses, neurotoxicity and oxidative stress, and iii) determining adverse effects in multiple organs of the mussels through a whole-body histopathological appraisal. In particular, it is aimed at assessing the toxicological effects of the substance at tissue and subcellular levels while determining the ability to deploy biochemical responses that are acknowledged defences towards Ahr (aryl hydrocarbon receptor)-linked organic toxicants and neurotoxicants, such as acrylamide, has been described for vertebrate models.

2. Material and methods

2.1. Chemicals

Acrylamide ($\geq$ 99% purity), Bradford reagent; phosphate-buffered saline (PBS); bovine serum albumin (BSA); ethylenediamine tetraacetic acid (EDTA); reduced glutathione (GSH); Dulbecco's phosphate-buffered saline; 1-chloro-2,4-dinitrobenzene (CDNB); Triton-X 100; Trizma base; acetylthiocholine iodide (ACTI); acetylcholinesterase (AChE) from electric eel, dithionitrobenzoic acid (DTNB); 7-ethoxyresorufin; resorufin, nicotinamide adenine dinucleotide phosphate (NADPH); glycine; malondialdehyde (MDA); thiobarbituric acid (TBA); glacial acetic acid; Purpald; potassium periodate; haematoxylin; eosin; xylene; Trolox; hydrogen peroxide, myoglobin and bovine liver catalase were acquired from Sigma-Aldrich (USA). Ethanol and histology-grade pelleted paraffin were purchased from Panreac (Spain). The DPX mounting agent was acquired from BDH (England) and ABTS from Alfa-Aesar (USA). Methanol, sodium hydroxide (NaOH), trichloroacetic acid (TCA) and potassium hydroxide were purchased from Merck Millipore (Germany). All necessary solutions and dilutions were prepared using ultrapure water ($>$ 16.2 MΩ cm).

2.2. Equipment

All spectrophotometric biomarker analyses were performed using 96-well microplates (Greiner, Germany) and absorbance recorded using an Infinite 2000 Microplate reader (Tecan, Switzerland). All histological analyses were performed with a DMLB model microscope coupled with a DFC480 digital camera, from Leica Microsystems (Darmstadt, Germany).

2.3. Experimental design

Mediterranean mussels, *M. galloprovincialis*, (ca. 36×20 mm^2 shell size) were collected from the intertidal zone of a clean site in NW Portuguese coast (ca. 250 individuals) and acclimatised in 50 L polystyrene tanks for one week, arranged in a closed circuit system with clean seawater (pH 7.65 ± 0.2, temperature 19 ± 1 °C, and salinity 31 ± 1) under a 12 h light:12 h dark photoperiod, with continuous aeration. The same water was used in the bioassays.

The acute toxicity assay consisted of a duplicate static array of 15 L capacity tanks within each were randomly allocated 15 mussels per replicate and test concentration. The concentrations of acrylamide ranged between 100 and 2000 mg/L, in face of the absent information on acrylamide LC_{50} for bivalves. During the experiment, mussels were fed ad libitum with *Dunaliella salina*. The assay lasted 96 h, during which mortality and morbidity were recorded continuously. The half-maximal lethal concentration (LC_{50}) at 96 h was estimated using the US EPA Probit software v3.2.

The chronic exposure assay consisted of a semi-static disposition of three 15 L tanks, each holding 13 randomly-selected mussels (in total 39 individuals). The tanks correspond to exposure to 1 and 10 mg/L acrylamide plus the control tank, during 21 days. Water changes (100%) were done every 48 h to ensure constancy of water parameters. Feeding was provided as mentioned above. At the end of experimental period, mussels were collected and dissected. The soft tissue was excised and divided in halves, producing two similar whole-body sections for both histological and biochemical analyses. The following sections refer to sample preparation and analyses on animals subjected to the chronic exposure assay.

2.4. Preparation of tissue homogenates

Whole soft-body mussel samples (13 individual samples per experimental condition) were homogenised in 600 μL of 1XPBS (phosphate buffered saline). Afterwards samples were centrifuged at 12,000*g* for 20 min at 4 °C and the pellet was discarded to obtain the cleared homogenates. The supernatants were stored at −80 °C for subsequent biomarker analysis. Total protein concentration in samples was determined by the Bradford assay, using BSA as standard (Bradford, 1976). All biochemical biomarker responses were normalised to total protein concentration.

2.5. Activity of acetylcholinesterase (AChE)

Determination of AChE activity was performed via quantification of thiocholine, following Ellman et al. (1961). On a 96-well microplate, 50 μL of the sample was mixed with 150 μL DTNB (270 μM in 50 mM sodium phosphate, pH 7.4). Afterwards, 50 μL of 3 mM ACTI was added to the mixture and the absorbance at 412 nm was recorded during 15 min, taking AChE from electric eel (Sigma-Aldrich) as a standard.

2.6. Discontinuous ethoxyresorufin-O-deethylase (EROD) spectrophotometric assay

The activity of phase I enzyme EROD was measured through the discontinuous method, adapted from De Almeida et al. (2011). The reaction mixture was prepared