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# Integrated approach of eco-epigenetics and eco-metabolomics on the stress response of bisphenol-A exposure in the aquatic midge *Chironomus riparius*

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ABSTRACT

The stress response mechanisms of Bisphenol A (BPA), an endocrine disrupting compound, remain to be elucidated. In this study, we explored the effects of BPA on the non-biting midge *Chironomus riparius* through basic ecotoxicity assays, DNA damage (comet assay), eco-epigenetics (global DNA and histone methylations) and non-targeted global metabolomics (NMR based) approaches. The reproduction failure, increase in DNA damage, global DNA hyper-methylation, and increased global histone modification (H3K36) status were evident due to BPA exposure at 10% lethal concentration ($LC_{10}$: 1 mg/L, based on 48 h acute toxicity). Moreover, non-targeted global metabolomics followed by pathway analysis identified alterations of energy metabolism, amino acids, and methionine metabolisms etc. Most importantly, we found a potential cross-talk between altered epigenetics and metabolites, such as, increase in methionine and o-phosphocholine metabolites corresponds with the phenomena of global hyper-methylation in DNA and H3K36 mark. Overall, our results suggests that the crosstalk of global metabolomics and epigenetic modification was fundamental of the underlying mechanisms in BPA-induced stress response in *C. riparius*.

## 1. Introduction

Bisphenol A (BPA), a monomer of polycarbonate plastics and epoxide resin, is a widly used chemical, having endocrine disrupting property. Being an industrially important chemical material for the manufacture of engineering plastics (e.g., epoxy resins/polycarbonate plastics), food cans (i.e., lacquer coatings), dental composites/sealants etc., BPA pollutes freshwater and marine ecosystems via industrial and municipal effluents, leachates from landfill sites, and litter (Huang et al., 2012). Recently, BPA pollution hotspots were identified, further reflecting the importance of determining the effects of current and predicted levels of BPA pollution on ecosystem health (Little and Seebacher, 2015). BPA disrupts several receptor-mediated pathways including thyroid hormone, estrogen-related, and glucocorticoid receptors in various species, including aquatic organisms (Little and Seebacher, 2015; Canesi and Fabbri, 2015). Animal studies and human epidemiological studies indicate that BPA is an endocrine disrupting chemical (EDC) that has been implicated as a potential carcinogen and epigenotoxicant (Weinhouse et al., 2015). Moreover, BPA exposure has been linked to adult metabolic pathologies, but the pathways through which these disruptions occur is unknown (Veiga-Lopez et al., 2015).

Epigenetics represents how the environment interacts with genes and modulates gene expression/activity without changing the DNA sequence of the cell (Head et al., 2012; Casati et al., 2015; Vandegehuchte and Janssen, 2011). The commonly studied epigenetic mechanisms involved chromatin remodeling (DNA methylation and histone modification) and RNA-mediated modifications (non-coding RNA and microRNA) (Ho and Burggren, 2010). Alterations in epigenomes could cause mitotically or meiotically heritable changes in gene function and pass through subsequent generations (Vandegehuchte and Janssen, 2011) or could result in stable transcriptional changes of health susceptibility to develop diseases (from cancer to mental disorders), which might not necessarily be heritable (Casati et al., 2015). At present, it is widely recognized that exposure to environmental factors (chemical, diets, temperatures etc.) alters gene expressions and affects health by affecting genome and modulating epigenome. Growing evidence indicates the use of epigenetic tools in ecotoxicology; however, the knowledge and applications in ecologically relevant organisms are not as comparable as mammalian model organisms (Suarez-Ulloa et al., 2015).

Metabolomics usually reflects the combined effects of multiple upstream factors, such as transcriptomes, proteomes, and the nutritional environment (Wilmes et al., 2013). Metabolic profiling not only permits candidate biomarkers identification but also would elucidate the

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induced toxicity mechanisms of the compound (Long et al., 2015). Environmental metabolomics, the application of metabolomic techniques to analyze the interactions of organisms with their environment, can be attributed to generate hypotheses involving nontargeted metabolomics of environmental stressors with an unknown mode of action (Lankadurai et al., 2013).

Whether environmental levels of BPA have adverse effects on aquatic organisms remain controversial (Huang et al., 2012) and BPA induced mechanisms of toxicity, specifically epigenotoxicity and metabolomics pathways, in aquatic species remain elusive.

In the present study, we explored the effects of BPA on the crosstalk of eco-epigenetic and metabolomic modification in 4th instar larvae of the non-biting midge *Chironomus riparius* (*C. riparius*). First, acute and chronic toxicity tests were conducted and thereafter, assays for DNA damage, global DNA methylation and histone methylation modifications were performed to elucidate the genetic-epigenetic alteration mechanisms in BPA-treated *C. riparius*. Finally, global metabolomic profiling with nuclear magnetic resonance (NMR) was performed to elucidate not only altered metabolomic pathways but also the role of epigenetics-metabolomics crosstalk in BPA-induced toxicity.

