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Hippocampal metabolomics reveals 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity associated with ageing in Sprague-Dawley rats

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ABSTRACT

Metabolomics, the exponentially developing technique, could provide a systemic mapping in toxicology by directly measuring small molecular metabolites. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was found to be neurotoxic in mammalian animals. In this study, we employed liquid chromatography/quadrupole time-of-flight mass spectrometry for non-targeted analysis of metabolic profiling in hippocampal sample sets of the rats exposed to TCDD. Hippocampal metabolome from different ages of the healthy rats (4-week, 12-week and 20-week) was also deciphered. The relationship between the two tested cases was unlocked to delineate TCDD toxicity associated with ageing. Tandem mass spectrometry fragmentation in conjunction with metabolic database searching and compared to authentic standards was utilized for metabolite identification. As a consequence, the reduced levels of phenylalanine and leucine/isoleucine as well as the up-regulation of inosine and hypoxanthine were highlighted for understanding of TCDD toxicity related to age in rats and the trajectory was depicted by principal components analysis.

1. Introduction

The environmental dispersion and subsequent accumulation in the food chain seem to be the major pathways of the general population exposed to polychlorinated dibenzo-*p*-dioxins (PCDDs). PCDDs, commonly known as dioxins, have high lipophilicity [1]. Therefore, these chemicals, exemplified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are readily bioconcentrated in animal fat depots [2]. Exposure of humans to high levels of dioxins may be linked to impairment of the immune system, the developing nervous system, the endocrine system and reproductive functions, and several types of cancer [3,4]. Of particular interest is the neurotoxicity induced by the potent toxicant TCDD that alters normal brain development and produces cognitive disability and motor dysfunction. For example, lipid peroxidation, DNA damage, elevated intracellular calcium levels and tau phosphorylation were caused by TCDD in a neuroblastoma cell line [5]. In another cell line, the apoptotic process in neural growth factor-differentiated PC12 cells induced by TCDD was also addressed [6]. It was found that oxidative stress could be elicited by TCDD exposure in organisms or cells. TCDD exhibited its extreme toxicity via aryl hydrocarbon receptor (AhR) and produced mitochondrial reactive oxygen which was regulated by glutathione redox state. Concomitantly, mitochondrial DNA damage occurred [7,8]. Moreover, the concentrations of the environmental toxicants associated with neurotoxicity resulting in a spectrum of adverse outcomes were also investigated, which changed over time to reflect the industry efforts and lifestyle patterns [9].

TCDD, the notorious toxicant, poses its toxic threat on human or animal health upon AhR activation [10]. The metabolic signatures were manifested with the different responses to TCDD in AhR-high affinity C57BL/6J mice and low affinity DBA/2J mice [11,12]. Metabolomics has been demonstrated to move from the information upon analytical platforms to knowledge. The metabolite changes in concentration could reveal the disturbed metabolic pathways in the different tested groups and indicate the underlying mechanisms induced by genetic modifications and/or environmental influences. For instance, small molecular metabolites and trace elements in rat amniotic fluid were measured to broaden the understanding of biological variations associated with fetal neurodevelopment induced by environmental perturbation [13].

The aims of the current work were to apply metabolomics to measure the metabolic signatures and investigate the possible relationship between TCDD toxicity and ageing of the healthy rats. Liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/QTOFMS) was used to analyze the small molecular metabolites in the hippocampus tissues of rat brains by using non-targeted approach. Principal components analysis (PCA) was

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performed to describe the trajectory for understanding TCDD toxicity development related to age.

2. Materials and methods

2.1. Chemicals

Authentic standards of inosine, hypoxanthine, L-phenylalanine, L-leucine and L-isoleucine were purchased from Sigma–Aldrich (St. Louis, MO, USA). TCDD (purity > 99%) was obtained from AccuStandard Inc. (New Haven, CT, USA). HPLC grade acetonitrile was obtained from Tedia (Fairfield, OH, USA) and pure water was prepared from a Milli-Q Ultrapure water system (Millipore, Billerica, MA, USA). Other reagents were of analytical grade.

