



Full length article

Induction of brown cells in *Venerupis philippinarum* exposed to benzo(a)pyrene

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ABSTRACT

Benzo(a)pyrene is an important polycyclic aromatic hydrocarbon (PAH) commonly present in the marine environment and responsible for carcinogenic, teratogenic and mutagenic effects in various animal species. In the present study, we investigated by both histochemical and immunohistochemical approaches the effect of an acute exposure to different concentrations of B(a)P in the Manila clam *Venerupis philippinarum*. The general morphology of the different clam tissues, which was investigated histologically, evidenced a significant increase in the number of intestinal brown cells after B(a)P exposure. An increasing trend response to B(a)P was detected. The histochemical analysis for lipofuscin revealed the presence of lipofuscin-like substances inside the cytoplasm of intestinal brown cells. The same cells exhibited a PAS positivity and a reactivity to Schmorl's solution for melanin pigment. Moreover, intestinal brown cells exhibited an immunopositivity to HSP70 antibody confirming the increasing trend response to B(a)P detected by the histochemical analysis. Our results suggest that histological tissue changes resulting from exposure to B(a)P can be an useful marker in biomonitoring studies.

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1. Introduction

Among the numerous polycyclic aromatic hydrocarbons (PAHs) that appear at the highest concentrations in most urbanized coastal areas, mainly as a result of industrial activities, benzo(a)pyrene (B(a)P) is considered one of the most important indicators because it usually occurs in mixtures of PAHs. PAHs are ubiquitous in the environment and once released into the marine environment, they become bioavailable to aquatic vertebrates and invertebrates through the food chain, causing a series of harmful effects including genetic damage, immune and endocrine dysfunction, malformations, fibrosis and cancer [1–6].

Histological tissue changes, resulting from exposure to contaminants, can be an useful marker in biomonitoring studies [7]. Among the different histological markers, lipofuscin deposition is considered as indicative of contaminant exposure, revealing a general response to pollution [8,9]. It has been demonstrated that the cellular lipofuscin content of mollusks increases not only with

organism age but also with exposure to pollutants [10]. Lipofuscin formation takes place from cellular components through peroxidation of their unsaturated neutral lipids and its accumulation in granules within cells [11–14].

The expression of several proteins is usually employed to detect the exposure to contaminants. Heat shock proteins (HSPs), also called *stress proteins*, are a family of cellular proteins detected in all life forms and are highly conserved in the evolutionary scale [15–17]. Among the numerous heat shock proteins, HSP70 plays an important biomarker role protecting cells against harmful conditions by binding and refolding damaged proteins. HSP70 proteins are divided in constitutive members (HSC70), which play important chaperoning role in unstressed cells, and inducible (HSP70) forms, which are expressed at detectable levels after acute stressor insults [15,18]. In aquatic species, the expression of HSP70 has been studied in fish after exposure to heat shock, pesticides, virus, metals and other toxic compounds [19–22]. In our previous work, an immunolocalization of inducible HSP70 in different tissues of sea bass (*Dicentrarchus labrax*) subjected to transport stress revealed the presence of the protein only in skeletal muscle of stressed animals [23]. Moreover, in *Cyprinus carpio*, the inducible form (HSP70) was evidenced in the epithelia of renal tubules, gills and skin of

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common carp (*C. carpio*) after transport stress [24], suggesting the potential use of HSP70 expression as a marker for biomonitoring studies.

In mussel, an increased expression of HSP70 has been detected after exposure to contaminants [25,26]. Moreover, an upper regulation of HSP70 has been observed in the clam (*Venerupis decussata*) upon *Perkinsus olsenii* infection [27].

The Manila clam (*Venerupis philippinarum*) represents an important economic resource in the Venice Lagoon, where this species is fished and farmed. It is a filter-feeding bivalve living in soft bottoms and used as sentinel model in biomonitoring programs aimed to investigate the water/sediment pollution in coastal lagoon ecosystems.

The aim of this study was to evaluate the acute effect of B(a)P on the general morphology of the different clam tissues as well as on the immunolocalization of HSP70 protein in the clam *Venerupis philippinarum* exposed to different concentrations of B(a)P for 24 h. B(a)P determination in exposed clams was carried out by HPLC-FLD analyses.