## 2. Materials and methods

### 2.1. Test organisms and media

*C. riparius* were obtained from the Toxicological Research Center of the Korea Institute of Chemical Technology and have been reared in our laboratory for more than 10 years. The larvae were reared on an artificial diet of fish food flakes (Tetramin; Tetrawerke) in glass chambers containing dechlorinated tap water and acid-washed sand, with aeration at 20 ± 1 °C under a 16:8-h light:dark photoperiod. All experiments were performed in US Environmental Protection Agency moderately hard water (EPA-MHW) (US EPA, 2002).

### 2.2. BPA exposure condition

The effect of BPA (purity > 99%; Sigma-Aldrich Chemical, St. Louis, MO) exposure on groups of 4th instar larvae collected from the rearing aquaria was assessed. At the beginning of the experiment, 1 mL of an acetonic solution of the compounds was placed in the 1 L experimental tanks. Thirty larvae (of sublethal toxicity) were randomly introduced into each test aquarium. The exposure was carried out at a constant temperature (20 ± 1 °C) within a 16:8 h (light:dark) photoperiod in all the experiments. The comet assay, metabolomics assay, global DNA methylation assay and primary screening assay of histone methylation were conducted in sublethal concentration of BPA (48 h at 1 mg/L: $LC_{10}$ BPA).

### 2.3. Acute toxicity test

A mortality test was conducted using a modified OECD guideline (TG 235) (OECD, 2011). A group of 10 larvae was exposed to four concentrations of BPA (1, 2.5, 5, and 7 mg/L), and the other was the control group. It was determined that acute toxicity occurs after 48 h exposure, and that the condition eventually leads to death. Log-probit (epa probit analysis program version 1.5) data transformation was used to estimate 48 h 10%, 50%, and 90% lethal concentration ($LC_{10}$, $LC_{50}$, and $LC_{90}$) values and the corresponding 95% confidence intervals.

### 2.4. Chronic toxicity tests (reproduction and development tests)

For the chronic toxicity test, a modified OECD guideline (TG 219) (OECD, 2004) was used. Exposure aquariums were prepared by adding 400 mL of EPA-MHW to 100 mL of acid-washed sand. The thirty 4th instar larvae were introduced and BPA concentration (1 mg/L BPA: concentration of $LC_{10}$) were spiked to the treatment conditions. Emergence and reproduction were monitored for 25 days until all treated and control organisms were dead. The emerging adults from each vessel were counted and retained with steel-wire mesh until emergence was complete in all treatments. The egg masses oviposited by the emerged adults in the control and treated vessels were counted and used for reproduction parameters. Every 2 days, 50 mg tetramin fish food flakes was supplied to each aquarium. Test solutions were not renewed. All data were recorded at daily intervals.

### 2.5. DNA damage measurement by the comet assay

Ten larvae of *C. riparius* were collected from the control and the 48-h BPA treatment (at 1 mg/L) for the comet assay as described previously (Park and Choi, 2007). Briefly, 100 µl 1% low melting point (LMP) agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4 °C for 5 min to allow solidification. The samples were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 NaCl, 10% DMSO (only organisms), 10% Triton X-100, pH 10), and subsequently exposed to alkali conditions (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow the DNA to unwind and the alkaliliable sites to be expressed. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min, after which, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place prior to image analysis. Before their analyses, the slides were stained with 50 µl ethidium bromide (5 µg/mL), and then observed under a fluorescence microscope (Nikon, Kanagawa, Japan) equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm at 400 × magnification. Approximately, 50 cells per slide (3 slides per treatment) were examined. DNA damage was expressed as the tail moment using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

### 2.6. Global DNA methylation

Total DNA from BPA-treated samples (for 48 h at 1 mg/L) was extracted using a DNA extraction kit (NucleoSpin, Macherey-Nagel) and the quantity and quality of DNA were detected in Nano-drop. Next, global DNA methylation assays were performed according to the manufacturer's instructions (MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric), Epigentek Group Inc. NY, USA). Briefly, 100 ng DNA and other provided standards were added to a 5-Methylcytocine (5-mc) coated well and incubated for 90 min at 37 °C. Next, after adding the antibodies (anti-5mc and secondary antibody in provided ELISA buffer), enhancers and stop solutions sequentially, absorbance at OD450 nm was measured and the 5-mc % was calculated from the standard curve. Appropriate negative and positive controls were used to prepare the standard curve.

### 2.7. Screening assay of global histone methylation modifications

Total histone from the control and BPA-treated samples (for 48 h at 1 mg/L) was extracted using a histone extraction kit (EpiQuik Total Histone Extraction Kit, Epigentek Group Inc. NY, USA) and the quantity was detected in a spectrophotometer. The primary screening assay of histone methylation was carried out in BPA-treated *C. riparius* (EpiModifier™ Epigenetic Histone Modification Profiling, Epigentek Group Inc. NY, USA). The methylated fraction (K4, K9, K27, and K36) of histone H3 was detected using specific antibodies (0.5 µg/mL of each antibody) against histone H3 with these methylated lysine sites, and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of methylated histone H3 is proportional to the OD450 intensity measured. The volume for each sample was 300 µl with the histone concentrations ranging from 0.112 to 0.319 µg/µl.