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RESEARCH ARTICLE

NMR- and LC–MS/MS-based urine metabolomic investigation of the subacute effects of hexabromocyclododecane in mice

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Abstract In the present study, both untargeted and targeted metabolomics approaches were used to evaluate the subacute effects of hexabromocyclododecane (HBCD) on mice urine metabolome. Untargeted metabolomics based on ^{1}H NMR showed that HBCD exposure disturbed mice metabolism in both dosed groups, especially in high dosed group. The low-dose HBCD led to a decrease in alanine, malonic acid, and trimethylamine (TMA). High-dose HBCD-treated mice developed high levels of citric acid and 2-ketoglutarate, together with decreased alanine, acetate, formate, TMA, 3-hydroxybutyrate, and malonic acid. Targeted metabolomics for metabolic profiling of 20 amino acids identified alanine, lysine, and phenylalanine as significantly disturbed metabolites. These results indicated that subchronic exposure to HBCD caused a disturbance of mice metabolism, especially in TCA cycle, lipid metabolism, gut microbial metabolism, and homeostasis of amino acids, and the application of untargeted and targeted metabolomics combined with conventional toxicology approaches to evaluate the subacute effects of pollutants will provide more comprehensive information and aid in predicting health risk of these pollutants.

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Introduction

HBCD is a globally produced brominated flame retardant with an annual production of 23,000 t (Committee POPR 2011; de Wit 2002). It has been extensively used in building industry, upholstered furniture, textiles, and electronics, which makes the exposure to this compound ubiquitous. More importantly, HBCD has attracted considerable attentions mainly due to its characteristics of persistent organic pollutants, and it has been detected in almost all environmental media, including air, dust, sediments, soils, animals (Allchin and Morris 2003; Covaci et al. 2006; Johnson-Restrepo et al. 2008; Morris et al. 2004), and even in human tissues (Covaci et al. 2006; Shi et al. 2009). In spite of many strict regulatory legislations being enacted to reduce or ban the use of HBCD, the residues in the environment will still pose an enduring risk on human health (Committee POPR 2011).

From a toxicological perspective, HBCD was thought to be a weak toxicant based on its low acute toxic effect (Darnerud 2003). However, subacute and chronic animal studies highlighted its perturbation in hepatic metabolism (Cantón et al. 2008), the endocrine gland (van der Ven et al. 2009), reproduction (Saegusa et al. 2009), the central nerve (Eriksson et al. 2006; Lilienthal et al. 2009) and immunity (Koike et al. 2013). The thyroid was reported to be disrupted by HBCD in both in vivo and in vitro animal models (Schriks et al. 2007; van der Ven et al. 2006). The liver was another deeply influenced organ, with downregulated lipid metabolism-

related gene expression in female rat liver (Cantón et al. 2008). Though many studies based on conventional toxicology have been tried to understand the mechanisms underlying HBCD-induced subacute toxicity, it is not sufficient to provide a comprehensive information; systems toxicology approach such as metabolomics is considered as a powerful tool to further our understandings while complementing traditional approaches.

Metabolomics is defined as the quantitative measurement of the dynamic metabolic changes within a cell, tissue, or biofluid of an organism in response to external or internal stimuli (Nicholson et al. 1999). Metabolomics analyses can be categorized into nontargeted and targeted. Nontargeted metabolomics means an analysis of all metabolites by nonbiased methods in a biological sample. On the contrary, targeted metabolomics focuses only on several or a specific group of metabolites (Beckonert et al. 2007). Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are two major analytical platforms used in metabolomics. For NMR, owing to its wide range compatibility and nonselectivity, it has been extensively used for environmental metabolomics studies (Issaq et al. 2008). However, NMR is powerless for detection of trace levels of metabolites. Mass spectrometry, with much higher sensitivity, makes the exploration into trace metabolome possible. The datasets obtained from metabolomics studies contain a large number of variables; in order to simplify the analysis and maximize the identification of potential biomarkers, multivariate analysis approaches, such as principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are necessary tools (Holmes et al. 1998; Trygg et al. 2007).

