A Metabonomic Investigation of Zebrafish exposed to Bisphenol A

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Abstract—Metabonomics approach based on the GC-MS technique was used to characterize the perturbation of volatile and semi-volatile components in zebrafish embryos induced by bisphenol A (BPA) for 8 d since fertilization. The exposure concentrations of BPA were 0, 0.5, 1.5 and 4.5 mg/L, around assumed a “safe level” (1.5 mg/L) that stated by US EPA. Results indicated that, the relative proportions of 9,12-Octadecadienoic acid and myo-sitosterol were decreased dramatically at 1.5 mg/L BPA, and those of saturated fatty acid (SAF), amino acid and D-myo-inositol were increased significantly. Therefore, 1.5 mg/L BPA would disturb the regular metabolizing of zebrafish. These alterations were related with the physiological and histological changes found in the exposure stage. The integration of metabonomics approach and conventional toxicology study may provide invaluable information in assessing the risk of environment pollutants.

Keywords—metabonomics; GC-MS; bisphenol A; zebrafish; toxicity

I. INTRODUCTION

Metabonomics, which defined as “the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” [1], recently has been applied to various biomedical and toxicological studies [2-3]. The extra-cellular environment of biological cells would be adjusted under the toxic stressors in order to maintain constancy of their internal environment, and these perturbations of the concentrations and fluxes of endogenous metabolites would be detected by metabolic techniques and characterized as the nature or site of this toxic compound. Therefore, metabonomic study has been proposed as a promising resolution for understanding the toxic mechanisms of environmental pollutions.

Bisphenol A (BPA), an estrogenic chemical used in the manufacture of epoxy resins and polycarbonates, is a substance ubiquitously found in consumer products [4]. BPA could bedetected from some beverage and baby bottles to the lining of food cans, and can leach out into food, and might have potential effects on the development of babies. Defined by US Environmental Protection Agency (US EPA) and the European Commission Scientific Committee on Food (EC SCF), the “safe” level of BPA was a TDI of 50 µg/kg bw/day. These assumptions allow a maximal Bisphenol A concentration in drinking water of 1.5 mg/L (60 kg adult, intake of 2 L drinking water) (A WWWF European Toxics Programme Report, April 2000). However, toxic alterations have been observed in some investigations on BPA exposure at doses far below the current accepted NOAEL of 50 µg/kg [5-7]. Meanwhile, current knowledge on bisphenol A toxicity for the development of aquatic species, especially fish, is limited to very few studies [8-10].

In this study, a metabonomics approach based on the GC-MS technique was used to characterize the perturbation of volatile and semi-volatile components in larval zebrafish induced by bisphenol A (BPA) for 8 d since fertilization. The aim was trying to assess the toxic effects of BPA in the biochemical system and explore some toxic mechanisms under metabonomics level.

II. MATERIALS AND METHODS

A Sample Preparation

Three nominal concentrations of bisphenol A (0.5, 1.5 and 4.5 mg/L) were used for exposure. The exposure was carried out in standard 100 mm Petri dishes which were rinsed 3 times with acetone and incubated over-night with the exposure solution prior to the exposure start, and each dish contained 50 selected fertilized embryos. Exposure media
were changed daily through the experiment by relocating the embryos or larval fish into a Petri dish that contained fresh solution. When exposure lasted until 8 d past fertilization, all the fish in each dish were collected as single sample. All exposure experiments were repeated 3 times on different days. All samples were frozen dry and stored at -80 °C until use.

B Extraction Procedure

The sample was extracted with 500 µL of a mixture chloroform/methanol (2:1, v/v, in a 1.5 mL Falcon tube), and then ultrasonically extracted for 10 min, and followed by centrifugation 10.0 min at 2500 rpm. The underlying organic phase was transferred to a spherical flask and the process was repeated twice. After the combination of all three chloroform/methanol phases, they were evaporated to dryness under a stream of nitrogen gas. 50 µL of ethyl acetate was added and shaken until completely dissolved. The 50 µL of a mixture which containing N, O-bis(trimethylsilyl) trifluoroacetamide, pyridine and ethyl acetate (3:1:1, v/v/v) was added to above 50 µL of the extract. After the silylation for 5 min at room temperature, the sample was ready to be injected into the GC-MS.

