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Environmentally relevant concentrations of di(2-ethylhexyl)phthalate exposure alter larval growth and locomotion in medaka fish via multiple pathways

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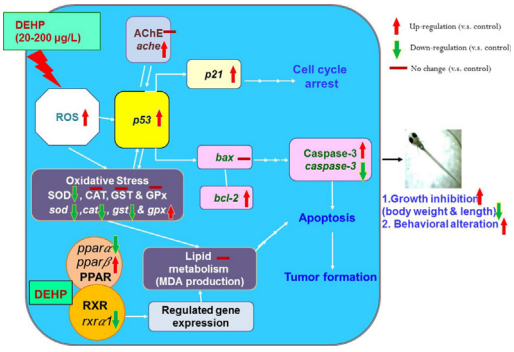
HIGHLIGHTS

- Medaka larvae growth was retarded after DEHP exposure.
- Decreased expression of antioxidants with DEHP indicated an impaired antioxidant mechanism.
- High p53 and p21 gene expression indicated cell cycle arrest with DEHP exposure.
- DEHP-altered *caspase-3* expression may lead to abnormal cell apoptosis.
- Altered fish locomotion and *ache* expression reveal the neurotoxic effects of DEHP.

GRAPHICAL ABSTRACT



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ABSTRACT

Di(2-ethylhexyl)phthalate (DEHP) is a commonly used plasticizer, with evidence of ubiquitous human exposure and widespread occurrence in the aquatic environment. It is an emerging environmental pollutant with regulatory priority; however, most studies have focused on the toxicity of DEHP related to endocrine disruption and reproduction in mammals. The ecotoxicological impact of phthalates (e.g., DEHP) on early life stages of fish under environmentally relevant concentrations of chronic exposure remains unclear. In this study, 7-day post-hatching fry of medaka fish (*Oryzias latipes*) underwent 21-day continuous exposure to DEHP solutions at 20, 100 and 200 μg/L to assess the effects on fish development and locomotion and related toxic mechanisms. Larval mortality was low with DEHP (20–200 μg/L) within 21 days, but such exposure significantly reduced fish body weight and length and altered swimming behavior. At 21 days, DEHP exposure resulted in specific patterns of larval locomotion (e.g., increased maximum velocity and absolute turn angle) and dose-dependently increased the mRNA expression of *acetylcholinesterase* (*ache*) but did not alter AChE activity. Transcriptional expression of antioxidants such as *superoxide dismutase*, *catalase*, *glutathione peroxidase*, and *glutathione S-transferase* and *peroxisome proliferation-activated receptor* and *retinoid X receptor* genes was significantly suppressed with 21-day DEHP exposure (20–200 μg/L), with marginal alteration in reactive oxygen species levels and antioxidant activities within the dosing period. As well, DEHP altered the mRNA expression of p53-regulated apoptosis pathways, such as up-regulated *p53*, *p21* and *bcl-2* and downregulated *caspase-3* expression, with increased enzymatic activity of

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AP-1, activator protein-1; ARE, antioxidant response element; CAT, catalase; DEHP, di(2-ethylhexyl)phthalate; dpe, day(s) post-exposure; ERK, extracellular signal-regulated kinase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione *S*-transferase; LSD, Fisher's least significant difference; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; MEHP, mono(2-ehylhexyl) phthalate; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator-activated receptors; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; RPL7, ribosomal protein L7; RXR, retinoid X receptor; SOD, superoxide dismutase; TRHr, thyrotropin releasing hormone receptor; VC, vehicle control.

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caspase-3 in larvae. Our results suggest that toxic mechanisms of waterborne DEHP altered fish growth and locomotion likely via a combined effect of oxidative stress, neurotoxicity and apoptosis pathways.

1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) is the most commonly used synthetic plasticizer added in daily-use products such as medical equipment, food packaging, and toys (Magdouli et al., 2013; Zolfaghari et al., 2014). Because DEHP is massively produced and used worldwide (Koch et al., 2003), it has been widespread detected in the aquatic environment around the world, particularly in North America, Europe, and Asia (Magdouli et al., 2013; Zolfaghari et al., 2014). Thus, increasing studies are investigating the physicochemical properties, fate and biological toxicity of DEHP in the aquatic ecosystem (Magdouli et al., 2013; Nugegoda and Kibria, 2017; Zolfaghari et al., 2014).

DEHP is a fat-soluble artificial chemical with low water solubility (1.9×10^{-3}–0.4 mg/L) and high bioaccumulation potency (USEPA, 2002b). DEHP can be stably persistent in the aquatic environment due to its resistance to photolysis and biodegradation (Lertsirisopon et al., 2009). In rivers worldwide, DEHP is frequently detected at 8–25 μg/L in Japan (Yuwatini et al., 2006), 0.33–97.87 μg/L in Germany (Fromme et al., 2002) and 18.5–81.7 μg/L in Taiwan (Yuan et al., 2002). The average concentration of DEHP detected in river sediments ranges from 0.21 to 8.44 mg/kg in Germany (Fromme et al., 2002), 1.97 to 47.3 mg/kg in Taiwan (Lin et al., 2009; Yuan et al., 2002) and 7.89 to 115 mg/kg in the United Kingdom (Magdouli et al., 2013). DEHP is likely bioaccumulated in fish tissues (e.g., Log BCF = 2–5 in fish) (USEPA, 2002b) and is detected at 0.025–1.68 mg/kg in fish from Taiwan rivers (Huang et al., 2008).

