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Assessing biological effects of fluoxetine in developing zebrafish embryos using gas chromatography-mass spectrometry based metabolomics

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HIGHLIGHTS

- GC-MS based metabolomics was used to assess fluoxetine toxicity in zebrafish embryos.
- 31 endogenous metabolites were positively identified.
- 11 metabolites were significantly altered in fluoxetine exposed embryos.
- Amino acids, fatty acids and glucose were downregulated in exposed embryos.
- Citric acid was upregulated in exposed embryos.
- Pathway analysis revealed perturbation of energy and amino acid metabolism.

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ABSTRACT

Continuous low-dose exposure of pharmaceutically active compounds (PhACs) in aquatic ecosystems is a concern worldwide. In this study, we utilized a gas chromatography mass spectrometry (GC-MS) based metabolomics approach to assess endogenous metabolite changes in developing zebrafish embryos exposed to different concentrations of the widely used antidepressant, fluoxetine. Embryos were exposed from 2 h post fertilization (hpf) until 96 hpf. Using the Fiehn GC-MS library, a total of 31 metabolites were positively identified in embryos. Statistical analyses revealed significant dysregulation of 11 metabolites in fluoxetine exposed embryos. Metabolite classes that were significantly altered included, amino acids, monosaccharides, glycerophosphates, fatty acids, carboxylic acid derivatives and sugars. Concentrations of amino acids, maltose, D-malic acid, 3-phosphoglycerate and D-glucose were significantly reduced in exposed embryos. Conversely, concentrations of citric acid were in some cases significantly elevated in exposed embryos. Metabolic pathway analysis revealed perturbation of five main pathways, including (i) alanine, aspartate and glutamate metabolism, (ii) phenylalanine, tyrosine and tryptophan biosynthesis, (iii) phenylalanine metabolism. (iv) tyrosine metabolism and (v) starch and sucrose metabolism. The results indicate fluoxetine exposure causes perturbation of energy and amino acid metabolism, which may adversely impact embryogenesis due to depletion of energy reserves during this period. Also, the observed alterations in aspartic acid, phenylalanine and tyrosine in fluoxetine exposed embryos suggests potential disruption of normal neurobehavioral and liver function. The results further demonstrate that GC-MS based metabolomics is an effective approach for assessing toxicodynamics and threshold effect levels of environmental pollutants in aquatic organisms.

1. Introduction

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) that is the active pharmaceutical ingredient (API) of the antidepressant drug Prozac, one of the most widely prescribed antidepressants globally (Grohol, 2012). Fluoxetine, (±) -*N*-methyl-3-phenyl-3-[(α,

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α, α-trifluoro-ρ-tolyl) oxy] propyl amine, exists as a racemic mixture of two enantiomers R- and S- fluoxetine. Conventional wastewater treatment plants (WWTPs) are unable to completely remove fluoxetine, with residues frequently detected in WWTP effluent and sludge (Kinney et al., 2006; Gardner et al., 2013). Consequently, fluoxetine is routinely detected in aquatic environments receiving WWTP discharge, including samples of surface water (Barnes et al., 2008; Gros et al., 2009) and sediments (Schultz et al., 2010). Reported concentrations of fluoxetine in surface water range between approximately 10 ng/L and 0.5 μg/L (Kolpin et al., 2002; Brooks et al., 2003a; Schultz et al., 2010). In addition to households, hospitals are potentially important point sources of fluoxetine. A recent survey of psychiatric hospitals in Beijing, China reported relatively high concentrations of fluoxetine in hospital WWTP influent (0–17 ng/L), as well as secondary effluent (10 ± 1 ng/L), (Yuan et al., 2013).

As pharmaceuticals are generally designed for a specific mode of action (MOA) in humans, there is concern regarding adverse effects in non-target organisms (Berninger and Brooks, 2010). Several studies have reported biological effects of pharmaceuticals in aquatic organisms, including shrimp, mussels, zooplankton, fish, benthic invertebrates and algae (Crane et al., 2006; Diniz et al., 2015; González-Ortegón et al., 2016).

Fluoxetine readily bioaccumulates in fish (Nakamura et al., 2008). Chronic fluoxetine exposure in teleosts can result in impacts to the endocrine system (0.54 μg/L), (Silva de Assis et al., 2013), delay in onset of their sexual development (71 μg/L), (Henry and Black, 2008), changes in feeding behaviour (5 μg fluoxetine/g body weight), (Mennigen et al., 2009), altered predator avoidance behaviour (25 ng/L −1 μg/L), (Painter et al., 2009; Weinberger and Klaper, 2014), decreased growth, survival and delays escape responses (0.03 μg/L and 0.5 μg/L), (Pelli and Connaughton, 2015), developmental abnormalities (0.1 μg/L-5 μg/L) (Foran et al., 2004), and induction of plasma vitellogenin synthesis in sexually mature males (28 ng/L), (Schultz et al., 2011).