In a previous study, a metabolomics approach has been used to evaluate a single oral HBCD exposure effects in infantile mice (Szabo et al. 2011). But to our knowledge, the subacute effect of HBCD in adult mice with longer exposure time has not been reported. Therefore, in the present study, we used a ^{1}H NMR-based untargeted metabolomics approach to determine the globally metabolic changes of mice exposed to HBCD for 28 subacute days. Furthermore, 20 amino acids were selected for quantitative analysis. Amino acids were kind of very important molecules, which not only played their roles as building blocks of proteins but are also necessary for growth, immunity, and reproduction (Wu 2009; Rhoads and Wu 2009). Many papers had demonstrated the effect of amino acids on nutrient metabolism, cellular signaling, immune function, reproduction, and lactation (Jobgen et al. 2006; Li et al. 2007, Rhoads and Wu 2009; Wu et al. 2008). Therefore, we then used a targeted metabolomics approach based on LC-MS/MS to quantitate the concentration of 20 amino acids in urine.

2 Materials and methods

2.1 Chemicals

Technical hexabromocyclododecane (95 % purity) was purchased from DELTACHEM (QINGDAO) CO. LTD with alpha (10 %); beta (10 %); gamma (80 %) three stereoisomers. L-Alanine, L-Arginine, L-Asparagine, L-Aspartate, L-Cysteine, L-Glutamate, L-Glutamine, L-Glycine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine were obtained from Aladdin. Algal amino acid mixture-^{13}C, ^{15}N (98 atom % ^{13}C, 98 atom % ^{15}N) used as internal standards were purchased from Sigma. Other solvents were purchased from commercial sources.

Animals and treatment

All animal experiments were performed in accordance with the current Chinese legislation and approved by the independent Animal Ethical committee at China Agricultural University. Eight-week-old female CD-1 mice were obtained from Vital River Laboratory Animal Company (Beijing, China). Females were selected for their more sensitive response after exposure to the commercial HBCD in previous toxicity studies (Cantón et al. 2008; van der Ven et al. 2006). During the experiment, animals were maintained at a controlled temperature (22 °C) and 12 h light/dark cycle. They were housed individually with free access to water and diet and allowed to acclimate for one week before experiment.

Fifteen mice were randomly divided into three groups, including one control group and two dosed groups, thus each group consisted of five mice. Mice were administrated by oral gavage for consecutive 28 days with 10 mg/kg and 50 mg/kg body weight (bw) in dosed groups. HBCD was dissolved in corn oil via primary solution in acetone, and then evaporated. The high dose of this study was derived from the usual range between 0.1 and 200 mg/kg bw for HBCD subacute toxicity research and slightly higher than the previous dose in infantile mice (30 mg/kg) to the level of 1/200 LD50 (Cantón et al. 2008; van der Ven et al. 2006). The control group was treated with an equivalent volume of corn oil. Individual urine samples of 24 h following the final dose of 28 days were collected by metabolic cages. Then, the supernatant liquors were separated by centrifuge at 12,000 rpm for 5 min and urine samples were stored at −20 °C for further NMR and LC-MS/MS analysis.

^{1}H NMR analysis

Two hundred microliters (200 μL) of phosphate buffer (PH = 7.4) was mixed with 400 μL urine to minimize variation

in urine PH, and the mixture was allowed to centrifuge at 12, 000 rpm for 5 min. Then, 50 μL TSP-d4/D_2O (29.02 mM) solution was added to the supernatant. The TSP acted as a chemical shift reference (δ0.0) and the D2O provided a lock signal. NMR spectrums of these samples were recorded on a BRUKER AVANCEIII600 spectrometer at 298 K. All 1D ^1H NMR spectrums were obtained using a 1D NOESY pulse sequence with the spectral width of 20 ppm. The water resonance was preferentially presaturated during relaxation delay. For each spectrum, a relaxation delay of 4 s, an acquisition time of 2.66 s, and a mixing time of 0.01 s was performed to collect 128 transient scans into 64 K data points. Spectrums were automatically Fourier transformed, using a line broadening of 0.3 Hz and zero filling to 128 k points.