Phthalates including DEHP have a variety of adverse effects on reproduction, development and essential systems including endocrine, nervous, immune, respiratory and circulatory systems in mammals (Magdouli et al., 2013; Zolfaghari et al., 2014). DEHP is a known endocrine disruptor (Magdouli et al., 2013; Ye et al., 2017), affecting the reproductive endocrine and sex hormones (Magdouli et al., 2013; Nugegoda and Kibria, 2017; Ye et al., 2014). DEHP can also cause lesions in the rat thyroid, with an important role in growth, development and differentiation in vertebrates (Botelho et al., 2009; Desouza et al., 2011; Magdouli et al., 2013; Nugegoda and Kibria, 2017; Zolfaghari et al., 2014). DEHP has liver carcinogenic potency in rodents (Magdouli et al., 2013; Zolfaghari et al., 2014). It can increase reactive oxygen species (ROS) levels, oxidative stress, lipid peroxidation and DNA damage (Erkekoglu et al., 2010; Jain et al., 2009; Magdouli et al., 2013; Mankidy et al., 2013; Srinivasan et al., 2011). DEHP can also regulate the activities of certain receptors including peroxisome proliferator-activated receptors (PPARs) and pregnane X receptor, involving energy homeostasis and xenobiotics metabolism (Magdouli et al., 2013). As well, it is a potential neurotoxicant because it decreases the density of mature and immature neurons in the male rat hippocampus (Smith et al., 2011). Most DEHP toxicity studies have focused on mammalian systems and reproduction; however, few have investigated the ecotoxicologic effects of phthalates (e.g., DEHP) on aquatic organisms, particularly the developmental effects of early life stages.

DEHP-impaired reproduction was reported in aquatic organisms. Adult zebrafish (*Danio rerio*) exposed to DEHP (0.2 and 20 μg/L) for 3 weeks showed increased DNA fragmentation in sperm cells and decreased embryo production (Corradetti et al., 2013). Similar results were observed in marine medaka (*Oryzias melastigma*), showing decreased egg production and fertilization with DEHP exposure (0.1 and 0.5 mg/L for 6 months) (Ye et al., 2014). In the early life stages, liver detoxification and the blood–brain barrier are not well developed, so organisms may be more sensitive to environmental toxicants such as DEHP. Embryos of fathead minnow (*Pimephales promelas*) showed increased mortality and lipid peroxidation with 1 mg/L DEHP exposure for 96 h (Mankidy et al., 2013). The body length and weight of guppy fry (*Poecilia reticulata*) were reduced after exposure to DEHP (0.1, 1, and 10 μg/L) for 91 days (Zanotelli et al., 2010). Abnormal embryogenesis and fry development including increased mortality, changes in hatching time and weight loss were observed in early life stages of fish with DEHP exposure (Chikae et al., 2004; Mankidy et al., 2013; Ye et al., 2014; Zanotelli et al., 2010).

Medaka (*Oryzias latipes*) is an important fish model teleost in the field of physiology, medicine, embryonic development, toxicology and environmental science because it has a complete genomic database that provides sufficient bioinformation for molecular studies (Kasahara et al., 2007; Wittbrodt et al., 2002). In the present study, to elucidate the effects of DEHP on fish development under environmentally relevant concentrations, we continuously exposed early life stages of medaka larvae to DEHP solutions (20–200 μg/L) for 21 days and assessed organismal effects on growth and locomotion and related toxic mechanisms including oxidative stress, apoptosis and neurotoxicity. The findings will extend our understanding of the potential toxic impact of DEHP in early life stages of aquatic organisms and provide basic information for risk assessment of human health and ecological safety.

2. Materials and methods

2.1. Preparation of dosing solutions and concentration characterization

DEHP (D201154, >99.5% pure; Sigma-Aldrich, St Louis, MO, USA) was dissolved in acetone to make stock solutions of 20, 100 and 200 mg/L. From each stock solution, 0.1 mL was added into 1 L EPA artificial surface water (containing 1.14 mM $NaHCO_3$, 0.50 mM $MgSO_4 \cdot 7H_2O$, 0.05 m MKCl, 0.35 mM $CaSO_4 \cdot 2H_2O$) that mimics the natural river water of medium hardness (USEPA, 2002a) to achieve final concentrations of 20, 100 and 200 μg/L DEHP for fish exposure. A vehicle control (VC) containing 0.1% (v/v) acetone and blank water control (WC, EPA artificial water only) were included. The tested concentrations referred to the reported environmentally relevant levels (10 and 100 μg/L) described previously (Fromme et al., 2002; Yuan et al., 2002; Yuwatini et al., 2006) and a two-fold increase (200 μg/L) was included as the worse-case scenario concentration. Before exposure, all solutions were aerated to minimize the residual acetone.

The DEHP dosing solutions (50 mL) were filtered through 0.45-μm filters and acidified to pH 2.0 for solid-phase extraction with Oasis HLB cartridges (200 mg, 6 cm^3, Waters, Milford, MA, USA) conditioned with dichloromethane, methanol and MilliQ water. The cartridges were dried for 30 min, and absorbed DEHP was eluted with methanol and dichloromethane. The extracts were evaporated under an N_2 stream and suspended. The actual concentrations of DEHP in dosing solutions were determined by a high-performance liquid chromatography system equipped with a Purospher SRAR RP-18e column (5 μm, 4.6 mm × 250 mm) and a diode array detector set at 228 nm (HPLC-DAD, Hitachi, L2130). The isocratic methanol/water mixture (95:5, v/v) at a flow rate of 1 mL/min was used as the eluent. The measured concentrations of DEHP in each dosing solution were as anticipated, with 102% to 120% of their nominal values (Table S1, supporting information [SI]).

2.2. Fish culture and care

Adult reddish-orange medaka fish were bred in dechlorinated tap water at 27 ± 1 °C with a daily 14-h:10-h light:dark photoperiod. The water was continuously circulated and partially renewed every day.