Zebrafish are physiologically and genetically homologous to mammals. Approximately 86% of human drug targets have been reported to be evolutionally conserved in zebrafish (Gunnarsson et al., 2008). Previous studies of fluoxetine exposure in zebrafish have shown neuroendocrine dysfunction due to inhibition of natural increase in plasma cortisol levels, which may lead to increased predation and mortality (Abreu et al., 2014).

Conventional toxicological endpoints such as reproductive defects, behavioural changes and mortality are commonly used to assess effects of waterborne contaminants of concern (De Coen and Janssen, 2003; Ankley et al., 2007). However, these endpoints do not provide pertinent information regarding specific biochemical changes at the cellular or tissue level or insight regarding toxic mode of action. While conventional observational endpoints can help to describe manifestation of symptoms related to chemical exposure, the data are relatively ineffective for distinguishing between different toxicants that produce similar phenotypical endpoints.

In recent years, metabolomics based approaches have been employed to evaluate biological impacts in aquatic organisms related to exposure of environmental contaminants (Flores-Valverde et al., 2010; Southam, 2011; Xu et al., 2015), as well other environmental stressors such as temperature (Kullgren et al., 2013) and anoxia (Podrabsky et al., 2007). Metabolomics based toxicity assays may be particularly useful, as metabolic stress is the first response to physical and chemical stressors. Perturbations of endogenous metabolites in biofluids, cells or tissues can occur following xenobiotic exposure or other physiological stress periods (Bundy et al., 2008). Detection of early stage disruptions at the molecular level and profiling of differential metabolites can aid ecological risk assessment initiatives (Lin et al., 2006).

Several studies have employed metabolomics to assess the effects of environmental contaminants in zebrafish, including investigations of polycyclic aromatic hydrocarbons (PAHs) and oxy-PAHs (Elie et al., 2015), fipronil (Wang et al., 2016), microcystins (Pavagadhi et al., 2013), polychlorinated biphenyls (PCBs) (Xu et al., 2016) and 17α-ethinylestradiol (EE2), (Teng et al., 2013). Huang et al. (2016) recently reported metabolite profile changes in zebrafish larvae exposed to various pharmaceuticals and personal care products (including fluoxetine), heavy metals, petroleum-derived compounds, performance chemicals and endocrine disruptors. However, there are relatively few studies of effects of fluoxetine exposure in embryonic zebrafish at the molecular level, especially at environmentally relevant concentrations. Early life stage fish generally have weaker xenobiotic metabolizing potential compared to equivalent life stages of mammals (Andersson and Förlin, 1992), which may result in greater sensitivity to contaminant exposure compared to adults.

In the present study, we utilize a gas-chromatography mass spectrometry (GC-MS) based metabolomics approach to assess biological effects of fluoxetine in developing zebrafish embryos. The specific objective of the study was to conduct a series of experiments to evaluate metabolite profile changes in embryos exposed to a wide range of fluoxetine concentrations (ng/L to μg/L). The study aims to provide important information regarding the toxicodynamics and threshold effect level of fluoxetine in developing fish embryos.

2. Materials and methods

2.1. Chemicals

Fluoxetine hydrochloride (product number F132, CAS No-56296-78-7) was purchased from Sigma Aldrich. HPLC grade methanol (Sigma Aldrich) was used as solvent. Stock solution of fluoxetine was made in methanol at a concentration of 7 g/L. However for static exposures, solutions were made in egg water with a 0.01% final methanol concentration. Fatty acid methyl esters (C_8-C_{28} FAMEs), myristic acid (d_{27}) and derivatization agents were all procured from Sigma.

2.2. Zebrafish maintenance and embryo collection

Zebrafish embryos were procured from zebrafish adults of wild type strain. The adults were reared under standard laboratory conditions in the aquarium of the Department of Biological Sciences, National University of Singapore. The use of zebrafish embryos were carried out in accordance with the relevant laws & institutional guidelines, as approved by the Institutional Animal Care and Use Committee (IACUC). Embryos were collected from spawning adult fish after 0.5 h post fertilization (hpf). Embryos from different adult pairs were pooled in order to avoid batch effects. After collection, embryos were rinsed in egg water and maintained at 28 ± 0.5 °C in an incubator until 2 hpf (hours post fertilization), at which time exposure was initiated.

2.3. Chemical exposure

The selected fluoxetine test concentrations ranged from a low-dose (12 ng/L), which is typical of surface waters (Kolpin et al., 2002) to a high-dose (700 μg/L), which is the reported LC50 value for fluoxetine in fathead minnow (Brooks et al., 2003b). Specifically, exposure concentrations were set at 12 ng/L, 120 ng/L, 12 μg/L, 70 μg/L and 700 μg/L. Control groups consisted of egg water controls and solvent controls (0.01% methanol in egg water). All of the