Data reduction and multivariate statistical analysis

All ^1H NMR spectrums were baseline and phased corrected using TopSpin2.1 (Bruker Biospin, Germany), then spectrums were integrated between 0.3 and 10.0 ppm over a series of 0.04 ppm integral regions using AMIX (version 3.9.11). The δ6.1 − 4.7 region was excluded prior to statistical analysis due to the residue of water resonance. To account for dilution or bulk mass variations between urine samples, the integral regions were normalized to the total spectra area. Data sets were then imported into SIMCA package (Version 11, Umetrics, Sweden) for multivariate statistical analysis. PCA and PLS-DA were respectively performed to reveal the intrinsic clusters and model the treatment-related differences. The values of all NMR data were mean centered and pareto scaled prior to PCA or PLS-DA. Score plot based on NMR data was used to visualize the separation between the experimental and control group. Loading plots identified the spectral regions that contribute most to the separation of samples in the score plots. To validate the PLS-DA model, a sevenfold cross-validation was used to estimate the predictive ability (Q^2 parameter) of this model. VIP (variable importance in the projection), a statistical parameter ranking the variables according to their ability to discriminate different groups, was applied to discriminate special regions. Then, an ANOVA followed by post-hoc Fisher's LSD was used to determine which metabolite was significantly different between groups. Combined VIP and P value of ANOVA, metabolites with VIP values of greater than 1 and P values less than 0.05 were thought significantly distinct.

The metabolite resonances were identified according to information from HMDB (Human Metabolome Database) and MMCD (Madison Metabolomics Consortium Database). The biological pathway analysis was conducted based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

Determination of 20 amino acids

The quantitation of 20 amino acids was modified according to the previous described procedures (Kaspar et al. 2009). Briefly, 20 μL of the internal standard mixture was added to 50 μL of urine. Then, the sample was diluted to 200 μL with 0.1 % formic acid in water. Next, a derivation process was performed with propyl-chloroformate. To be specific, 80 μL reagentI (1-propanol/3-picoline 77/23) and 50 μL reagentII (chloroform/iso-octane/propyl-chloroformate 71.6/11/17.4) was added to 200 μL prepared urine sample, and the mixture was vortexed for 1 min. Two hundred fifty microliters of ethyl acetate was used to extract the derivatives. The supernatant was transferred to a new vial and was evaporated to dryness under a stream of nitrogen and redissolved in 200 μL 0.1 % formic acid in water.

An UltiMate 3000 systems coupled with TSQ Quantum Access Max mass spectrometer was used to determine the concentrations of 20 amino acids. The separation of 20 amino acids was performed on EZ:faast 4u AAA-MS (Phenomenex) column maintained at 25 °C. The mobile phase consisted of eluent A (acetonitrile with 0.1 % formic acid) and eluent B (water with 0.1 % formic acid). The elution program started at an initial mobile phase composition of 62 % A and 38 % B and then was ramped linearly to 79 % A and 21 % B in 12 min, then returned to 62 % A in 1 min. The final composition was used to equilibrate for 3 min. Injection volume was 20.0 μL and flow rate was 0.3 mL/min. Experiments were processed using the Xcalibur 2.2. ESI in positive mode and scheduled MRM were used (Table S2).

Statistical analysis

One-way analysis of variance (ANOVA) followed by post-hoc Fisher's LSD was used to determine the changes of body weight and amino acids between treatment groups and control group. A $P < 0.05$ was thought significantly different.

Results

Body weight

Figure S1 displayed the average body weights for control and dosed groups on days 1, 7, 14, 21, 28. No obvious toxic symptoms or changes in body weight and organ weight (data not shown) were observed after a 28-day oral exposure.