2.2. Animal experiments

Male Sprague-Dawley (SD) rats were purchased from Chinese University of Hong Kong, Hong Kong SAR, China. All animal experiments were conducted according to the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals. Rats were acclimatized for two weeks in the animal room. The rats were separated into 2 categories, one for the TCDD-associated experiment and the other for the investigation on ageing comparison. For the later category, different ages of rats living under normal conditions ($n = 8$ for 4-week, $n = 8$ for 12-week and $n = 9$ for 20-week) were used for the collection and analysis of hippocampus tissues. For the category of TCDD experiment, the rats were treated with either vehicle or TCDD dissolved in corn oil consecutive six days by oral administration of 20 $\mu g\,kg^{-1}$ body weight each day for high-dose group or 2 $\mu g\,kg^{-1}$ body weight each day for low-dose group ($n = 9$ for each group). Body weight was examined at pre-dose (day 0), day 1, day 3, day 6 and day 10 afterward administration. Rats in the TCDD toxicity investigation were sacrificed at 14-week of the ages and the hippocampus tissues in the brains from the rats were harvested and stored at −80 °C.

2.3. Hippocampal sample preparation for LC/MS analysis

The tissue sample of each hippocampus was weighed (~20 mg wet), homogenized with 800 μL cold methanol and stored at −20 °C for 1 h. Then the samples were centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant was dried under the gentle nitrogen stream till dryness at 37 °C, re-dissolved with 200 μL solvent mixture (acetonitrile/water = 10:90, v/v) and stored at −20 °C for 1 h, then clarified at 14,000 × *g* for 10 min at 4 °C. The supernatant was collected and stored at −20 °C prior to LC/MS analysis.

2.4. LC/QTOFMS analysis

Chromatographic separation was performed on Agilent 1100 HPLC system equipped with G1389A autosampler (Agilent Technologies, Palo Alto, CA, USA) by using a XTerra MS C_8 column (2.1 mm × 100 mm, 3.5 μm) (Milford, MA, USA). Aliquot of 8-μL of each sample was injected onto the HPLC column and eluted with solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) with a linear gradient program at a flow rate of 0.2 mL min^{-1} in positive ion mode. For negative ion mode data, 5 mM ammonium acetate was substituted for solvent A and solvent B as the mobile phase. The initial condition was 5% B, held for 5 min, increased to 95% B for 20 min, held at 95% B for 5 min, and a re-equilibration at 5% B for 10 min.

ESI-MS analysis was conducted on a QTOF mass spectrometer (API QStar Pulsar i, MDS Sciex, Toronto, Canada) with a TurboIonspray source. The MS parameters for positive ion mode were optimized as follows: ionspray voltage (IS) 5400 V, declustering potential I (DPI) 55 V, declustering potential II (DPII) 15 V, focusing potential (FP) 120 V. The mass range was set from m/z 100 to 1000. The ion source gas I (GSI), gas II (GSII), curtain gas (CUR) and collision gas (CAD) were set at 30, 15, 25 and 3, respectively. The temperature of GSII was set at 350 °C. For negative ion mode data, IS −4100 V, DPI −70 V, DPII −15 V, FP −160 V, other parameters were same to those in positive ion mode. Data acquisition and processing was performed on a personal computer with Analyst QS software.

2.5. Histopathological examination

Six rats treated with vehicle and high-dose TCDD ($n = 3$ for each group) were anaesthetized for heart perfusion. The liver and kidney were dissected free of fat and adjacent tissue, weighed, and dehydrated with alcohol and xylene for the tissue lesion. Representative samples for histology were embedded in paraffin wax, cut into 4-μm sections, stained with hematoxylin-eosin (HE), and examined by light microscopy.

2.6. Data analysis

The LC/MS data in instrument-specific format were converted to CDF format files. The program XCMS was implemented in R language for nonlinear retention time alignment, automatic integration and extraction of the peak intensities. The output tables were imported into MATLAB software R2009b (The MathWorks Inc.), where normalization was performed prior to multivariate data analysis. PCA was performed in MATLAB programming environment. The *P*-values of One-Way ANOVA were recalculated using Bonferroni correction by OriginPro software version 8.0 (OriginLab Co., MA).

3. Results and discussion

3.1. General information of TCDD toxicity in rats

The change of rat body weight over time after TCDD administration is shown in Fig. 1a. Interestingly, it was found that TCDD could cause the rats to lose body weight after exposed to either the low-dose or high-dose compared to the control group. The diet consumption at the 7th, 8th and 9th day after the first dosage of TCDD indicated that the food uptake might be affected by TCDD exposure depending on the dosage (Fig. 1b). By taking the histopathological examination into account, the pronounced abnormality in liver and kidney exposed to high-dose TCDD was observed compared to control group (Supplementary data, Fig. S1), indicating that swelling and severe hepatocellular vacuolation, cell necrosis and inflammation in the liver, and the vacuolated cells and necrosis within the renal tubular in the kidney. Collectively, TCDD administration could cause acute toxicity in various tissues of SD rats and might induce anorexia and wasting syndrome. The underlying mechanism might be associated with the expressions of the orexigenic and anorexigenic neuropeptides in the hypothalamic area [14].

