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Vitamin E reduces endosulfan-induced toxic effects on morphology and behavior in early development of zebrafish (*Danio rerio*)

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ABSTRACT

The aim of this study was to investigate if vitamin E (α-TOC) modulates the developmental toxicity of the pesticide endosulfan (ESF), using a modified zebrafish embryotoxicity test (ZET). Zebrafish (*Danio rerio*) embryos were exposed from 6 to 72 h post fertilization (hpf) to either ESF (0.1–50 mg/L) or α-TOC (0.01–3 mM) alone or in combination. The effects of these exposures on embryonic morphology, larval behavior and antioxidant gene expression were analyzed. Phenotypic analysis at 48 hpf showed that ESF led to a dose-dependent increase in embryonic deformities, including axis malformations, pericardial edema and reduced pigmentation. Co-exposure of ESF with α-TOC (1–3 mM) significantly ($p < 0.05$) reduced ESF-induced embryonic malformations. Exposure to solely α-TOC did not affect rates of survival or malformations. Behavior studies showed that ESF caused hyperactivity at 5 days post fertilization, indicating a developmental neurotoxic effect. The ESF-induced hyperactivity was ameliorated by α-TOC. Elevated ESF concentrations caused down-regulation of the antioxidant genes *cuzn-sod*, *gpx1a* and *cat*, suggesting that ESF promoted oxidative stress in the embryos. α-TOC did not prevent the ESF-induced dysregulation of these genes. These results demonstrate that α-TOC protects against phenotypic and behavioral effects caused by ESF but did not rescue ESF-induced aberrations in antioxidant gene expression.

1. Introduction

Nutrients can modulate the toxic effects of contaminants. For example, pesticide-induced oxidative damage can be reduced by nutrients such as vitamin E, C and selenium (Abdollahi et al., 2004; Garg et al., 2011; Ozkan et al., 2012). In aquaculture feed, vegetable-based ingredients are increasingly utilized as replacements for marine products (Søfteland et al., 2014). This affects the feeds' nutrient composition and exposes fish to new contaminants such as agricultural pesticide residues (Olsvik et al., 2015). However, the interactive effects between the contaminants and altered nutrient profiles in current aquaculture feeds remain to be determined.

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzadioxathiepin-3-oxide) is a fat-soluble organochlorine insecticide that can persist in the environment (Singh, 2012). Commercial ESF is usually a mixture of two isomers: α-ESF (70%) and β-ESF (30%) (Zervos et al., 2011). ESF was classified as a persistent organic pollutant (POP) in 2012 (Dong et al., 2013), but is still widely used across the world and remains a serious environmental concern. ESF is regarded as extremely toxic to fish, especially to the younger life stages, and may cause acute and chronic harmful effects in aquatic species at concentrations as low as 0.22 μg/L and 0.056 μg/L, respectively (Dar et al., 2015; Moon et al., 2016). ESF has been found to affect many processes, including embryo development, reproduction and signal transduction (Silva and Gammon, 2009; Xu et al., 2016), but is primarily regarded as a neurotoxin. The primary mode of action in the nervous system is blockage of GABA (γ-aminobutyric acid) receptors in neural cells, which prevents uptake of chloride ions and leads to uncontrolled excitation and brain damage (Klaassen and Watkins,

Abbreviations: α-TOC, Alpha-tocopherol; CAT, catalase; CuZn-SOD, Copper-Zinc superoxide dismutase; dpf, days post fertilization; DMSO, dimethylsulfoxide; ESF, Endosulfan; hpf, hours post fertilization; GABA, γ-aminobutyric acid; GCLC, Glutamate cysteine ligase catalytic subunit; GPx, Glutathione Peroxidase; NFE2L2, Nuclear factor (erythroid-derived 2)-like 2; ROS, Reactive oxygen species; PRDX, Peroxiredoxin; ZET, Zebrafish embryotoxicity test.

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2001; Lee et al., 2015). Finally, many studies have shown that ESF might increase the production of reactive oxygen species (ROS), potentially leading to oxidative stress (Dar et al., 2015; Du et al., 2015; Kim et al., 2015). ROS signaling plays an essential role in embryonic development (Timme-Laragy et al., 2013), particularly during neural development. ROS are important for regulating processes such as cell proliferation, differentiation, migration and death. Different concentrations of specific reactive molecules can evoke contrasting effects within cell development (Covarrubias et al., 2008). Any imbalance in ROS levels or the internal redox status at this stage is therefore detrimental to the organism. Cells have several mechanisms for protection against oxidative damage and endogenous enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (PRDX) constitute one part of this antioxidant defense. The non-enzymatic antioxidant glutathione (GSH) is also an essential component of the cellular antioxidant system, as it is the main cellular redox buffer. Furthermore, the endogenous antioxidant defense also relies on exogenous nutrients including vitamin E, vitamin C and carotenoids (Bouayed and Bohn, 2010). Vitamin E is fat-soluble and can reduce oxidized lipids, preventing oxidative chain reactions in the polyunsaturated fatty acid (PUFA) rich cell and organelle membranes (Hamre, 2011; Hamre et al., 2010). For most vertebrates, including fish, alpha-tocopherol (α-TOC) is the most abundant and important form of this vitamin (Hamre et al., 1997). Lipid-soluble antioxidants such as α-TOC are known to help protect against pesticide-induced oxidative stress (Du et al., 2015; Garg et al., 2011; Sohn et al., 2004; Takhshid et al., 2012), and α-TOC has previously been suggested to inhibit ESF-induced oxidative stress in mice (Mansour et al., 2014; Wu et al., 2012).