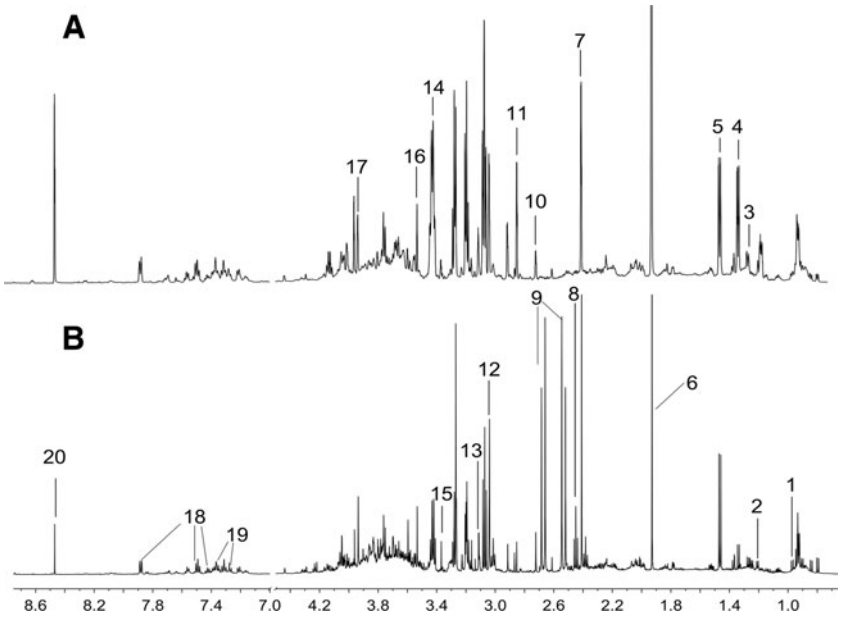

Fig. 1 Representative 600 MHz ^{1}H NMR spectrums of urine samples from control group (**a**) and high dosed group (**b**). The chemical shift assignments are given as follows. Key: 1 valine, 2 3-hydoxybutyrate 3 methylmalonate, 4 lactate, 5 alanine, 6 acetate, 7 succinate, 8 2-ketoglutarate, 9 citrate, 10 dimethylamine, 11 trimethylamine, 12 creatine, 13 malonic, 14 taurine, 15 scyllo-inositol, 16 glycine, 17 glycolate, 18 benzoic acid, 19 phenylalanine, and 20 formate. See also Table S1

^{1}H NMR spectroscopic and pattern recognition analysis

Representative 600 MHz ^{1}H NMR spectrums of urine samples from control group (A) and dosed group (B) were presented in Fig. 1. One mouse in the control group died due to improper operations. Therefore, four samples in the control groups and five samples in both treatment groups were used for further analysis. A trend for unsupervised separation between control and HBCD treatment samples was found in the PC1 vs PC2 scores plot of PCA (Fig. 2), suggesting that HBCD disturbed the mice urinary metabolome after 28 days of consecutive exposure.

The PCA allowed a primary unsupervised separation between groups. We then used a supervised PLS-DA model to augment the separation to further identify the differential metabolites that account for the separation between groups. For the three experiment groups, a comparison analysis generated a PLS-DA model with $R^2Y = 0.867$ and $Q^2 = 0.679$, displaying the faithful representation and excellent predictive capacity, respectively. A robust model was linked to a Q^2 value > 0.40 (McCombie et al. 2009). In fact, this was the case for this study. The score plot (left panel A) and loading plot (right panel B) of PLS-DA was shown in Fig. 3. The score plot of the PLS-DA based on entire urine datasets displayed a clear separation between the control group and the HBCD treatment groups, then loading plot was plotted to get the discriminate variables. On the loading plot, 45 variables were obtained with a VIP value > 1.0, and 47 buckets were statistically different by ANOVA. Combined the VIP and ANOVA results, eight discriminate metabolites were identified in the metabolites profiles according to HMDB and MMCD. Endogenous metabolite variations induced by HBCD exposure showed increased citrate (2.54 ppm, 2.66 pm), 2-ketoglutarate (2.46 ppm) compared with control samples, conversely, accompanied by the decreased alanine (1.46 ppm), acetate (1.94 ppm), formate (8.46 ppm), TMA (2.86 ppm), 3-hydroxybutyrate (1.18 ppm), and malonic acid (3.10 ppm). As shown in Table 1, the low dosed group associated with the decreased alanine, malonic acid, and trimethylamine. Mice treated with high dosed HBCD, under more severe influences, developed high levels of citric acid and 2-ketoglutarate, together with low levels of alanine, acetate, formate, trimethylamine, 3-hydroxybutyrate, and malonic acid.

