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Adverse effects induced by ecgonine methyl ester to the zebra mussel: A comparison with the benzoylecgonine

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ABSTRACT

Cocaine and its metabolites are the prevalent psychotropic substances in aquatic environment. However, to date the knowledge on their adverse effects to non-target organisms is inadequate. The aims of this study were to investigate sub-lethal effects induced by the ecgonine methyl ester (EME) to the freshwater bivalve *Dreissena polymorpha* and to compare its toxicity to that by benzoylecgonine (BE), the other main cocaine metabolite. EME sub-lethal effects were investigated by 14 days *in-vivo* exposures and a multi-biomarker approach. Slight variations in biomarker responses were found at 0.15 μg/L treatment. 0.5 μg/L EME treatment induced destabilization of lysosome membranes, an overall inactivation of defense enzymes, increases in lipid peroxidation, protein carbonylation and DNA fragmentation, but no variations in fixed genetic damage. The use of a biomarker response index (BRI) showed that at 0.5 μg/L both cocaine metabolites had the same toxicity to zebra mussels specimens.

1. Introduction

Illicit drugs have been recently identified as a group of emerging pollutants, attracting the interest of analytical and environmental chemistry (Richardson, 2009; Zuccato and Castiglioni, 2009). An increasing number of monitoring surveys have showed the occurrence of cocaine (COC), amphetamines (AMP), Δ^9-tetrahydrocannabinol (THC), ecstasy (3,4-methylenedioxy-N-methylamphetamine; MDMA), opiates (heroin, morphine and codeine), as well as of their corresponding metabolites, in both surface and wastewaters worldwide in ng/L concentrations (Zuccato et al., 2008; Postigo et al., 2010; Castiglioni et al., 2011), matching levels of common pharmaceuticals used for therapeutic purposes (Santos et al., 2010). Cannabis (119–224 million people) and amphetamine-type stimulants (14.4–52.5 million people) are the two most used illicit drugs worldwide (UNODC, 2012) and global COC use has remained stable at 0.3–0.4% of the population aged 15–64 (between 13.2 million and 19.5 million users) during the 2008–2010 period, with stable or declining trends in the prevalence of its use in several countries (UNODC, 2012); despite these evidences, concentrations of COC in aquatic environment are higher than those of other psychotropic substances. Although current environmental levels of COC are quite low, considering its high pharmacological activity and its interaction with residues of many other therapeutics, this drug may cause toxic effects to aquatic organisms. A recent investigation by Binelli et al. (2012), confirmed this suggestion, showing that environmentally relevant COC concentrations induced remarkable sub-lethal effect to the zebra mussel *Dreissena polymorpha*. However, since in humans COC is largely excreted in urine as metabolites (45% benzoylecgonine and 40% ecgonine methyl ester of the administered dose) and only a small percentage as the unchanged drug (1–9% of the administered dose; Baselt, 2004), in aquatic environment the levels of the benzoylecgonine (BE) and ecgonine methyl ester (EME) are higher than those of the parental compound (Castiglioni et al., 2006, 2011; van Nuijs et al., 2009; Postigo et al., 2010). Despite of these evidences, the ecotoxicology of COC metabolites is greatly inadequate. Recently, Parolini et al. (2013) showed that *Dreissena polymorpha* specimens exposed to a BE concentration similar to the mean level measured in the influents of sewage treatment plants (1 μg/L) suffered a situation of marked oxidative stress, as highlighted by the alteration of antioxidant activity and the increase of both lipid peroxidation and protein carbonyl content, whit the consequent rise of genetic damage. The involvement of oxidative stress in BE toxicity to zebra mussel specimens was also confirmed by a redox proteomics analyses applied to bivalve gills that showed an increase in protein carbonylation after 14 days of exposure to the same BE concentration. Moreover, oxidative modifications in different classes of proteins, such as those of the cytoskeleton, energetic metabolism and stress response were found (Pedriali et al., 2012). In contrast, the knowledge of possible adverse effects induced by the second CO main metabolite, the ecgonine methyl ester, to non-target

