Aquatic Toxicology 199 (2018) 21–29

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Effects of bisphenol A and its analogs bisphenol F and S on life parameters, antioxidant system, and response of defensome in the marine rotifer *Brachionus koreanus*

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ARTICLE INFO

Keywords:
Bisphenol analogs
Defensome
Antioxidant system
Brachionus koreanus
Rotifer

ABSTRACTS

To understand the adverse outcome in response to bisphenol A and its analogs bisphenol F and S (BPA, BPF, and BPS), we examined acute toxicity, life parameter, and defensome in the marine rotifer *Brachionus koreanus*. Among the bisphenol analogs, BPA showed the highest acute toxicity and then BPF and BPS, accordingly in the view of descending magnitude of toxicity. In life parameters including life span and reproduction, BPA, BPF, and BPS were found to cause adverse effect. Both intracellular ROS level and GST activity were significantly increased ($P < 0.05$) in response to each dosage of bisphenol analogs exposures. In response to bisphenol analogs, defensomes of phase I, II, and III detoxification mechanism demonstrated inverse relationship between the lipophilicity of bisphenol analogs and the expression patterns of defensomes. BPA and BPF were found to have significant modulation ($P < 0.05$) in the expression of cytochrome P450 (*CYP*) and *GST* genes. In phase III, BPS with comparatively lower lipophilicity demonstrated highly diversified expressional pattern, suggesting that BPS is likely caused less toxicity compared to BPA and BPF. In this study, via phase I, II, and III detoxification mechanism, bisphenol A and its analogs F and S demonstrated specific detoxification mechanism in rotifer.

1. Introduction

Bisphenol A (BPA; 2,2-bis [4-hydroxyphenyl] propane) is ubiquitous in both aquatic and terrestrial environment due to increasing anthropogenic pollution, as demand for its usage in the production of polycarbonate plastics (e.g. epoxy resins) and many consumer products including food containers, paper products, flame retardant, toys, and medical equipment every year (Staples et al., 1998; Vandenberg et al., 2007). The overwhelming demands and consumptions of BPA has endangered many organisms, including humans, and become vulnerable to BPA and its analogs via dietary and non-dietary route of sources (Vandenberg et al., 2007; Geens et al., 2012). It is critical to consider the aquatic environment as an important ecosystem for analyzing the effect of BPA, as BPA accumulation not only occurs through migration of BPA-based products into rivers and the ocean but also via effluent from wastewater treatment plants and landfill sites (Kang et al., 2007). Therefore, it is inevitable that aquatic organisms, including humans, are vulnerable to toxic and endocrine-disruptive effects by BPA. Indeed, a large body of research has reported adverse effects of BPA on reproduction, development, neural networks, cardiovascular, metabolic, and immune systems in rodents and human (Richter et al., 2007; Bonefeld-Jorgensen et al., 2007; vom Saal et al., 2007; Crain et al., 2007). For example, in the amphibian *Xenopus laevis*, BPA exposure has caused microcephaly, abnormal physical formation, and edema (Sone et al., 2004). In addition, BPA demonstrated its detrimental effect as endocrine-disrupting chemical where even small concentration of BPA (0.078 μg/L) exhibited retardation in the emergence of both male and female of the midge *Chironomus riparius* in the second generation (Watts et al., 2001) and reduced the sperm density in the brown trout *Salmo trutta f. Fario* in response to 1.75–2.4 μg/L BPA (Lahnsteiner et al., 2005).

Due to the adverse outcome of BPA usages, environmental move has shifted the uses of bisphenol A alternatives, bisphenol F (BPF; 2,2-bis [4-hydroxyphenol] methane) and bisphenol S (BPS; 2,2-bis [4-hydroxyphenol] sulfone) from the detrimental BPA (Seltenrich, 2015; Rochester and Bolden, 2015). However, due to similar structures with two parahydroxyphenyl moieties shared among bisphenol analogs, all analogs could be expected to elicit similar adverse effect in living

