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Exposure to low concentration of fluoxetine affects development, behaviour and acetylcholinesterase activity of zebrafish embryos

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ABSTRACT

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor (SSRI) antidepressant widely used in clinics and very often found in environmental samples of urban aquatic ecosystems in concentrations ranging from ng/L to μg/L. Fish populations might be especially susceptible to FLX due to the presence of conserved cellular receptors of serotonin. Neurotoxic effects on fish biota of polluted water bodies may be expected, but there are no sufficient studies in the current literature to elucidate this hypothesis. Batteries of embryo larval assays with zebrafish were performed to evaluate the potential effects of FLX exposure, including environmentally relevant concentrations. Evaluated parameters included survival, development, behaviour and neuronal biochemical markers. Regarding acute toxicity, a 168 h-LC_{50} value of 1.18 mg/L was obtained. Moreover, hatching delay and loss of equilibrium were observed, but at a concentration level much higher than FLX measured environmental concentrations (> 100 μg/L). On the other hand, effects on locomotor and acetylcholinesterase activity (≥0.88 and 6 μg/L, respectively) were found at levels close to the maximum reported FLX concentration in surface waters. Altogether, these results suggest that FLX is neurotoxic to early life stages of zebrafish, in a short period of time causing changes in important ecological attributes which can probably be linked from molecular to population level.

1. Introduction

Pharmaceuticals are a highly diverse group of compounds, widely used and not totally eliminated from domestic effluents by current methods of wastewater treatment (Aus Der Beek et al., 2015). Thus, many of them are often detected in surface waters (Hernando et al., 2006). Water contamination by pharmaceuticals has been mainly attributed to the constant discharge of treated or untreated domestic effluents in receiving water bodies (Heberer and Heberer, 2002). Psychiatric drugs are among the most used and detected contaminants in aquatic ecosystems, but their potential risk to aquatic biota is a growing concern, since recent studies have reported behavioural changes in aquatic species, especially fish, exposed to environmentally relevant concentrations of those chemicals (Ford and Fong, 2015).

Discovered in 1975 and approved for commercialization by the Food and Drug Administration (FDA) in 1987, Fluoxetine (FLX) was the first antidepressant used in the treatment of clinical depression (Henry and Black, 2008). Nowadays, FLX is also used in the treatment of major

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depressive disorder, obsessive-compulsive disorder, panic disorder, and bulimia, and has been one of the most heavily prescribed antidepressant drugs worldwide (Stewart et al., 2014). Consequently, FLX has been reported in aquatic environmental samples at concentrations ranging from 0.012 µg/L to 0.93 µg/L (Martínez Bueno et al., 2007; Kolpin et al., 2002; Lister et al., 2009; Metcalfe et al., 2003; Weinberger and Klaper, 2014). Belonging to the group of selective serotonin reuptake inhibitors, this antidepressant is a potent selective inhibitor of the transporter enzyme for serotonin reuptake at the presynaptic membrane, increasing serotonin concentrations at postsynaptic receptor sites (Costagliola et al., 2008).

Unlike other contaminants, pharmaceuticals are designed to trigger a specific therapeutic response in humans (Fent et al., 2006), but many of their molecular targets are also present in other orthologous species (Gunnarsson et al., 2008). Thus, biological effects on non-target organisms might be expected. For instance, FLX has been described as neurotoxic to aquatic organisms, affecting their central nervous system and causing neuroreceptor and neurotransmitter modulation, behavioural changes, reproductive impairment and death (Berg et al., 2013; Weinberger and Klaper, 2014; Weis, 2014). All these biological effects are directly or indirectly related to the drug's designed mode of action to act as an antidepressant for humans. In spite of the increasing number of studies suggesting the potential aquatic environmental risk of psychiatric drugs, standard approaches and endpoints for ecotoxicological assessment of these compounds are not clearly defined, and the link among the observed effects and ecologically relevant parameters remains unclear, especially for fish populations.

Zebrafish (*Danio rerio*) early life stages are widely used as a model organism to assess the toxicity of environmental contaminants in fish populations (Scholz et al., 2008). The species has many advantages, such as a sequenced genome, abundant spawning, rapid embryonic development, transparent embryos and available standard protocols for acute and chronic assessment (ISO, 2007; Prieto et al., 2012; OECD, 2013). Moreover, in the last decade, the assessment of zebrafish behavioural and biochemical neuromarkers (e.g. cholinesterase) has been increasingly used as an endpoint to assess the sub-lethal effects of pollutants, proving to be a sensitive and reliable measure of stress exposure (Domingues et al., 2010; Andrade et al., 2015; Henriques et al., 2015; Klüver, 2015).