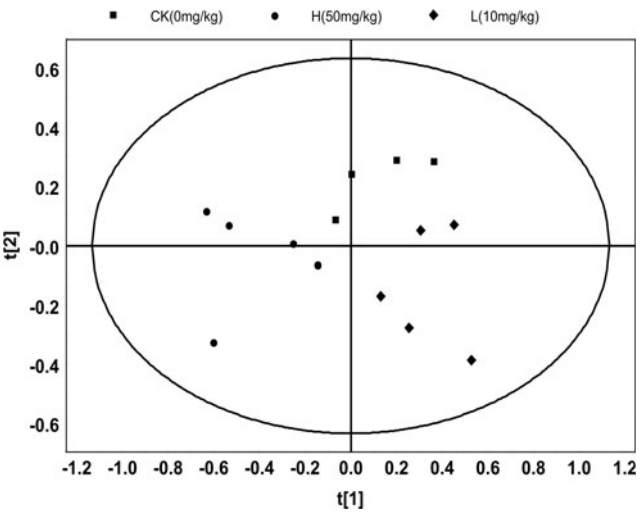

Fig. 2 PCA score plots along with the PC1 vs PC2 components based on the ^{1}H NMR spectra of the urine of mice orally treated with HBCD at doses of (**a**) 0 mg/kg (■), (**b**) 10 mg/kg (◆), and (**c**) 50 mg/kg (●)

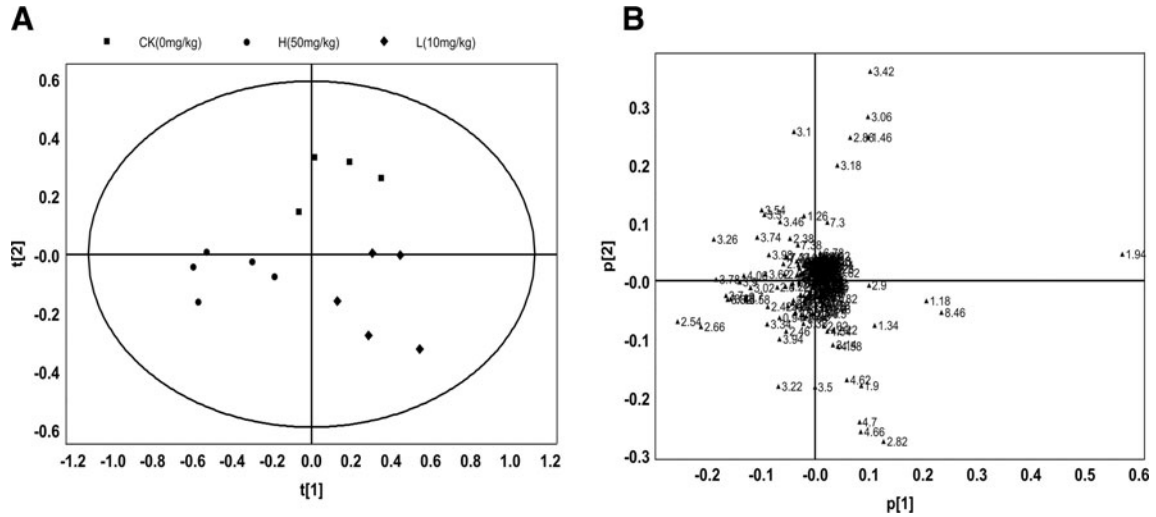

Fig. 3 PLS-DA score plots (**a**) and corresponding loading plots (**b**) of urine samples from mice orally treated with HBCD at doses of (a) 0 mg/kg (■), (b) 10 mg/kg (◆), and (c) 50 mg/kg (●)

Analysis of 20 amino acids

Each amino acid was quantitated by its respective isotope-labeled internal standard. The amino acids' lack of isotope-labeled counterparts used the adjacent labeled amino acids as their internal standards. The use of stable isotope-labeled AAs enabled us to correct for drift in instrument performance and for variation that arose downstream of sample preparations. All amino acids obtained good linearities with the coefficients of correlation over 0.99, and the levels of amino acids detected in urine samples were above LQDs. The representative chromatography of amino acids and corresponding labeled internal standards were showed in Fig. 4. The ANOVA analysis results showed that three amino acids, including alanine, lysine, and phenylalanine, were significantly altered by HBCD exposure (Fig. 5). Among those, Alanine was disturbed in high dosed group, whereas lysine and phenylalanine decreased in low dosed group. Most other amino acids have not been significantly disturbed after HBCD administration. The decreasing alanine demonstrated the result of NMR recognitions.