Zebrafish is a popular model species due to its short generation time, cheap housing and embryo transparency, the latter being important for developmental studies (Kimmel et al., 1995). The zebrafish embryotoxicity test (ZET) has become an important toxicological test strategy (Brannen et al., 2010), and as pesticide-induced oxidative stress can be detrimental to early stages of development, analyses of embryonic stages are central in understanding the toxic effects of pesticide exposure.

The present work aimed to investigate ESF-induced toxicity in embryo development, by exposing zebrafish embryos to graded concentrations of ESF. We identified adverse effects on embryo morphology, locomotor activity and antioxidant gene expression after ESF exposure. A further objective was to identify possible alleviating effects to ESF toxicity from co-exposure with the antioxidant nutrient vitamin E (α-TOC). We found that α-TOC decreased the extent of ESF-induced morphological malformations and larval locomotor hyperactivity, but did not prevent ESF-induced dysregulation of antioxidant gene expression.

2. Materials and methods

2.1. Zebrafish

Zebrafish were maintained in compliance with Norwegian Animal Welfare Act guidelines (Ministry of Agriculture and Food, 2009). Adult zebrafish were kept in 3 L tanks in a recirculating system (Aquatic Habitats, Apopka, FL, USA). The system was maintained under standard conditions: 28.5 ± 1 °C, pH 7.5 ± 0.3, electrical conductivity 500 ± 50 μs, 10% daily water exchange and a 14:10 h light:dark photoperiod. Zebrafish embryos were obtained by performing a group mating with two different wild-type zebrafish lines: female AB and male TLF, at a ratio of 2:1. Mating and egg incubation were performed as previously described (Westerfield, 2007). Fertilized eggs were staged according to Kimmel et al. (1995) and were exposed to treatments from 6 hpf.

2.2. Pesticide and antioxidant waterborne exposures

Endosulfan (PESTANAL®, analytical standard, α+β ≈ 2 + 1) and α-tocopherol (≥95.5% purity) were both purchased from Sigma-Aldrich Norway AS. Exposure solutions were prepared fresh from stock solutions of 50 mg/mL of ESF or 10 M of α-TOC, dissolved in 100% DMSO (Sigma Aldrich, Oslo, Norway). Stock solutions were kept in glass vials, protected from light at 4 °C. Exposure solutions were renewed every 24 h and contained 1% (v/v) DMSO in E3 medium, a standard zebrafish embryo medium (Cold Spring Harbor Laboratory Press, 2011). Exposures were performed in 24 well plates (Thermo Scientific, Oslo, Norway), with 15 embryos/well with each well containing 1 mL of solution. Exposures were performed in triplicates from 6 to 72 hpf. To explore the dose-response effect from ESF, we chose to perform exposures with graded concentrations. To study if α-TOC could prevent ESF toxicity, we used an ESF exposure concentration that causes a significant phenotypic effect, and utilized graded concentrations of α-TOC to reach the α-TOC concentration where the rescue effect was significant, but where the α-TOC concentration had no visible phenotypic effect alone. Exposure concentrations used were 0.1, 0.5, 5, 10, 20, 30, 40 and 50 mg/L ESF and 0.01, 0.1, 0.5, 1.0 and 3.0 mM α-TOC for phenotype and gene expression analyses, while 0.1, 0.5 and 1.0 mg/L ESF and 3.0 mM α-TOC were utilized for behavior studies. α-TOC was studied both alone and in co-exposure with ESF. Control groups were E3 medium (ctrl) and E3 medium with 1% DMSO (ctrl/D or DMSO). Larvae that were utilized for behavior studies were maintained in E3 medium from 72 hpf until analysis at 120 hpf (5 dpf).

2.3. Phenotype analyses

The survival rate and phenotypes of zebrafish embryos were recorded at 48 hpf using a Nikon SMZ645 microscope (Nikon Cooperation, Tokyo, Japan). Phenotypes were only assessed in viable embryos, where axis malformations, pigmentation level and pericardial edema were assessed. Embryos containing one or more of these malformations were regarded as deformed. Representative embryos were fixed in 4% paraformaldehyde (20 min, 4 °C) and mounted on microscope slides. Pictures were taken using the Nikon microscope, and a Nikon Digital Sight DS-Fi1 camera with NIS Elements Basic Research software (V. 3.2, Nikon).

2.4. Behavior analyses

Zebrafish larvae utilized for behavior studies were first exposed to ESF and/or α-TOC from 6 to 72 hpf before being transferred to E3 medium until analysis at 5 dpf. Prior to analysis, individual larvae were transferred to wells of a 12-well plate filled with E3 medium (2.2 mL). Only larvae free of any morphological abnormalities were utilized for the behavior analyses. Twelve measurements were performed per exposure group (Three biological replicates with 4 larvae/replicate). An automated video-tracking system (Zebrabox, ZebraLab, Viewpoint, Lyon, France) was utilized for the analyses. The plates were placed in the Zebrabox and left for 1 min to allow the water surface to settle. Individual larval movements were recorded using the tracking mode. Within the ZebraLab system, the following arbitrary units were utilized: small (1 mm/s) and large (3 mm/s) movements, and light conditions of 5 min with 100% light (full white light) and 5 min of full darkness (0%), with an instant change in light conditions. The data was binned into 5 min blocks, corresponding to each light period. Behavior parameters measured were time spent and distance travelled within each activity zone, and the number of times larvae changed between activity zones. The three activity zones were based on the speed of the moving larvae: inactive state (<1 mm/s), low activity (1–3 mm/s) and high