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Experimental exposure of blue mussels (*Mytilus galloprovincialis*) to high levels of benzo[*a*]pyrene and possible implications for human health

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are lipophilic compounds able to accumulate in the food chain. Mussels showed to bioaccumulate contaminants, such as PAHs, so that recurrent consumption of such contaminated food represents a risk for human health. This study was aimed to elucidate if acute exposure of Mediterranean blue mussel (*Mytilus galloprovincialis*), a bivalve of great economic importance in several countries, to a PAH, benzo[*a*]pyrene (B[*a*]P), at doses able to induce cytochrome P450 1 A (CYP1A) and pathological changes in mussel gills, can produce accumulation in soft tissue. We explored the cytotoxic effects (cell viability, DNA laddering, and glutathione levels) of in vitro exposure of human peripheral blood mononuclear cells (PBMCs) to organic extracts obtained from blue mussels previously exposed for 12 and 72 h via water to B[*a*]P (0.5–1 mg/L). In our experimental conditions, B[*a*]P induced CYP1A induction and morphological changes in mussel gills and a significant B[*a*]P accumulation in soft tissue. Conversely, exposing PBMCs to organic extracts obtained from contaminated mussels, resulted in a significant reduction of cell viability and cell glutathione content, and in an increase in DNA laddering. This confirms that consumption of mussels from B[*a*]P polluted waters might affect human health. Our data lead us to suggest that CYP1A activity in mussel gills may be useful (more than the amount of detected PAHs in the mussel edible tissue) as a marker in assessment of risk for health of consumers exposed to PAHs through ingestion of shellfish.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a class of over 200 different chemicals containing two or more aromatic rings. They are generally formed from incomplete combustion or pyrolysis of organic material and, at a lower extent, are of petrogenic origin (Wang et al., 2001). PAHs belong to the food and environment contaminants (Wang et al., 2001; Lerda, 2011) and are a class of ubiquitous and persistent organic pollutants (POPs) that are not readily degraded in the environment (Lerda, 2011). They represent a major concern for human health (EFSA, 2008) since some of them are known to be highly carcinogenic and mutagenic. However, benzo[*a*]pyrene (B[*a*]P) is the only PAH classified by the IARC as a recognized carcinogen (group 1) to humans (Tarantini et al., 2009), and is routinely employed as a representative of this class of pollutants due to its carcinogenic potency, prevalence and correlation with other PAHs. PAHs, including B[*a*]P, are amongst the most omnipresent organic pollutants in aquatic environments because of the many mechanisms causing their production and dissemination, such as industrial waste and sewage leakage, natural and industrial combustion of organic materials, natural oil seepage, and crude oil spills. Aquatic organisms are able to grow and reproduce in polluted milieus and thus have a great capacity to accumulate pollutants (Ramalhosa et al., 2012; Gomes et al., 2013), so that recurrent contaminated seafood consumption can predispose consumers to health risk (Kalogeropoulos et al., 2012; Martorell et al., 2010; Ramalhosa et al., 2012; Gomes et al., 2013; Binelli and Provini, 2004). Mussels are good indicators utilized in environmental-monitoring programmes, since they are widely distributed, sedentary, and tolerant to many environmental conditions; furthermore, they are filter feeders with low metabolism and thus accumulate chemicals, such as organic pollutants including PAHs, in their tissues (Cajaraville et al., 2000; Kamel et al., 2012; Mercogliano et al., 2016; Widdows et al., 2002; Viarengo and Canesi, 1991; Vidal-Liñán et al., 2010). In particular, *Mytilus galloprovincialis* (Mediterranean blue mussel) attaches to hard substratum and filters through the water column, thus it is mainly exposed to the dissolved and particulate matter rather than sediments, making it a good

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bioindicator for water pollution (Monteduro et al., 2007). Mussels are also important elements of the food chain and (as well as also oysters, scallops, clams, etc.) in several countries are extensively farmed, and are part of the human diet, representing an important socio-economical resource. Although fish and seafood represent only a small percentage (about 10%) of the human diet, it has been demonstrated that they are one of the major routes of contaminants, including POPs, into the human body. In particular, mussels for human consumption may represent an important source of human exposure to PAHs. In fact, although both vertebrates and invertebrates possess mixed-function oxygenase (MFO) enzymes making them able to metabolize petroleum products, this ability is reduced in invertebrates with respect to vertebrates. Thus, finfish rapidly and efficiently metabolize and excrete PAHs (Ramalhosa et al., 2012; Gomes et al., 2013; Ortiz-Delgado et al., 2007), so that they pose a risk to human health only when consumers eat fish tissues (such as liver and gall bladder) accumulating higher levels of PAHs. Conversely, marine invertebrates, comprising many shellfish, metabolize really slowly petroleum products, and thus they accumulate important levels of PAHs (Glad et al., 2017; Guéguen et al., 2011). Furthermore, people often eat the entire invertebrate, thus ingesting all the accumulated PAHs. Thus, the European authorities have established the legal limit for PAHs in shellfish to control and ensure the quality of this food. On the basis of Commission Regulation (EU) n.835/2011 maximum level of PAHs in bivalve mollusks cannot be higher than 5 μg/kg for B[*a*]P, and 30 μg/kg for the sum of 4 hydrocarbons B[*a*]P, chrysene, benzo[*a*]anthracene, and benzo[*b*]fluoranthene (EFSA, 2008; European Union, 2011).