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https://doi.org/10.1016/j.aquatox.2018.03.024
Received 30 January 2018; Received in revised form 19 March 2018; Accepted 20 March 2018
Available online 22 March 2018
0166-445X/

organisms (Tišler et al., 2016). In addition, the uses of BPF and BPS has evoked yet another problem, as comparatively high concentration were detected in both environment (Fromme et al., 2002; Song et al., 2014; Yang et al., 2014) and human urine sample (Liao et al., 2012a; Zhou et al., 2014; Thayer et al., 2016). Furthermore, BPA, BPF, and BPS were recently reported to be the predominant bisphenol analogs form in sediments and indoor dust (Liao et al., 2012a, 2012b). Up to date, studies on the effects of BPS and BPF are mostly limited in the zebrafish *Danio rerio* on estrogenic, developmental, teratogenic effects, lacking molecular studies (Moreman et al., 2017; Tišler et al., 2016). Recent studies have investigated the adverse outcomes on hormonal and endocrine system in the zebrafish *D. rerio* (Huang et al., 2016; Naderi et al., 2014) in response to BPF and BPS. Although the threshold concentration to be in-effect were much higher than that of the BPA, having structural similarity as its analogs form, BPF and BPS require further studies in their toxicity to be safely applied in daily products.

Among aquatic invertebrates, the rotifers are widely distributed and occupy crucial niche as energy transmitter between producers and consumers in aquatic food webs. The rotifer genus *Brachionus* (e.g. *Brachionus koreanus*), in particular, has been considered as suitable model species for ecology, gerontology and ecotoxicology research, as they have several advantages (e.g. small size [≈200 μm], short generation cycle [≈24 h], high fecundity, and easy laboratory maintenance) (Snell and Janssen, 1995; Dahms et al., 2011). Furthermore, extensive RNA-seq information of *B. koreanus* allowed a better understanding of physiological effects at cellular and molecular levels in response to abiotic and xenobiotic stressors (e.g. gamma radiation, microplastic and methylmercury [MeHg]) in recent studies (Han et al., 2014; Jeong et al., 2016; Lee et al., 2017a,b). With the whole-genome sequencing of the rotifer *B. koreanus*, genes involved in detoxification, namely phase I (cytochrome P450; Kim et al., 2013), II (glutathione *S*-transferase; Han et al., 2013), and III (ATP binding cassette transporter; Jeong et al., 2017) were successfully identified and analyzed their function in response to xenobiotics such as benzo[α]pyrene, BDE-47, atenolol, trimethoprim (Kim et al., 2013; Park et al., 2017a; Rhee et al., 2012). To investigate how bisphenol analogs cause toxic effects and display differences in their toxicities, the life parameters, oxidative stress with antioxidant enzymatic activities, and defensomes (i.e. gene and protein sets in relation to defense mechanism in response to environmental stressors) of the three detoxification phase systems were assessed in the marine rotifer *B. koreanus*.

2. Materials and methods

2.1. Culture and maintenance

The monogonont rotifer *B. koreanus* was collected at Uljin (36°58′43.01″ N, 129°24′28.40″ E) in South Korea. For laboratory culture, a single rotifer was isolated, reared, and maintained in filtered artificial seawater (TetraMarine Salt Pro, Tetra, Cincinnati, OH, USA). The strain was preserved by serial transfer of asexual populations at 25 °C under a light: dark 12:12 h photoperiod with 15 practical salinity units (psu) of salinity. The green alga *Tetraselmis suecica* was used as a live diet (approximately 6×10^4 cells/mL). Species identification was confirmed by morphological analysis and sequencing of the mitochondrial DNA *CO1* gene (Hwang et al., 2013; Mills et al., 2017).

2.2. Reagents

The chemicals and reagents used in this study were from Sigma-Aldrich Co. (St. Louis, MO, USA), Qiagen (Hilden, Germany), or Invitrogen (Carlsbad, CA, USA) as molecular biology grade. For exposure study, BPA (molecular weight 228.29 g/mol, purity ≥99%), BPF (molecular weight 200.23 g/mol, purity ≥98%), and BPS (molecular weight 250.27 g/mol, purity ≥98%) were purchased from Sigma as analytical grade. Bisphenol analogs were dissolved in dimethyl sulfoxide (DMSO) and the solvent concentration of the exposure groups did not reach over 0.1% DMSO (v/v).