Discussion

In a previous study, a single oral gavage administration of HBCD altered the mice serum metabolome in infantile mice, which demonstrated the susceptibility of infantile mice to environment pollutants (Szabo et al. 2011). Results concluded the metabolic perturbations primarily in amino acid and energy metabolism, in

Table 1 Changes in endogenous urine metabolites in mice treated with a low or high dose of HBCD

Metabolites	HMDB ID	Chemical shift (ppm)	ANOVA analysis *P* value	Exposure groups	
				Low (10 mg/kg)	High (50 mg/kg)
Acetic acid	HMDB00042	1.94 (s)	5.99E-04	–	↓
Alanine	HMDB00161	1.46 (d)	7.82E-03	↓	↓
2-ketoglutarate	HMDB00208	2.46 (t)	3.78E-02	–	↑
Citric acid	HMDB00094	2.54 (d),2.66 (d)	1.47E-03	–	↑
Formate	HMDB00142	8.46 (s)	1.12E-02	–	↓
Malonic acid	HMDB00691	3.10 (s)	1.32E-02	↓	–
3-Hydroxybutyrate	HMDB00357	1.18 (d)	3.81E-03	–	↓
Trimethylamine	HMDB00906	2.86 (s)	6.64E-03	↓	↓

The integral values for each segmented region of chemical shift were represented for each metabolite resonance. Significant changes ($P < 0.05$) are relative to control groups

