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Developmental toxicity of synthetic phenolic antioxidants to the early life stage of zebrafish

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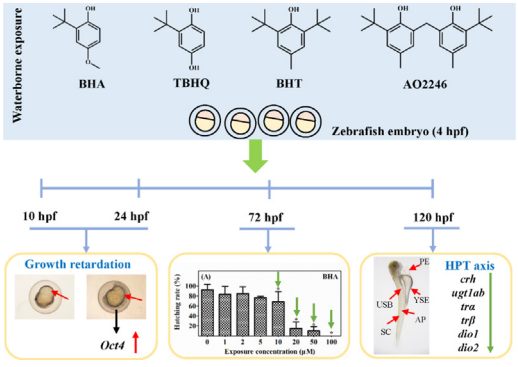
HIGHLIGHTS

- SPA exposures induced developmental toxicities in zebrafish embryos and larvae.
- Diverse deformities were caused in zebrafish larvae by SPA exposures.
- The toxicity order of SPAs to zebrafish based on their 96 h LC_{50} values was AO2246 > TBHQ > BHA > BHT.
- SPAs caused dysfunctions of HPT axis, GH/PRL synthesis, and *hh* pathway in zebrafish larvae.
- BHA and TBHQ caused early developmental retardation of zebrafish embryos.

GRAPHICAL ABSTRACT



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ABSTRACT

Synthetic phenolic antioxidants (SPAs) have gained high concerns due to their extensive usages and unintended environmental release via various routes. Their contamination in water system could pose potential threat to aquatic organisms, therefore, the studies on the aquatic toxicology of this kind of chemicals are of high importance. In this research, the developmental toxicities of four commonly used SPAs, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ), and 2,2′-methylenebis (6-tert-butyl-4-methylphenol) (AO2246) were investigated using the zebrafish embryo toxicity test (ZFET). The results showed that these four SPAs exerted different acute toxicities to zebrafish, and the toxic order, based on their 96 h LC_{50} values, was AO2246 > TBHQ > BHA > BHT, and decreased hatching rates were induced for the embryos in BHA, TBHQ and AO2246 exposure groups. Non-lethal exposures of BHA (≤20 μM), TBHQ (≤20 μM), BHT (≤200 μM) and AO2246 (≤2 μM) decreased the heart rates and body lengths of zebrafish in exposure concentration-dependent manners. Diverse morphological deformities, including uninflated swim bladder, pericardial edema, spinal curvature, severe yolk deformation, or abnormal pigmentation, were induced in zebrafish larvae upon SPA treatments. The transcriptional levels of the related genes, examined by quantitative PCR, indicated that the interferences of SPAs with hypothalamic-pituitary-thyroid axis (HPT axis), GH/PRL synthesis and Hedgehog (*hh*) pathway contributed to their developmental toxicities in zebrafish. The up-regulation of pluripotency biomarker, *Oct4*, caused the developmental retardation during the early stages of

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zebrafish embryos in BHA and TBHQ exposure groups. The results obtained herein provided important information on the developmental toxicity of SPAs, which could be very helpful in guiding the risk assessment on their aquatic toxicology.

1. Introduction

The antioxidants are compounds that inhibit or retard the chain reactions in the oxidation of other molecules. There are two different groups of antioxidants, i.e. industrial chemicals, and natural substances, and the former is widely added to consumer products to extend their shelf-lives due to its superior antioxidation activity and easy accessibility (Makahleh et al., 2015). Synthetic phenolic antioxidants (SPAs) are one of the commonly used man-made antioxidants, which have been extensively applied in a variety of products, such as cosmetics, plastics, pharmaceuticals, foodstuffs, and fish food (Liu et al., 2015; Wang et al., 2016). Of various SPAs, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butyl hydroquinone (TBHQ) are the most commercially used ones in foodstuffs with the maximum allowable addition level of 200 mg/ kg in food, beverages or oil products regulated by the U.S. Food and Drug Administration (FDA), the European Union (EU) and CODEX STANDARD (Freitas and Fatibello-Filho, 2010; Zhou et al., 2015). Another SPA antioxidant, 2,2′-methylenebis (6-tert-butyl-4-methylphenol) (AO2246), has the high similarity in the chemical structure to the above ones, and is commonly applied in rubber and plastic industries to prevent oxidation (Takahashi and Oishi, 2006). The production, usage, disposal and other unintended release of SPAs provided the potential contamination sources for the environment, and ubiquitous detectable levels of SPAs were found in aquatic system, including river, rainfall, surface runoff and ground water (Nieva-Echevarría et al., 2015; Soliman et al., 2007). Some farmed fish, such as salmon, trout, and halibut, were reported to have BHA and BHT contamination with the concentrations ranging from 0.019 to 3.9 mg/kg (Lundebye et al., 2010). As a concomitant of SPA contamination in the water system, increasing concerns have been raised about their potential deleterious effects on aquatic organisms, like fish.