3.2. Feature selection and metabolite identification

LC/QTOFMS analysis in both positive and negative ion modes was conducted for investigating metabolite profiles from the sample sets. XCMS program [15] was implemented for peak extraction, alignment and matching. When finishing the program performance, the extracted ion chromatograms were yielded for all ions including the fragment ions. The features (ions) sorted with *P*-values and fold-changes in the resulting delimited table and feature selection was performed based on the criteria ($P \leq 0.01$ and fold-change ≥ 1.5). It should be noted that we attempted to establish the possible patterns of relationship between TCDD toxicity and

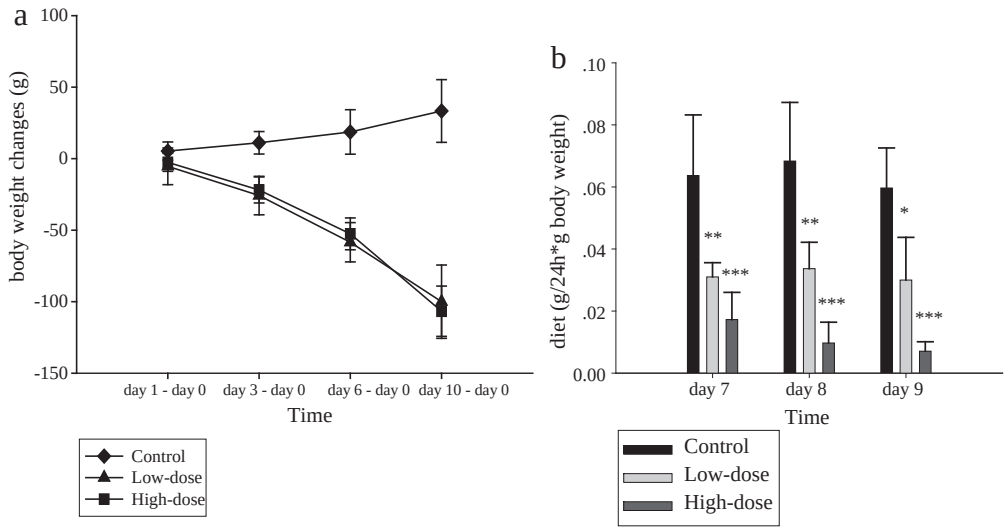

Fig. 1. (a) Graph of the body weight changes in TCDD investigation groups over time (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, significant difference with the control group; each time point presents mean ± S.D. and $n = 9$ per group). (b) Graph of the diet consumption for the TCDD investigation groups from day 7 to day 9 (each time point presents mean ± S.D. and $n = 5$ per group).

ageing. Therefore, only the high-dose TCDD rats compared to control group were utilized to perform non-targeted metabolomic analysis as well as HE histopathological examination. As a result, more than 200 features in each table for either age comparison (4-week, 12-week and 20-week) or TCDD exposure comparison (vehicle and high-dose TCDD exposure group) in positive or negative ion mode were acquired by LC/QTOFMS measurement. In that case, each given molecule may be represented by several different features, such as the naturally occurring components of its isotopic cluster and fragment ions in the resulting delimited table [16]. This would be helpful in metabolite identification that was considered to be an important task in any non-targeted analysis. As the routine approach, high resolution MS data, MS/MS spectrum, database searching and authentic standard comparison were performed. For example, the ions at *m/z* 103.054, *m/z* 120.081 and *m/z* 166.086 in TCDD comparison from positive ion mode LC/MS analysis displayed the similar fold-changes (*P*-values) as 2.31 (0.0053), 2.64 (0.0074) and 2.47 (0.0030), respectively. The retention times of the three ions were observed at 270 s (Fig. 2a-1–a-3), postulating that the molecule might be phenylalanine from the MS spectrum in Fig. 2b and searching against the METLIN database (http://metlin.scripps.edu). The identification was confirmed further from the MS/MS analysis and that of authentic standard. Another example of metabolite identification was asso-