2. Materials and methods

2.1. Organisms, B(a)P exposure and tissue samples

The experimental protocol is detailed in Boscolo Papo et al. [28]. In brief, the adult clams (*V. philippinarum*; valve length = 3–4 cm) were obtained from a local depuration plant “Consorzio Cooperative Pescatori del Polesine, Scardovari” and transferred to the laboratory in refrigerated bag. Animals were acclimated in tanks (1 L for animal) with daily renewed artificial sea water (distilled water plus Ocean Fish Marine Salt, Prodac International, Italy) and fed with a mix of microalgae, for one week before the exposure test. After the acclimation, animals were randomly divided into four experimental groups with two replicates and exposed to different concentration of B(a)P: 0 mg/l, 0.03 mg/l, 0.5 mg/l and 1 mg/l. B(a)P concentration of 0.03 mg/l, 0.5 mg/l are usually reported for acute stress experiments, whereas the concentration of 1 mg/l has been employed in order to induce a clear response in clams subjected to high exposure of B(a)P. We did not look at the lethal concentration (LC50) as we wanted to investigate effects of lower concentrations that are more environmental realistic. Moreover, several papers reporting the effects of contaminants and their toxic mechanisms in water environments do not consider the evaluation of the (LC50) [29–31].

For histochemistry and immunohistochemistry, the whole body from 40 animals was fixed for 24 h at 4 °C in 10% neutral buffered formaldehyde.

2.2. Histochemistry

After fixation, samples were dehydrated in a graded series of ethanol and then paraffin-embedded. Dewaxed serial sections (4 µm-thick) were stained with hematoxylin and eosin (H & E) sequential stain to determine structural details, with Schmorl's solution [32] to detect the melanin pigment, with lipofuscin stain [32] to determine the lipofuscin accumulation and with PAS (Periodic Reactive Schiff) stain [32] to detect glycogen, mucin, mucoprotein, and glycoproteins. Following visual examination of the sections by a light microscope (Olympus Vanox photo-microscope, Japan), a quantitative assessment of brown cells was made using a computerized image analyzer system (Olympus CellB, Japan) on sections of intestine since it was the organ which exhibited the highest number of brown cells. The count proceeded as follows: (1) Each haul was represented by 3 sections from each intestine collected from 40 randomly selected clams (10 for each

experimental condition). (2) Three fields from each intestine section were analyzed and the number of brown cells was recorded.

2.3. Immunohistochemical localization of HSP70 protein

Immunohistochemistry was carried out by an automated immunostainer (Autostainer link 48 Dako, Italy). Sections were deparaffinized in xylene, rehydrated in graded ethanol and rinsed in distilled water. Heat-induced antigen retrieval was performed in 10 mM citrate buffer (pH = 6.0) at 97 °C for 15 min. Endogenous peroxidases were neutralized by incubating the sections with the EnVision FLEX Peroxidase-Blocking Reagent (SM801, Dako), for 10 min at RT; serial sections were incubated overnight at +4 °C with a mouse monoclonal HSP70 antiserum, dilution 1:200 (Stressgen Biotechnologies, USA). Sections were then incubated with the detection system EnVision FLEX/HRP (Dako), whereas the EnVision FLEX Substrate Buffer EnVision FLEX DAB (Dako) was used as chromogen. The sections were then counterstained with the EnVision FLEX Hematoxylin (Dako). The specificity of the immunostaining was verified by incubating the sections with PBS instead of the specific primary antibody.

2.4. Statistical analysis

Statistical analysis was carried out with STATISTICA 9.1 (StatSoft, inc.). Differences in brown cells number between animals exposed to different B(a)P concentration, were assessed by mean of Kruskal–Wallis one-way analysis of variance followed by multiple comparison test. In all analyses a $p < 0.05$ value was accepted as significant. All data are reported as mean $\pm$ standard deviation (SD).

3. Results

3.1. B(a)P concentrations

The significant effect of B(a)P exposure concentration on B(a)P contents in *Venerupis philippinarum*, after 24 h exposure, was previously reported in Boscolo Papo et al. [28]. Tissue B(a)P concentrations levels were maximal (6910 ± 1009 ng/g ww) after animal exposure to 1 mg/l of B(a)P and minimal (1.5 ± 0.5 ng/g ww) in controls (Table 1).

3.2. Histochemistry and general morphology

Controls as well as exposed clams did not exhibit histopathological lesions in digestive gland, gills, kidney, heart, intestine and gonads (data not shown). Exposure to the different concentrations of B(a)P caused a haemocytic infiltration, typically consisting of brown cells, particularly distributed in the epithelium of intestine, where an increasing trend response to B(a)P was detected (Figs. 1 and 2A, C, E). The same cells exhibited a PAS positivity (Fig. 2(C and

Table 1

B(a)P concentrations (ng/g wet weight) in the whole tissue of the clams exposed to different B(a)P doses. Data are presented as means $\pm$ SD. Different letters indicate significant differences. Data from Boscolo Papo et al. [28].

Water B(a)P concentration mg/l	B(a)P in animals after exposition ng/g
0	1.5 ± 0.5^a
0.03	443.2 ± 148.7^a
0.5	5040 ± 2182^b
1	6910 ± 1009^c

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