Previous in vitro or in vivo studies have suggested that SPAs, like BHA, could exert endocrine disrupting effects, including weak estrogenic activity, perturbation in steroidogenesis, and interference with reproductive functions (Jeong et al., 2005; Kang et al., 2005; Soto et al., 1995; Yang et al., 2018). TBHQ and BHT could induce peroxide production and DNA damage, thus displaying potential carcinogenic effects (Eskandani et al., 2014; Meier et al., 2007). AO2246 was reported to cause testicular atrophy and reduce sperm production capacity in rats, showing apparent reproductive toxicity (Takahashi and Oishi, 2006). Despite of the toxicological data of SPAs based on mammal experimental models, the information on their aquatic toxicities remains blur, which limits the risk assessment on their increasing prevalence in aquatic environment.

The embryo of zebrafish (*Danio rerio*), as a popular experimental model, has been extensively used in aquatic toxicological studies due to the notable advantages of easy availability, rapid embryonic development, small size, high breeding rates, optically transparent embryo and high similarity in genome with human (McCollum et al., 2011). The embryogenesis of zebrafish is completed within 72 h post-fertilization (hpf), discrete organs and tissues are developed by 120 hpf (He et al., 2014), making it easy to follow chemical-induced effects during different developmental stages. The zebrafish embryo toxicity assay (ZFET) has been proposed as a standard test by OECD and the Environmental Protection Agency (EPA) to test the developmental toxicities of chemicals (Strahle et al., 2012). During the early life stages of zebrafish, thyroid hormones (THs) regulated by the hypothalamic-pituitary-thyroid axis (HPT axis), and pluripotency biomarkers, like pou5f1/Oct4, Nanog and Sox2, play important roles in embryonic organogenesis, development and energy metabolism (Chan and Chan, 2012; Lippok et al., 2014; Robles et al., 2011). They can serve as the vulnerable endpoints to explain chemical-induced embryonic developmental toxicities, and provide useful approaches for screening the hazardous effects of emerging chemicals of concern, like SPAs.

In the present study, four commonly used SPAs with similar chemical structures, i.e. BHA, TBHQ, BHT and AO2246, were investigated for their developmental toxicities using zebrafish embryo model. Based on the characterization of chemical-induced changes at morphological, physiological, and transcriptional levels during the embryonic and larval developmental window of 4–120 hpf, the developmental toxicities of the tested four SPAs were carefully evaluated, and compared. The findings could provide useful information on the potential aqua-ecosystem risks due to the environmental contamination of SPAs.

2. Materials and methods

2.1. Chemicals and regents

BHA (CAS NO. 121-00-6, >98%), TBHQ (CAS NO. 1948-33-0, >98%), BHT (CAS NO. 128-37-0, >99%), and AO2246 (CAS NO. 119-47-1, >99%) were all purchased from TCI (Tokyo, Japan). Dimethyl sulfoxide (DMSO, ≥99.9%) was purchased from Sigma (USA). The stock solutions of four SPAs described above were prepared by dissolving the corresponding chemicals in DMSO at the concentration of 200 mM, and stored at 4 °C till use. All other chemicals were of analytical grade.

2.2. Zebrafish experimental model

Wild-type adult zebrafish (AB strain) were obtained from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), and raised at 28 °C with the light/dark cycle of 14 h:10 h in an automatic circulation system. The dechlorinated water with the parameters of pH 6.9–7.2, hardness of 200 mg/L (as $CaCO_3$), dissolved oxygen concentration of 5–7 mg/L, and conductivity of 650 μS/cm was used for fish culture and exposure. When breeding, two adult females and two adult males were separately kept in the mating box overnight, and mixed together the next morning by pulling off the middle clapboard. After spawning and fertilization, the embryos of 2 hpf were collected and examined under the optical microscope (Olympus CKX31, Japan). The quick screening of the healthy embryos was based on the specific characteristics of abundant and compact blastomers in blastodisc during blastula period (OECD, 2006; Kimmel et al., 1995), and accomplished within 2 h. The healthy fertilized embryos of 4 hpf were submitted to the subsequent exposure experiments.

2.3. Acute toxicity tests for SPAs

The embryos used for the exposure experiments were randomly placed in the 12-well plates (Corning Inc., USA, 22 mm in diameter) with 20 embryos per well. The loading capacities in this test and the following ones were selected according to previous studies (Hu et al., 2009; Li et al., 2011), which was considered to be fine for the normal growth of zebrafish embryos and larvae, as no abnormal development problems were observed in the controls during the experiments. For testing the lethal effects of chemicals, the embryos were exposed to a series concentrations of BHA, TBHQ, BHT (0, 1, 2, 5, 10, 20, 50, 100,