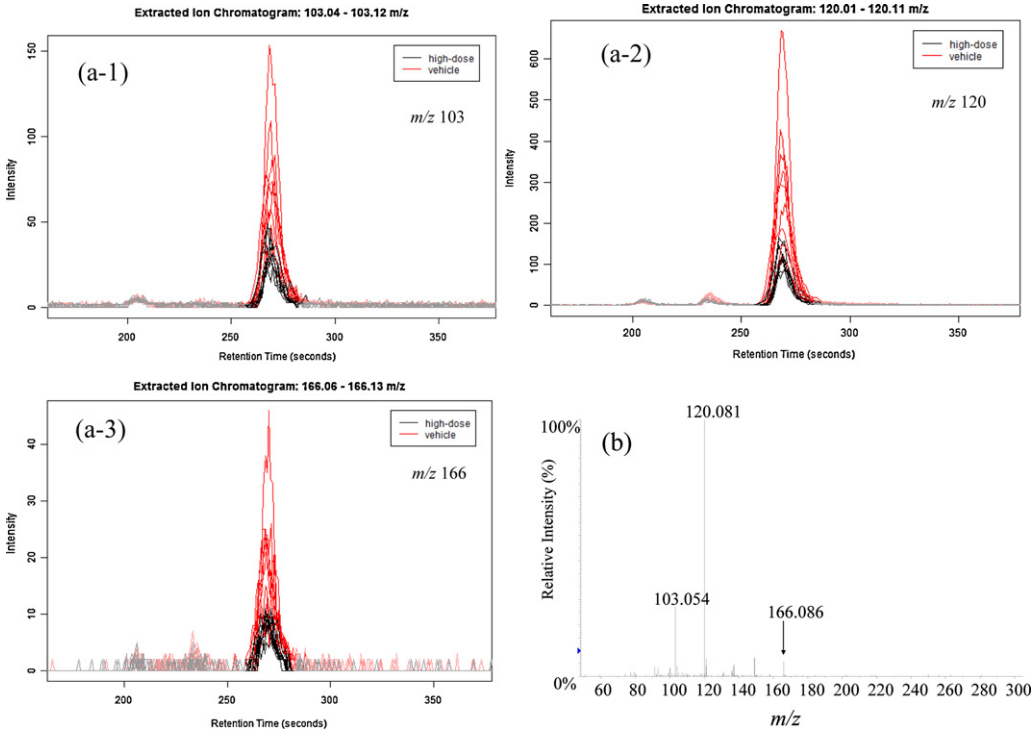

Fig. 2. Typical extracted ion chromatograms from XCMS program in R: (a-1) *m/z* 103, (a-2) *m/z* 120 and (a-3) *m/z* 166. (b) The MS spectrum of the peaks at retention time 270 s in the total ion chromatogram.

ciated with the ions at *m*/*z* 132.102 and *m*/*z* 86.098 observed in LC/MS analysis with ESI in positive ion mode. The identification was again compared and confirmed with the MS/MS analysis of the authentic standard L-leucine. It should be noted that L-leucine and L-isoleucine could not be differentiated under the current separation conditions. Thus, the compound in the sample sets was tentatively identified to be leucine (or isoleucine). Similarly, inosine and hypoxanthine were measured by MS/MS fragmentation in negative ion mode for identification. In summary, inosine, hypoxanthine, phenylalanine and leucine/isoleucine were highlighted in hippocampus tissues of SD rats exposed to TCDD for linking to ageing of healthy rats.

3.3. PCA trajectory for TCDD toxicity associated with ageing in rats

PCA is an unsupervised technique for data grouping which is useful for data mining and mapping the patterns of relationship. PCA analysis of the quality control (QC) sample could be performed for evaluation of the metabolic profiles generated from the LC/MS chromatograms. The QC sample was obtained by mixing all samples from the vehicle and high-dose groups. Each 10 samples was one batch, and total 6 batches were involved into the consideration. The raw data were imported for PCA analysis (Supplementary data, Fig. S2). The grouping was still revealed from batch to batch in QC samples, suggesting the instrumental drift occurred during the sample injections. Thus, samples were run in an order that alternated between the different tested groups and each sample was analyzed in duplicate to reduce error associated with instrumental condition variation. On the other hand, the grouping was performed again after data integration including the vehicle and high-dose TCDD-exposed groups. Then the 6 QC batches were observed to be overlaid together (Supplementary data, Fig. S3), indicating that the column separation and signal acquisition were stable to meet the semi-quantification of the metabolic profiling. The sample clusters could be revealed if the drastic artifacts occurred during the experimental procedure [17]. And the QC samples tightly clustered, suggesting carryover did not occur.