– no changes, ↓ decrease, ↑ increase, *s*, single, *d*, doublet, *t* triplet

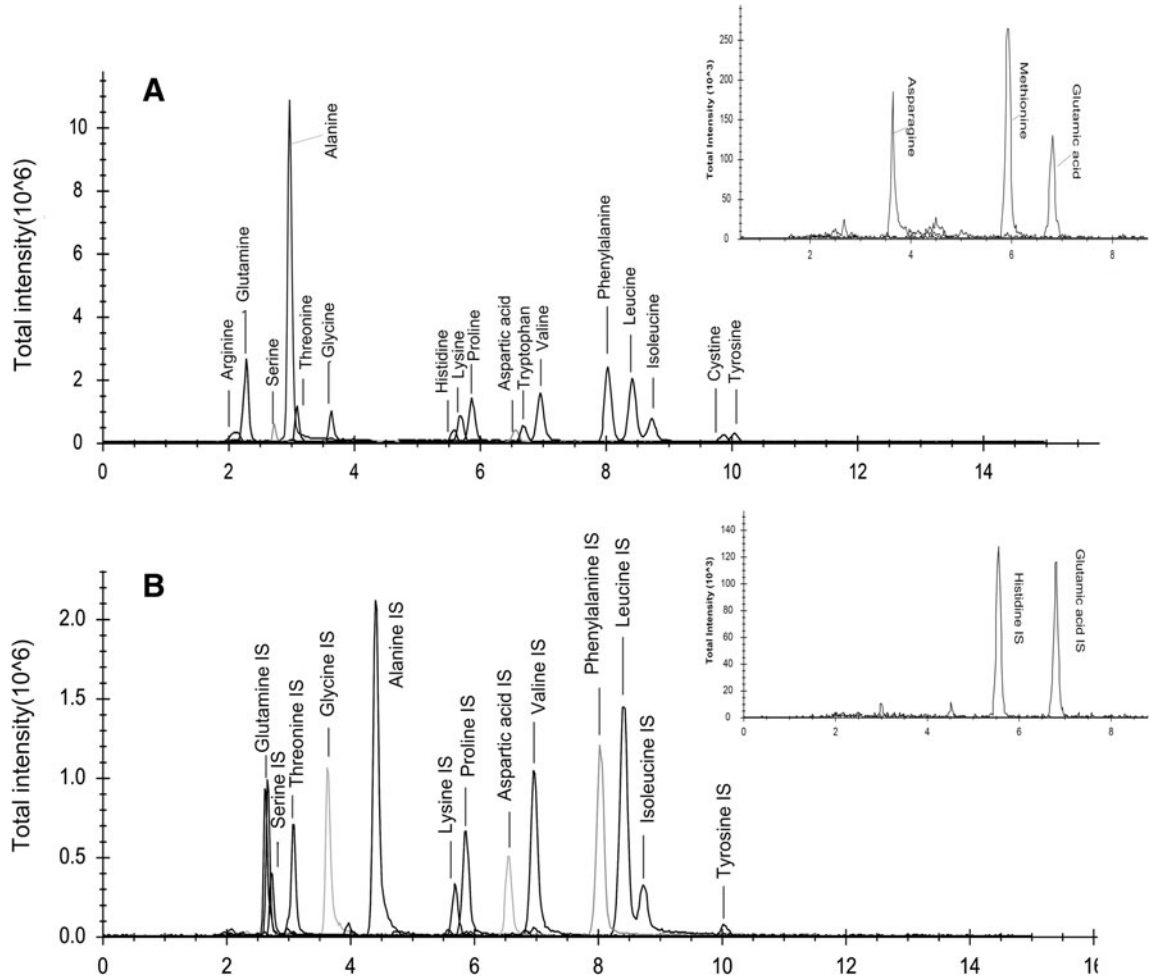

Fig. 4 Representative UPLC-MS/MS MRM chromatography of 20 amino acids (**a**) and corresponding internal standards (**b**). The internal standards were expressed as name of amino acids-IS, for example Alanine-IS. The 20 amino acids were separated with a ramp linearity procedure in 15 min

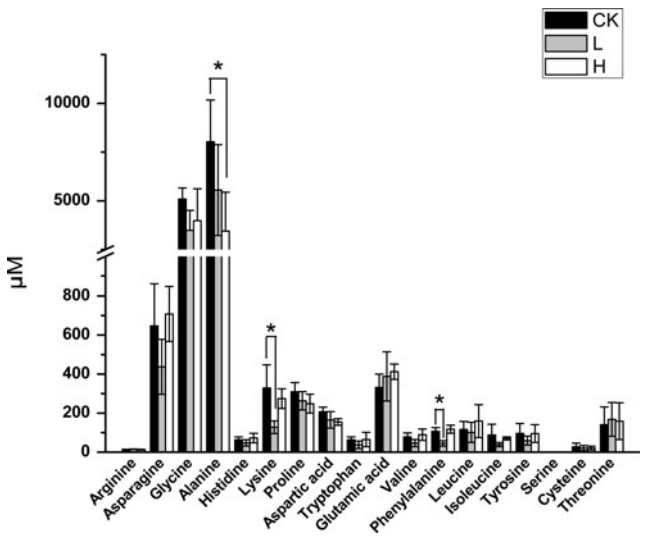

Fig. 5 Quantification of 20 amino acids by UPLC-MS/MS in control group and both dose groups (* $P < 0.05$, ** $P < 0.01$ compared to control group)

accordance with the present results. In the present study, adult mice with longer exposure time of 28 days is enacted to evaluate the subacute effects of HBCD in mice. Multivariate statistical analysis identified the alteration of many metabolites, and these metabolites were linked with many important metabolic process (Fig. 6).

The increased metabolites citrate and 2-ketoglutarate were important intermediates associated with TCA cycle; the elevated intensity of these two metabolites indicated that the activity of mitochondrial enzymes related to the TCA cycle may be activated by HBCD treatment. Acetate and formate were related to pyruvate metabolism. Alanine was a nonessential amino acid, but it played a key role in glucose–alanine cycle between tissues and liver, through this cycle, glucose was regenerated from pyruvate. The decreased alanine, acetate, and formate may display the reduced level of gluconeogenesis and pyruvate metabolism, therefore more glucose was metabolized by the flux of glycolysis and TCA cycle, leading to the excessive citrate and 2-ketoglutarate in high dosed group. 3-Hydroxybutyrate