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organisms is completely lacking. For these reasons, the first aim of the present research was to evaluate sub-lethal effects induced by EME to *Dreissena polymorpha* specimens. Thanks to its widespread diffusion, peculiar physiological characteristics and high sensitivity to several pollutants, including pharmaceutical and personal care products (Binelli et al., 2009a,b; Parolini et al., 2010, 2011a,b; Parolini and Binelli, 2012) and illicit drugs (Binelli et al., 2012; Parolini et al., 2013), this bivalve species is considered an excellent biological model in freshwater ecotoxicology. Zebra mussels were exposed to two environmentally relevant EME concentrations (0.15 and 0.5 μg/L) for 14 days and induced adverse effects were evaluated by using an *in vivo* multi-biomarker approach. The end-points of ten different biomarkers were measured and their integrated response was used both in detecting sub-lethal EME effects and in supposing its possible mechanism of action in zebra mussel specimens. In detail, EME cytotoxicity was evaluated by the Neural Red Retention Assay (NRRA), a simple indicator of general cellular stress in bivalves (Lowe et al., 1995). The activity of three antioxidant enzymes, namely catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), and the phase II detoxifying enzyme glutathione S-transferase (GST), the lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured as oxidative stress indices. In addition, primary (DNA strand breaks) and fixed (apoptotic and micronucleated cell frequency) genetic damage were investigated by the single cell gel electrophoresis (SCGE) assay, the DNA Diffusion assay and the micronucleus test (MN test), respectively. The second aim of this study was to compare the sub-lethal toxicity of COC metabolites. Since the main problem in the interpretation of the biomarker dataset is due to response variability, the application of approaches focusing on their integration into a synthetic index can allow an easier interpretation of complex ecotoxicological data (Sforzini et al., 2011). For this reason, we integrated all the biomarker responses into a synthetic biomarker response index (BRI) in order to compare and to rank the toxicity of these drugs.

2. Materials and methods

The ecgonine methyl ester (EME) standard (CAS number 7143-09-1; purity >99%) was purchased from Alltech-Applied Science (State College, PA, USA), while all the reagents used for biomarker analyses were purchased from Sigma–Aldrich (Steinheim, Germany). We diluted the methanol stock solution (1 g/L) to 10 mg/L in bi-distilled water (working solution), which was then used to obtain the desired EME concentration in experimental aquaria.

2.1. Exposure concentration selection

In order to give a marked ecological relevance to our research, we exposed mussels to two environmentally relevant EME concentrations, miming real conditions to which specimens might be subjected during their whole life span. Thus, according to the most recent European monitoring studies, we exposed mussels to 0.15 μg/L (0.75 nM, Low) and 0.5 μg/L (2.5 nM, High) of EME. The first concentration was similar to the EME levels found in European untreated wastewater (Castiglioni et al., 2011; Pal et al., 2012), while the second one was similar to the EME level measured in untreated wastewater from USA (Castiglioni et al., 2011). Moreover, in order to carry out a comparison between the sub-lethal toxicity of COC metabolites to zebra mussel, the highest concentration was the same used in our previous exposure to benzoylecgonine (Parolini et al., 2013).

2.2. Experimental design

Zebra mussel specimens were collected in March 2012 by a scuba diver at a depth of 4–6 m in Lake Lugano (Northern Italy), which is considered a reference site due to its low illicit drug pollution (Zuccato et al., 2008). The mussels were gently cut off from the rocks, quickly transferred to the laboratory in bags filled with lake water and placed in 15 L glass-holding aquaria filled with tap and lake water (50:50 v/v) to avoid a drastic change in the chemical composition of the water and to guarantee a food supply for the mussels during the first 24 h of acclimation. 200 specimens having similar shell length (20 ± 2 mm) were maintained in aquaria filled with 10 L of tap and deionized water (50:50 v/v), previously de-chlorinated by aeration, under a natural photoperiod with constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90% of saturation). The bivalves were fed daily with lyophilized algae belonging to the genus *Spirulina* spp. Water was regularly renewed every two days for 2 weeks to gradually purify the mollusks of any possible pollutants that had previously accumulated in their soft tissues. Only specimens that were able to reform their byssi and reattach themselves to the glass sheet were used in the experiments. Mussel viability was checked daily by the Trypan blue exclusion method, whereas biomarker baseline levels were checked weekly. Mussels were exposed to BE concentrations only when target biomarker levels were comparable with baseline ones obtained in previous studies (Parolini et al., 2010, 2011a,b, 2013). Exposure assays were performed under semi-static conditions for 14 days. Control and exposure aquaria were processed at the same time, and the whole water volume (10 L) was renewed on a daily basis. Exact volumes of working solution (10 mg/L) were added daily to the exposure aquaria until reaching the chosen concentrations. Considering that EME degradation does not occur in surface water after 35 h (van Nuijs et al., 2012), the complete water and chemicals renewal should guarantee a constant BE concentration over a 24-h period and prevent losses of contaminants, as well as the degradation of parental compound. Specimens were fed 2 h before the daily change of water and chemicals to avoid the adherence of the drugs to food particles and to prevent the reduction of their bioavailability. Several specimens ($n = 30$) were collected every 3 days for 14 days from each aquarium to evaluate EME-induced sub-lethal effects. Bivalve hemolymph was withdrawn and cyto-genotoxicity was evaluated on hemocytes. After the withdrawal, the soft tissues of mussel was immediately frozen in liquid nitrogen and stored at −80 °C until the analysis of LPO and PCC. Lastly, the soft tissue of other 15 specimens was frozen in liquid nitrogen and stored at −80 °C until the enzymatic activity was measured.

