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Environmental concentrations of cocaine and its main metabolites modulated antioxidant response and caused cyto-genotoxic effects in zebrafish embryo cells☆

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ABSTRACT

Illicit drugs have been recently identified as a serious environmental problem because of the growing evidence regarding their occurrence in aquatic environment and potential toxicity towards non-target organisms. Among them, cocaine (COC) and its main metabolites, namely benzoylecgonine (BE) and ecgonine methyl ester (EME), are commonly measured in freshwaters worldwide at levels that might cause diverse sub-lethal effects to aquatic organisms. Thus, the present study was aimed at investigating the potential adverse effects induced by the exposure to environmental concentrations (0.04, 0.4, 4 and 40 nM) of COC, BE, and EME on zebrafish (*Danio rerio*) embryos at 96 h post fertilization. Cytotoxicity was assessed by the Trypan Blue exclusion method, while primary and fixed genetic damages were evaluated by the Single Cell Gel Electrophoresis (SCGE) assay, and the DNA diffusion assay together with the Micronucleus test, respectively. The involvement of oxidative stress in the mechanism of action (MoA) of all tested drugs was assessed by measuring the activity of defense enzymes (SOD, CAT, GPx, and GST) and the expression of their encoding genes. Exposure to COC and both metabolites significantly reduced cell viability, increased DNA fragmentation and promoted the onset of apoptotic cells and micronuclei in zebrafish embryos. Results from oxidative stress-related endpoints and gene expression suggested that the observed genotoxicity may be caused by an overproduction of free radicals that imbalanced the oxidative status of embryos. The integration of biomarker responses into a synthetic index showed that at each tested concentration, BE and EME had a similar toxicity and were both more toxic than COC. Our data confirmed the potential toxicity of environmental concentrations of COC, BE, and EME, suggesting the need of further in-depth studies to shed light on their MoA and long-term toxicity towards non-target aquatic species.

1. Introduction

Illicit drugs have been considered for a long time as dramatic socio-economic and public health problems, but only recently they have been also identified as an environmental issue, attracting the interest of analytical and environmental chemistry (Zuccato and Castiglioni, 2009), as well as ecotoxicology (Binelli et al., 2012; Parolini et al., 2013a; Garcia-Cambero et al., 2015). The newest world drug report estimated that almost a quarter of a billion people aged between 15 and 64 years, corresponding to a global prevalence of 5.2%, used one illicit drug in 2014 (UNODC, 2016). This report depicts a particularly alarming scenario because even if illicit drugs' use remained stable in the past four years, the estimated number of drug users actually risen by 7 million to 247 million (UNODC, 2016). In Europe, cocaine (COC) is the most commonly used illicit stimulant drug and its market accounts for approximately one half of the global COC market (UNODC, 2015). Differently from other drugs, COC use declined worldwide as a result of the consumption trends in North America (particularly in the United States) and Europe, but it has been estimated that globally 18.3 million people aged 15–64 is still a cocaine user (UNODC, 2016). After human consumption, COC is metabolized by the liver

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and largely excreted by urine in the form of benzoylecgonine (BE, 45% of the administered dose) and ecgonine methyl ester (EME, 40%), while only a small percentage (1–9%) is eliminated unaltered (Baselt, 2004). Thus, both COC and its main metabolites reach the sewage and are usually identified in wastewaters; other metabolites, such as norcocaine and norbenzoylecgonine, are only occasionally detected (Bisceglia et al., 2010; Pal et al., 2013; Mendoza et al., 2014). Several monitoring studies showed that the concentration range of COC, BE and EME in wastewater treatment plants (WWTPs) was 4 - 4700 ng/L, 9 - 7500 ng/L and 4–36.6 ng/L, respectively (inlet), while it was 1–540 ng/L, 1–3425 ng/L and <0.56–7.5 ng/L, respectively (outlet) (Pal et al., 2013 and references therein). However, since WWTPs can only partially remove these substances (Zuccato et al., 2008), most of COC and its metabolites reach the surface waters, contributing to their contamination. Measurable levels of COC and BE were found in both European rivers and lakes, with concentrations up to 44 ng/L (range 0.5–44 ng/L) and 316 ng/L (range 1.6–316 ng/L), respectively (Pal et al., 2013 and references therein), while EME was detected only occasionally. According to COC metabolism in human body and its stability in water, the concentration of BE in aquatic ecosystems is always higher compared to that of the parental compound (van Nuijs et al., 2009; Postigo et al., 2010; Castiglioni et al., 2011). Although the current environmental concentrations of COC, BE and EME in freshwaters can be considered low, risks for the aquatic community cannot be excluded. In fact, psychotropic drugs have high pharmacological activities, and their presence in surface waters, in complex mixtures with residues of several other therapeutics, may result in unforeseeable interactions that could cause a variety of toxic effects to non-target aquatic organisms. Despite the growing awareness of the presence of these illicit drug residues in aquatic environments, just a few studies were focused on the evaluation of their ecotoxicity. For instance, planarians have been found to be very sensitive to COC and they have been used as reliable model organisms to assess the neurotoxic effects of this drug (Pagan et al., 2013). Nathaniel et al. (2013) showed that direct injections of COC (ranging from 2.5 to 10 μg/g body weight) altered the locomotor activity of crayfish, while Gay et al. (2013) demonstrated that the exposure to environmental concentration of COC affected the levels of brain dopamines, catecholamines, and pituitary activity in the European eel (*Anguilla anguilla*).