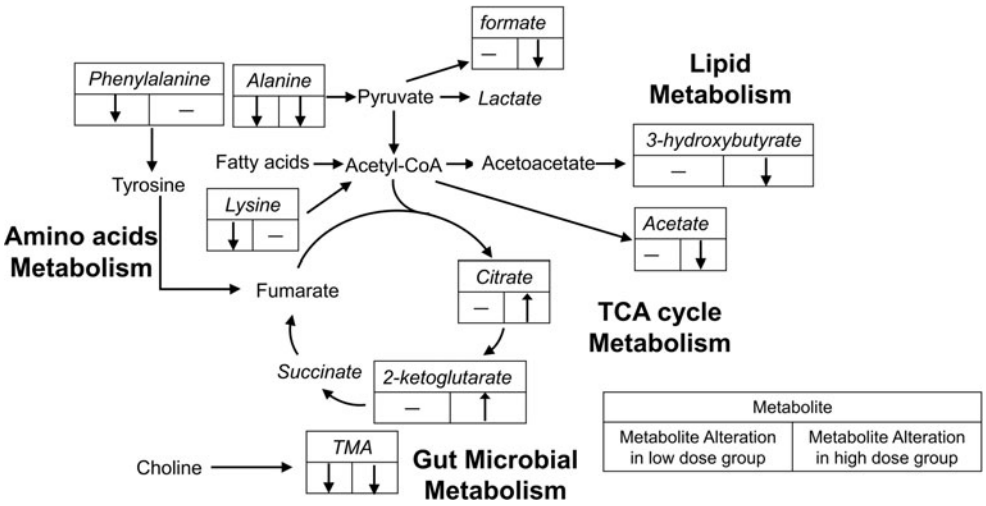

Fig. 6 Disturbance of metabolic pathway after HBCD treatment. The metabolites in italic were detected in this study. *Upward arrow* means significantly higher compared with control group;*downward arrow* means significantly lower compared with control group

was indicative of enhanced β-oxidation (Lei et al. 2008), the significantly reduced 3-hydroxybutyrate was an indicator of suppression of lipid metabolism, which was in accordance with the previous reported downregulated lipid metabolism at the messenger RNA (mRNA) level (Cantón et al. 2008). Phenylalanine was another significantly different metabolite, it was an essential amino acid, which cannot be synthesized de novo (from scratch) by the organism being considered, and therefore must be supplied in its diet. HBCD exposure may influence the absorption or ultilization of this essential amino acid, resulting in the decreased phenylalanine. Moreover, phenylalanine and its product tyrosine were substrates for productions of some neurotransmitters and hormones. The changes of trimethylamine (TMA) also provide evidence for the disruption of gut microbial homeostasis. TMA was the first metabolite of choline from the microorganisms, and the accumulation of TMA was reported to cause hepatic toxicity of mammals (Dumas et al. 2006). Overall results pointed out probable damages in the biochemical pathways especially in the TCA cycle, lipid metabolism, gut microbial metabolism, and homeostasis of amino acids (Fig. 6).

Of the disturbed metabolic pathways, amino acids have been selected for quantity analysis in this study. Three of the 20 amino acids has shown been significantly different between control and treated groups. Many environmental pollutants have also been reported to disturb the homeostasis of amino acid in metabolomics. For example, melamine, a synthetic nitrogenous product, was demonstrated to have an effect on the level of alanine, valine, leucine, and isoleucine (Sun et al. 2012). Diseases have also been linked to the amino acid in metabolomics. A chronic unpredictable mild stress model of depression had found the changes of valine and glutamine (Zheng et al. 2011). More importantly, some amino acids have been used as biomarkers to diagnose and predict diseases. Abnormal tryptophan catabolism has been linked to acute GVHD after human allogeneic stem cell transplantation (Landfried et al. 2011). Therefore, metabolic profiling of 20 amino acids can provide us with valuable and comprehensive information.

The doses used in this study is much higher than that in the environment, so the lower concentration close to the actual environmental concentration must be studied in further to explore the possible effect of HBCD on human beings and animals. Compared to the previous HBCD serum metabolomics, studies about each stereoisomer has been put on agenda to elaborate their different mechanisms.

Conclusions

In the present study, we used both targeted and nontargeted metabolomics approaches to evaluate the subacute effects of HBCD on mice after a 28-day oral gavage administration. Results showed that metabolic profiles of mice urine were significantly altered in both treatment groups compared to the control group. ^{1}H NMR based untargeted method demonstrated its interruption mainly in TCA cycle, lipid metabolism, gut microbial metabolism, and homeostasis of amino acids. LC-MS/MS-based targeted approach identified alanine, lysine, and phenylalanine as markers of 20 amino acids. Combined untargeted metabolic profiling with targeted metabolomics methods will provide more valuable and robust results for risk assessment of environmental pollutants.

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Compliance with ethical standards

Conflict of interest The authors report no declarations of interest.

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