2.3. Acute toxicity of BPA and its analogs in B. koreanus

To determine the acute toxicity, ten neonates from amictic *B. koreanus* were collected just after hatching (< 2 h) and exposed to different concentrations of BPA (0, 5 [21.9 μM], 10 [43.8 μM], 15 [65.7 μM], 20 [87.61 μM], 50 [219.02 μM], 100 [438.04 μM] mg/L), BPF (0, 5 [2.5 μM], 10 [4.99 μM], 15 [74.91 μM], 20 [99.88 μM], 50 [249.71 μM], 100 [499.42 μM] mg/L), and BPS (0, 5 [19.98 μM], 10 [39.96 μM], 15 [59.94 μM], 20 [79.91 μM], 50 [199.78 μM], 100 mg/L [399.57 μM]), respectively. The mortality was analyzed by counting the number of dead rotifers of non-motile individuals, under stereomicroscope (SZX-ILLK200, Olympus, Tokyo, Japan) at a time exposure 24 h in biological triplicate.

2.4. Effects of BPA and its analogs on life parameters in B. koreanus

To examine effects of BPA and its analogs on life span and daily reproduction rate, ten neonate (< 2 h post-hatch) was exposed to BPA (0 [control], 0.5 [2.19 μM], 1 [4.38 μM], 5 [21.9 μM], and 10 mg/L [43.8 μM]), BPF (0 [control], 0.5 [2.5 μM], 1 [4.99 μM], 5 [24.97 μM], 10 [49.94 μM], and 15 mg/L [74.91 μM]), and BPS (0 [control], 0.5 [2 μM], 1 [4 μM], 5 [19.98 μM], 10 [39.96 μM], 50 [199.78 μM], and 100 mg/L [399.57 μM]), respectively, in each well with 1 mL test solution in a 24-well culture plate (SPL Life Science Co., Pocheon, South Korea). The newly born neonates in each well were counted and removed over 7 days with a stereomicroscope (SZX-ILLK200). Throughout the whole experiment, 100% of the test solution was renewed once every 24 h by replacing the original rotifer (F0) in a renewal solution containing well of the new plate. Each treatment was mixed with the marine microalga *T. suecica*, a dietary source, with a final concentration of 6×10^4 cells/mL.

2.5. Measurement of ROS, and glutathione S-transferase antioxidant enzymatic activity

To measure the oxidative stress induced by BPA and its analogs (0, 1, 5, and 10 mg/L) for 24 h in *B. koreanus*, approximately 6000 adult individuals were collected through 90 μm sieve prior to the experiment to acquire sufficient amount of protein extract. The samples were homogenized with Teflon pestle in a buffer (0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, and 0.4 mM PMSF at pH 7.4). The homogenate was centrifuged at 10,000 x g for 20 min at 4 °C and the supernatant was used for the measurement.

ROS level was measured using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, OR, USA), which oxidizes to fluorescent dichlorofluorescein (DCF) by the intracellular ROS. The mixture of phosphate-buffered saline (PBS), probe (H_2DCFDA at a final concentration of 40 μM), and the supernatant fraction in a ratio of 170: 20:10, respectively, with the total volume of 200 μL was transferred to the Black 96-well plates (SPL Life Sciences). Each well was measured at an excitation wavelength of 485 nm and emission wavelength of 520 nm under a spectrophotometer (Thermo™ Varioskan Flash, Thermo Electron, Vantaa, Finland).

The intracellular GST activities were measured according to the protocols provided by Regoli et al. (1997). The increasing absorbance at 340 nm was measured for the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (extinction coefficient of CDNB is 9.6 mM^{-1} cm^{-1}) using a spectrophotometer at 25 °C. For quantification purpose, the total protein content of the supernatant for each experiment was determined by the dye-binding method (Bradford, 1976) using bovine serum albumin standard (0–200 μg BSA/mL in PBS) and presented as percentage relative to the control. Each experiment was performed using enzymatic assay kit purchased from Sigma-Alrich Co. in technical