This study was aimed to evaluate if acute exposure of Mediterranean blue mussel *M. galloprovincialis* to the prototypic PAH B[*a*]P, at doses able to induce cytochrome P450 1 A (CYP1A)-like protein increase and pathological alterations in gills (which are important absorption and excretion organs in direct contact with water pollutants), can induce B[*a*]P accumulation in the mussel edible tissue, so that this seafood may represent a risk for human health. At this aim we investigated the cytotoxic effects (as changes in cell viability, DNA laddering, and glutathione content) of in vitro exposure of human peripheral blood mononuclear cells (PBMCs) to organic extracts obtained from edible parts of blue mussels previously exposed via water to B[*a*]P at a level (0.5 and 1 mg/L, for 12 and 72 h); these B[*a*]P concentrations were chosen in order to simulate a high dose of exposure which may occur in case of spills. Results obtained by using PBMCs might be of great significance for human health because peripheral leukocytes are a main systemic target of food-borne xenobiotics; moreover, very few data about PAHs are reported on primary cell cultures, which usually yield more physiologically significant results.

2. Materials and methods

2.1. Reagents and chemicals

HPLC spectroscopic-grade solvents were obtained from T. Backer (Mallinckrodt Backer, Milan, Italy), whereas all other solvents were purchased from Carlo Erba reagents (Milan, Italy). All other reagents, unless otherwise specified, were purchased from Sigma-Aldrich (Milan, Italy).

2.2. In vivo experiments

A total of 150 individuals of commercial size (5 ± 0.7 cm) belonging to the species *M. galloprovincialis* were used in the experiments. The animals were obtained from a local aquaculture farm in Faro brackish pond (Messina, Sicily), and before the beginning of the experiments were acclimated for one week in the Experimental Center of Ittiopathology, University of Messina, in tanks containing aerated and daily renewed artificially reconstituted brackish-water. For B[*a*]P exposure transparent polyethylene bags (50 × 70 cm) were filled up with 15 L of water, continuously aerated, placed in plastic boxes (45 × 30 × 15 cm); each bag contained 30 individuals. Brackish water used in our experiments was artificially reconstituted by the opportune addition of salt to reverse osmosis-treated city water, according to procedures established at the Experimental Center of Ittiopathology, University of Messina; temperature (18.8 ± 0.2 °C), pH (7.5 ± 0.2), NO_2^- (< 0.1 mg/L), NO_3^- (< 8 mg/L), NH_4^+ (< 3 mg/L), dissolved oxygen (8.3 ± 0.3 mg/L), and salinity (32‰), were controlled daily in the course of the experiment. At the beginning of the experiments, B[*a*]P solutions were added into the tanks filled with water in order to obtain the opportune concentrations. Mussels were then divided into 5 equal groups, exposed to B[*a*]P (Sigma, Aldrich) or to the vehicle alone; group 1 and 2 received B[*a*]P 0.5 mg/L dissolved in DMSO (0.005% in water), respectively for 12 and 72 h; groups 3 and 4 received B[*a*]P 1 mg/L dissolved in DMSO (0.005% in water) respectively for 12 and 72 h; group 5, the control group, received only the vehicle (DMSO, 0.005% in water) for 72 h. During the exposure no mortality was observed.

After the exposure, gills were immediately removed from each organism and treated for histopathology and immunochemistry examination, while mussel tissues to be used for extract preparation were stored at −80 °C until further processing.

2.2.1. Histopathology and immunohistochemistry

Histopatology examination of gills was performed as elsewhere described (Lauriano et al., 2012) with a Zeiss Axioscope using Axiocam Zeiss digital camera. The immunohistochemical investigations by the indirect method of peroxidase were performed according to Calò et al. (2009). The detailed procedure is described in our previous paper (Zena et al., 2015).

2.2.2. Preparation of mussel organic extract (MOE)

The extraction procedure was performed according to Namiensik et al. (2010) with some modifications, as described in our previous paper (Zena et al., 2015). The MOE so prepared was used for the analytical quantification of B[*a*]P and for the treatment of human leukocytes.

2.2.3. B[a]P analysis

The quali-quantitative determination of B[*a*]P in the MOE was performed as elsewhere described (Cimino et al., 2014; Zena et al., 2015) on the dry extract obtained from 10 g (f.w.) of mussel tissue redissolved in 5 mL of acetonitrile, filtered through 0.22 μm filters, and stored at −80 °C until use. The concentration of B[*a*]P in the MOE samples was determined by using HPLC with a Varian instrument equipped with a UV/Vis detector (PROSTAR 240) and with a fluorescence detector (Hewlett Packard 1046 A) and was quantified by the external calibration method. The limit of detection (LOD) and the limit of quantification (LOQ) were 1.02 ng/mL and 2.88 ng/g respectively. The spike-and-recovery assay, carried out by adding naive mussel samples with different concentrations (in the range 5–100 ng/mL) of B[*a*]P, revealed a B[*a*]P recovery of 93 ± 9% (mean ± RSD). To confirm the presence of PAHs in MOEs, a series of experiments were performed by HPLC/UV–VIS/diode array detector/mass spectrometry with a Waters instrument equipped with a 1525 Binary HPLC pump, a Micromass ZQ 2000 mass analyzer and a 996 Photo Diode Array Detector (PAD; Waters Corporation, Milford, MA).

2.3. Ex vivo experiments

2.3.1. Cell culture and treatments

PBMCs (1×10^6 cells/mL) isolated from whole blood (pool from 5 healthy, non-smoker human donors) with Histopaque® −1077, were exposed to 2 μL of MOE (dissolved in DMSO to obtain a final concentration corresponding to 20 mg/mL of fresh tissue) as elsewhere described (Zena et al., 2015; Cimino et al., 2014).