C GC-MS Analysis

A 2 µL aliquot of the derivate sample was injected with splitless mode by an Agilent 7683 Series autosampler (Agilent Technologies) into an Agilent 6980 GC system equipped with a fused-silica capillary column chemically (30 m x 0.25 mm i.d.) bonded with 0.25 µm ZB-5MS stationary phase (Phenomenex). The carrier gas used was helium (purity 99.999%) at a flow rate of 1.0 mL min⁻¹. The injector temperature was set at 250 °C. To acquire a well separation, the column temperature was initially maintained at 70 °C for 5 min, and then increased from 60 °C to 110 °C at rate of 10 °C/min. Then, the column temperature was increased to 240 at 10 °C/min. After that, the temperature was increased to 270 at 10 °C/min, and held for 3 min. The column effluent was introduced into the ion source of an Agilent 5973 mass selective detector (Agilent Technologies). The temperature of the ion source was set at 230 °C, and for the quadrupole, it was set at 150 °C. MS detection was implemented with electron impact mode and full scan monitoring mode (m/z 30-550). AMDIS software (National Institute of Standards and Technology, Gaitherburg, MD) was used for the peak deconvolution. Identification of the interested peaks was supported by NIST v1.0.0.12 ms spectra library.

The samples from the control group and exposed groups were blindly analyzed in a random order. Each sample was represented with a GC-MS total ion current (TIC) chromatogram, and the peaks with the intensity higher than 3-fold of the ratio of signal-to-nose were detected. Principal component analysis (PCA) was employed to process the acquired GC-MS data. The result of PCA displayed as score plots to represent the distribution of samples was able to identify the disease-representative biochemical compositions from the control samples [11].

D Data analysis

In all data, mean ± S.D. was calculated. Statistical comparisons among groups were carried out by one-way analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant different.

III. RESULTS AND DISCUSSIONS

A Multivariate Analysis of GC-MS Data.

As shown in Table 1, totally 25 metabolites were identified by GC-MS. They are saturated and unsaturated fatty acids, amino acids, and other metabolites such as glucose, lactose, cholesterol and so on. All representative peaks with their peak areas were used to construct specific vectors describing the biochemical compositions of each sample. The vector was normalized to the total sum of vector to partially compensate the different concentrations of the samples. After data standardization, PCA was carried out for the multivariate analysis.

As shown in Fig.1, from right to the left, it was clearly demonstrated 4 regions aggregated by the first principle component (t[1]), (1) the control group; (2) 0.5 mg/L BPA groups; (3) 1.5 mg/L BPA group; and 4.5 mg/L BPA group. Therefore, the metabolic component in 8 d zebrafish was dramatically altered when exposed to BPA, and the dose-response effect of BPA on the metabolic process was also significant. The regions of 1.5 and 4.5 mg/L BPA groups were very close, and that represented that the metabolic process of these two groups were a little similar.
Table 1 Main volatile and semi-volatile components validated by GC-MS

<table>
<thead>
<tr>
<th>No</th>
<th>Retention time (min)</th>
<th>Identified Compounds</th>
<th>No</th>
<th>Retention time (min)</th>
<th>Identified Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td></td>
<td></td>
<td>Other metabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.31</td>
<td>Tetradecanoic acid (C14:0)</td>
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<td>7.73</td>
<td>Phosphoric acid</td>
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<tr>
<td>2</td>
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<td>Hexadecanoic acid (C16:0)</td>
<td>2</td>
<td>8.17</td>
<td>Glucose</td>
</tr>
<tr>
<td>3</td>
<td>11.06</td>
<td>Heptadecanoic acid (C17:0)</td>
<td>3</td>
<td>8.58</td>
<td>Glucose</td>
</tr>
<tr>
<td>4</td>
<td>11.56</td>
<td>9,12-Octadecadienoic acid (C18:2)</td>
<td>4</td>
<td>9.83</td>
<td>Pantothenic acid</td>
</tr>
<tr>
<td>5</td>
<td>11.94</td>
<td>Octadecanoic acid (C18:0)</td>
<td>5</td>
<td>10.83</td>
<td>Uric acid</td>
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<tr>
<td>6</td>
<td>12.84</td>
<td>Arachidonic acid (C20:4)</td>
<td>6</td>
<td>11.61</td>
<td>Phenyl propane</td>
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<tr>
<td>7</td>
<td>14.23</td>
<td>4,7,10,13,16,19-Docosahexaenoic acid (C22:6)</td>
<td>7</td>
<td>11.99</td>
<td>myo-sinosterol</td>
</tr>
<tr>
<td>Amino acid</td>
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<td></td>
<td>8</td>
<td>13.82</td>
<td>D-Myo-inositol</td>
</tr>
<tr>
<td>1</td>
<td>5.36</td>
<td>Creatinine</td>
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<td>14.75</td>
<td>Lactose</td>
</tr>
<tr>
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<td>15.88</td>
<td>2-monostearin</td>
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<td>16.15</td>
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<tr>
<td>4</td>
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<td>Estra-1,3,5-L-Histidine</td>
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<tr>
<td>5</td>
<td>13.21</td>
<td>Uridine</td>
<td>13</td>
<td>19.47</td>
<td>β-sitosterol</td>
</tr>
</tbody>
</table>

Fig.1 PCA map of zebrafish metabolism components

Note. CN_1,2,3, 0.5_1,2,3, 1.5_1,2,3 and 4.5_1,2,3, indicated 3 replicated samples in solvent control and 0.5, 1.5 and 4.5 mg/L BPA groups, respectively. t[1] and t[2] represented the first principal component and the second principal component. Score plots which related to t[1] and t[2] denoted the distribution of samples and identified the disease-representative biochemical compositions.