Binelli et al. (2012) highlighted that a 96-h exposure to COC induced cellular stress and increased both primary and fixed genetic damage in the zebra mussel *Dreissena polymorpha*. In addition, the 14-day exposure to 1 μg/L BE determined an imbalance of zebra mussel oxidative status, as showed by the alteration of antioxidant activity and the increase of oxidative and genetic damage (Parolini et al., 2013a), while similar adverse effects were caused by 0.5 μg/L of EME (Parolini and Binelli, 2013). Thus, considering the potential cyto-genotoxicity of COC and its metabolites towards freshwater organisms, here we aimed at investigating the same adverse effects induced by the exposure to environmentally relevant concentrations of COC, BE and EME to zebrafish (*Danio rerio*) embryos. There is a growing interest in using this Vertebrate system as a tool to evaluate the presence and the toxicity of aquatic pollutants (Scholz et al., 2008). Indeed, zebrafish embryos are considered a powerful model to predict acute toxicity in fish (Busquet et al., 2014), and can be used to assess a series of diverse sub-lethal endpoints to identify the toxicity and the mechanisms of action of pollutants. In addition, embryonic stages are more sensitive than adults to the exposure to environmental contaminants and can highlight adverse effects even at low concentrations of xenobiotics (Ottinger et al., 2008). Our goal was reached by the application of a suite of biomarkers to zebrafish embryos at 96 h post fertilization (hpf); cytotoxicity was assessed by the Trypan Blue exclusion method, while primary (DNA strand breaks) and fixed (apoptotic, necrotic and micronucleated cell frequency) genetic damages were investigated by the single cell gel electrophoresis (SCGE) assay, and the DNA Diffusion assay and the micronucleus test (MN test), respectively. In addition, we investigated the possible involvement of oxidative stress in the mechanism of toxicity of COC and its main metabolites by evaluating the activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and of the detoxification enzyme glutathione S-transferase (GST), as well as the expression of their encoding genes (*sod1* and *sod2*, *cat*, *gpx*, and *gst*).

2. Materials and methods

Cocaine (COC - CAS number 53-21-4; molecular weight 303.35 g/mol), benzoylecgonine (BE - CAS number 519-09-5; molecular weight 289.33 g/mol) and ecgonine methyl ester (EME - CAS number 7143-09-1; molecular weight 199.25 g/mol) standards were purchased from Alltech-Applied Science (State College, PA, USA). COC, BE and EME standard solutions (1 g/L in methanol) were diluted in ultrapure water to obtain a 10 mg/L stock solution, corresponding to 33 μM for COC, 35 μM for BE and 50 μM for EME, respectively. The concentration of stock solutions was checked using solid phase extraction (SPE) and high performance liquid chromatography tandem mass spectrometry analysis (HPLC-MS/MS) according to previously validated methods (Castiglioni et al., 2006), and was 8.6 ± 0.6 mg/L, 10.4 ± 1.5 mg/L and 9.1 ± 0.8 mg/L for COC, BE and EME, respectively. Starting from each stock solution, we prepared four working solutions by serial dilutions (0.004 μM, 0.04 μM, 0.4 μM and 4 μM), which were then diluted in an appropriate volume of growth medium into 12-multiwell plates to expose zebrafish embryos to the same drugs' concentrations.

2.1. Concentration selection

In order to give a remarkable ecological significance to the research, the embryos were exposed to environmentally relevant concentrations of COC and its metabolites (0.04 nM, 0.4 nM, 4 nM and 40 nM, ranging from 0.01 μg/L to 10 μg/L). The lowest tested concentrations (0.04 nM and 0.4 nM) were similar to those currently found in surface waters and in outlets from European WWTPs (Pal et al., 2013), the intermediate one (4 nM) reflected the mean levels measured in the inlets of European WWTPs (Zuccato et al., 2008; Postigo et al., 2010; Castiglioni et al., 2011), while the highest one (40 nM) represented a possible 'worst case scenario' that could be achieved in surface waters as a consequence of the continuous use of COC worldwide. Because of the limited volume of exposure medium (3 mL) and the low tested concentrations, it was not possible to measure the actual levels of COC, BE and EME in the exposure wells. However, since no degradation of BE and a moderate decrease in COC and EME concentrations in wastewaters were previously reported (van Nuijs et al., 2012), the water and chemicals were daily renewed in order to provide constant exposure concentrations over a 24-h period.

2.2. Experimental procedure

Breeding wild-type zebrafish of the AB strain were maintained at controlled conditions into a thermostatic chamber (pH 7.5; 28 °C on a 14-hr light/10-hr dark cycle) located in our animal facility, which strictly adheres to the relevant Italian laws, rules and regulations (D.to L.vo 116/92), as also confirmed by the Autorizzazione Comunale of the city of Milan (D.Lvo 27.1.1992 n°116, art.10). Fertilized eggs were collected by natural spawning and raised at