2.3. NRRA, enzyme activity and oxidative stress biomarkers

The NRRA method followed the protocol proposed by Lowe and Pipe (1994). Slides were examined systematically thereafter at 15 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests finished when dye loss was evident in at least 50% of the hemocytes. The mean retention time was then calculated from five replicates. The activity of SOD, CAT, GPx, and GST was measured in triplicate ($n = 3$) in the cytosolic fraction extracted from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 100 mM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) using a Potter homogenizer. Specific protease inhibitors (1:10) were also added to the buffer: dithiothreitol (DTT, 100 mM), phenanthroline (Phe, 10 mM) and trypsin inhibitor (Try, 10 mg/mL). The homogenate was centrifuged at 15.000 g for 1 h at 4 °C. The sample was held in ice and immediately processed for the determination of protein and enzymatic activities. The total protein content of each sample was determined according to the Bradford method (1976) using bovine serum albumin as a standard. Enzymatic activities were determined spectrophotometrically as described by Orbea et al. (2002). Briefly, the CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 67 mM potassium phosphate buffer (pH 7). The SOD activity was determined by measuring the degree of inhibition of cytochrome c (10 μM) reduction at 550 nm by the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 μM) reaction. The activity is given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). The GPx activity was measured by monitoring the consumption of NADPH at 340 nm using 0.2 mM H_2O_2 substrate in 50 mM potassium phosphate buffer (pH 7) containing additional glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL), and NADPH (120 μM). Lastly, the GST activity was measured by adding reduced glutathione (1 mM) and 1-chloro-2,4 dinitrobenzene in phosphate buffer (pH 7.4) to the cytosolic fraction; the resulting reaction was monitored for 1 min at 340 nm. Lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured in triplicate ($n = 3$) from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 50 mM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) containing 1 mM DTT and 1 mM PMSF using a Potter homogenizer. LPO level was assayed by the determination of thiobarbituric acid-reactive substances (TBARS) according to Ohkawa et al. (1979). The absorbance was read at 532 nm after removal of any fluctuated material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of $1.56*10^5$ M/cm and expressed as nmol TBARS formed/g fresh weight. For carbonyl quantification the reaction with 2,4-dinitrophenylhydrazine (DNPH) was employed according to Mecocci et al. (1998). The carbonyl content was calculated from the absorbance measurement at 370 nm with the use of molar absorption coefficient of 22 000 mol/cm and expressed as nmol/(mg protein).

2.4. Genotoxicity biomarkers

Since methods and procedures of cyto-genotoxicity biomarkers applied in this study were well-described in detail by Parolini et al. (2010), only a brief description of the followed techniques was reported here. The alkaline (pH > 13) SCGE assay was performed on hemocytes according to the method adapted for the zebra mussel by Buschini et al (2003). Fifty cells *per* slide were analyzed using an image analysis system (Comet Score®), for a total of 500 analyzed cells *per* specimen ($n = 10$). Two DNA damage end-points were evaluated: the ratio between migration length and comet head diameter (LDR) and the percentage of DNA in tail. The apoptotic cell frequency was evaluated through the protocol described by Singh (2000). Two hundred cells *per* slide were analyzed for a total of 1000 cells *per* sample ($n = 5$). The MN test was performed according to the method of Pavlica et al. (2000). Four