B Fatty acid

According to the different frameworks, fatty acid was classified to be saturated fatty acid (SFA) and unsaturated fatty acid (UFA). As shown in Table 1, in this experiment 4 SFA and 3 UFA were identified by GC-MS in 8 d larval zebrafish. Among them, the proportions of 3 SFA (C14:0, C16:0 and C18:0) at 0.5 mg/L BPA were greatly increased to 21.74%, 37.76% and 21.22%, respectively. However, as shown in Fig.2, dose-response effects for all of them were not significant. Only 1 UFA (9,12-Octadecadienoic acid, C18:2) were dramatically decreased. The relative percent of
9,12-Octadecadienoic acid in control group was (0.15±0.046)%, but it was not detected in all the BPA exposure groups.

BPA has been shown to be metabolized to 5-hydroxybisphenol by cytochrome P450-dependent oxidizes and further converted to 4, 5, bisphenol-O-quinone [12]. In this process, reactive oxygen species (ROS) would also be induced by cytochrome P450, and produce oxidative stress by decreasing antioxidant enzymes and increasing lipid peroxidation [13]. UFA would be oxidized in this oxidative environment and the amount of it would be decreased as shown by this experiment. On the other hand, lipid peroxidation would destroy the cellular membrane, break the cellular functions, and induce abnormalities of living systems. It was reported that SFA could increase the cardiovascular risk [14]. In the exposure process of this study, blood circulation and pericardiac edema were also observed to be induced by BPA in the development of zebrafish [15]. It was accordance with the increase of 3 SFA found in this research.

C. Amino acid

In this experiment, 5 amino acids were identified by GC-MS. They are Creatinine, L-Phenylalanine, Glutamine, L-Histidine and Uridine. Among them, the proportions of Creatinine and Phenylalanine in 1.5 mg/L BPA-induced groups were significantly increased to 65.85% and 64.71% \( (P<0.05) \), and those of the other 3 amino acids were not greatly changed. As shown in Fig.3, as the concentration of BPA increased, the relative percents of Creatinine and Phenylalanine were also increased; therefore, the dose-response effects for both of them were significant \( (P<0.05) \).
amino acid.

Fig. 4 Hepatic toxicity of BPA to 8 d zebrafish

Note. Rounded nucleolus can be seen in the solvent control, but multiple stained fragments in the nucleus are visible at 4.5 mg/L BPA group. H&E staining, 1000× magnification.

C Other metabolites

Beside fatty acid and amino acid, 13 other volatile and semi-volatile metabolites were also detected by GC-MS in this study (Table 1). The level of D-myo-inositol as shown in Fig. 5 was below the detection limit at control and 0.5 mg/L BPA, but it was induced at 1.5 mg/L BPA, and increased dramatically at 4.5 mg/L BPA (P<0.05). Compared with control, myo-sitosterol was greatly decreased about 50% at 1.5 mg/L BPA, and the dose-response effects for it was significant (Fig. 5).

D-myo-inositol could be degraded in acute muscular movement. In the exposure stage, it was found that the fish exposed to BPA did not independently swim a lot. This may a reason for the accumulation of the D-myo-inositol in the fish exposed to BPA. It is reported that myo-sitosterol can effectively inhibit the preoxidation of hydroxyl radical (·OH) and superoxide anion (O2-) [16]. However, as presented above, BPA would produce oxidative stress by decreasing antioxidant enzymes and increasing lipid peroxidation [13]. Therefore, the proportions of myo-sitosterol would be decreased in BPA exposed fish. This was accordance with what we found in this study.

IV. CONCLUSIONS

In sum, a metabonomics approach based on the GC-MS technique was carried out to investigate the biochemical response of BPA. The result of multivariate analysis on metabolomic data showed that even fish exposed to 0.5 mg/L BPA, the proportions of several metabolites would be altered, and these alterations were related with the physiological and histological changes we found in the exposure stage. The TDI of 50 µg/kg bw/day defined by US EPA and EC SCF as safe level of BPA would cause some potential risk to the development of zebrafish. The integration of metabonomics approach and conventional toxicology study may provide invaluable information in assessing the risk of environment pollutants.

REFERENCES