After metabolite identification, peak area of each selected compound was extracted and normalized by the weight of its corresponding tissue. Data from the two groups of TCDD experiment category (vehicle and high-dose) as well as the three groups of the category under normal conditions with different ages (4-week, 12-week and 20-week) were imported into computation for pattern recognition. Consequently, PC1 accounts for 73.29% of the total variances in the PCA model, and PC2 accounts for 25.11%. The ageing trajectory was depicted in the PCA mapping (Fig. 3a). The discrimination of 4-week, 12-week and vehicle groups was not clear, demonstrating insignificant change referred to the ages below 14 weeks. Interestingly, the high-dose rats with TCDD (14 weeks old) were displayed toward the 20-week-old group compared to the vehicle group. The shift along with the arrow in Fig. 3a exhibited the

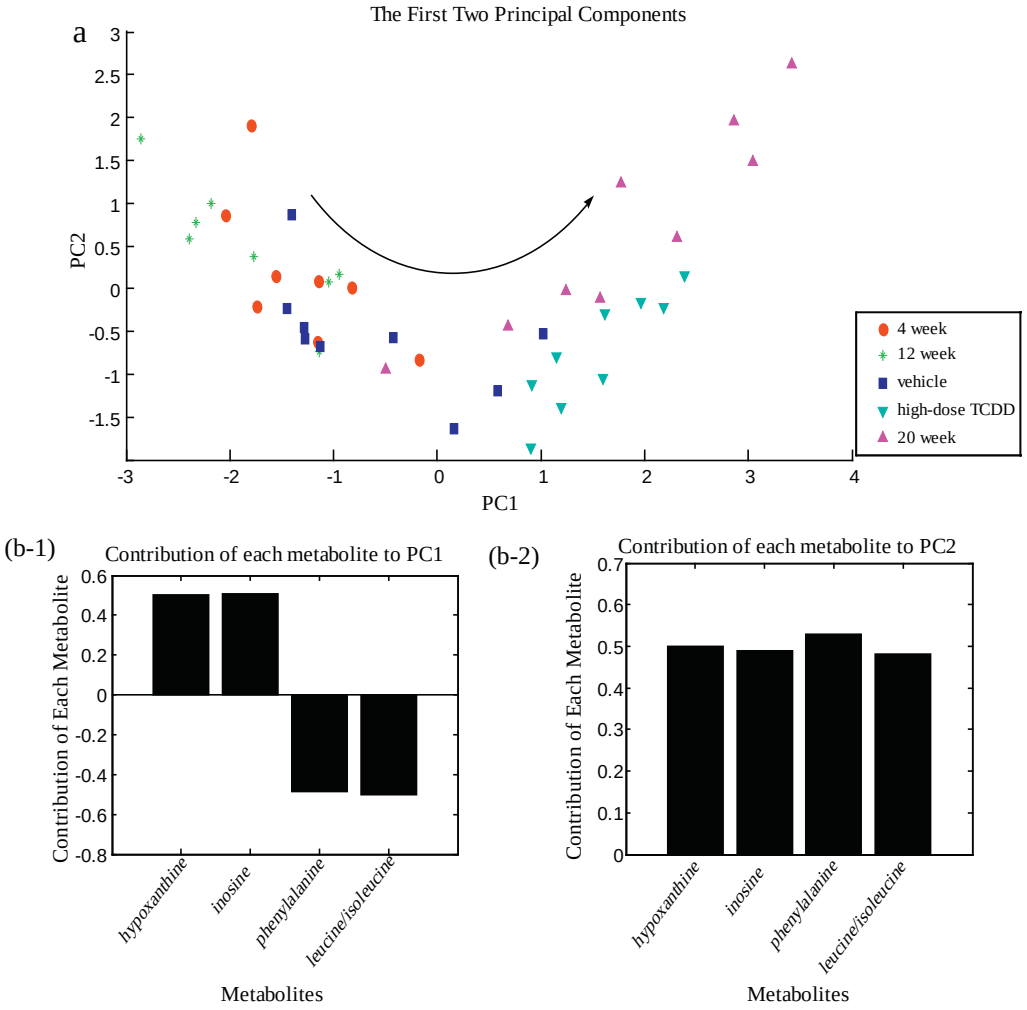

Fig. 3. PCA pattern recognition for the grouping of the five groups: (a) 4-week, 12-week, 20-week, vehicle group and high-dose TCDD. (b-1) The contribution of each metabolite (column) in PC1 and (b-2) the contribution of each metabolite (column) in PC2.