In this study, an integrated approach was conducted, using zebrafish embryos to evaluate the short-term toxicity of FLX. The parameters selected, comprising several organizational levels, were: i) mortality; ii) embryo development (including developmental delays and abnormalities); iii) fish behaviour (by measuring locomotor activity) and iv) acetylcholinesterase activity. Obtained data were compared with data from others studies with fish using a SDD analysis. Sub lethal responses, from molecular to population levels are discussed, and links between observed responses and parameters of ecological relevance are proposed.

2. Material and methods

2.1. Chemical

FLX 97% of purity was obtained from C&C Pharmaceutical industry, Amapa, Brazil (CAS Number: 56296-78-7, empirical formula: $C_{17}H_{18}F_3NO$).

2.2. Chromatographic analysis

To confirm the stability of FLX in test conditions, samples from test solutions were analysed using High Performance Liquid Chromatography (HPLC Shimadzu-Prominence). Samples were originally kept in the climate-controlled chamber where all toxicity tests were performed (SL-24 Solab Científica). Stability of FLX in the dilution water and climatic conditions of the tests were evaluated by HLPC, following the method described by Sabbioni et al., 2004 (see details in Suppl. Material-SM1, Fig. S1, Table S1).

2.3. Test organisms

Zebrafish were maintained in aquariums with reverse osmosis and activated carbon filtered water. Fish were raised in an aquatic facility (ZebTec - Tecniplast, Italy) with a photoperiod cycle of 12:12 h (light:dark) at the University of Brasilia (Brazil). The water parameters were strictly controlled: temperature was maintained at 27.0 ± 1 °C, conductivity at 650 ± 100 µS/cm, pH at 7.0 ± 0.5 and dissolved oxygen ≥ 95% saturation. The same water media, with similar physical-chemical parameters, was used to prepare the stock and exposure solutions in all the performed tests.

Zebrafish eggs were collected immediately after natural mating, rinsed in water, and checked under a stereomicroscope (Stereoscopic Zoom Microscope – Stemi 2000, Zeiss, Germany). The unfertilized eggs and those showing cleavage irregularities or injuries were discarded (< 15% of the total number of eggs).

2.4. Fish embryo toxicity (FET) test

Fish embryo toxicity test was based on the OECD guideline Protocol no. 236 “Fish Embryo Toxicity” (FET) test (OECD, 2013) with few adaptations including the extension of time of exposure from 96 h to 168 allowing a better evaluation of locomotor activity. Zebrafish embryos were exposed, immediately after fertilization, to seven different concentrations of FLX (0; 0.01; 0.27; 0.74; 2.02; 5.51; 15.0 mg/L) prepared by successive dilutions of stock solution direct in dilution water. The test was performed in 24-well microplates, 20 wells were filled up with 2 mL of the test solution and four wells with water (internal plate control, as required in the OECD guideline). A total of 60 eggs were used per treatment, divided in three independent replicates. The embryos were selected and distributed individually, one egg per well. The 24-well microplates with exposed embryos were kept in a climate chamber at 26 ± 1 °C and 12 h of light (SL-24 Solab Científica, Brazil). Embryos and larvae were observed daily under a stereomicroscope. Developmental parameters were evaluated in embryos over the test period, using a magnification of ×70 for eggs and ×40 for hatched embryos. Before hatching, the following parameters were evaluated: egg coagulation, otolith formation, general delay in development, eye and body pigmentation, somite formation, heartbeat, oedemas, detachment of the tail-bud from the yolk sac, yolk sac absorption and hatching. After hatching, spine malformation and posture (embryos side-lying in the bottom of the microplate well after mechanical stimulus) were also evaluated. All parameters were assessed and quantified as observed or not observed.

2.5. Sub lethal assays

For locomotor and Acetylcholinesterase activity assays a different range of concentrations were chosen considering both, the results of FET test, previously performed, (NOEC for Loss of equilibrium endpoint = 0.27 mg/L) and the maximum reported FLX concentration in the literature 0.93 µg/L (Bueno et al., 2007). Moreover, for both assays the exposed embryos were kept in a climate chamber at 26 ± 1 °C and 12 h of light.

2.5.1. Locomotor behaviour assay

Immediately after spawning, zebrafish embryos were individually exposed, one embryo per well, in 96-well microplates. The sub-lethal concentrations were 0.0, 0.88, 15.8, 281.2, 500 µg/L of FLX. A total of 48 embryos, divided in three independent replicates of 16, were used per treatment. Locomotor activity was measured at 120, 144 and 168 h, windows of exposure recommend by Padilla et al. (2011). Prior to the assessment of behaviour, dead larvae or larvae that exhibited physical