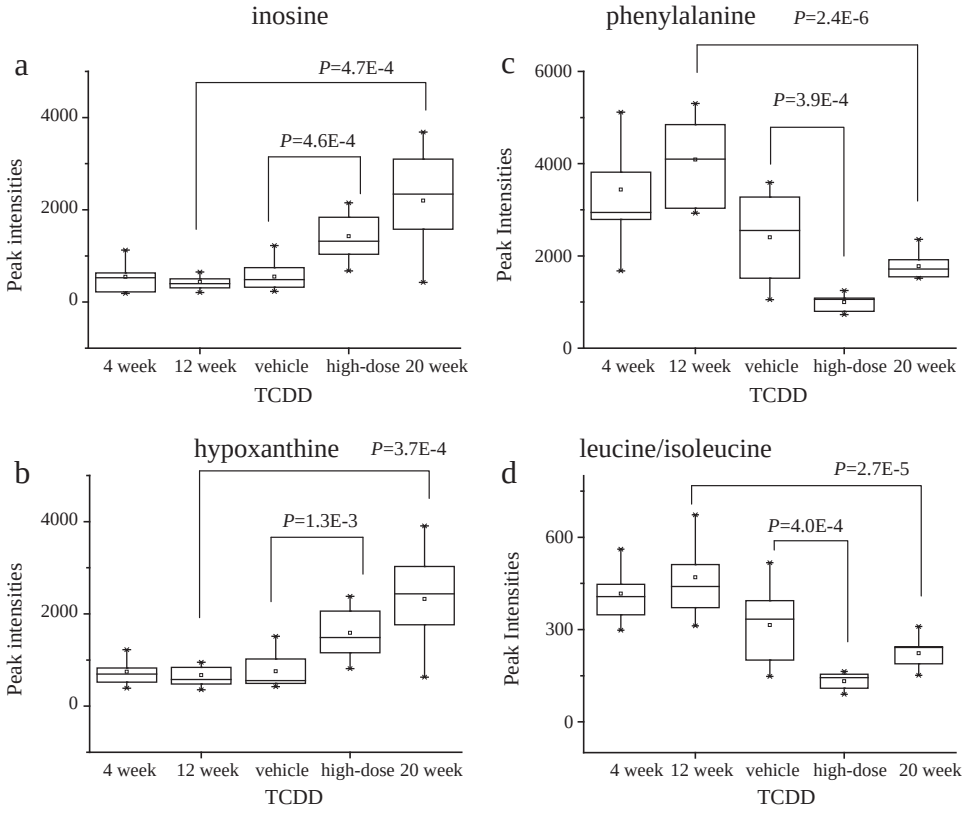

Fig. 4. Box plots calculated for inosine (a), hypoxanthine (b), phenylalanine (c) and leucine/isoleucine (d) in the five groups.

ageing trajectory for the TCDD-dosed rats. The accelerated ageing of the dosed rats occurred with the TCDD uptake because the real ages of both vehicle and high-dose TCDD exposure were both 14 weeks. To unveil the contribution of each metabolite in PCA model, Fig. 3b-1 and b-2 manifested the roles for PC1 and PC2, respectively. The concentrations of hypoxanthine and inosine were elevated by both factors, i.e., the TCDD exposure and normal ageing (positive contribution to PC1), while the concentrations of phenylalanine and leucine/isoleucine declined (negative contribution to PC1). On the other hand, the second principal component might characterize the individual variations. The four metabolites were basically positive contribution to PC2, suggesting that hypoxanthine, inosine, phenylalanine and leucine/isoleucine had similar effects on sample variation for distribution.

3.4. Metabolic annotation for the relationship between TCDD toxicity and ageing

To delineate the metabolic changes in hippocampus tissues, these four differentiating metabolites were shown in the box plots (Fig. 4). It was evaluated and recognized that the toxic effect of TCDD exposure could accelerate the male SD rats ageing in comparison with the normal ageing. Each metabolite exhibited the marked changes in the brain of rats induced by normal ageing or TCDD uptake, although the difference between 4-week and 12-week was not significant. For example, *P*-values were calculated to be 0.00047 and 0.00046 in the comparisons of 20-week versus 12-week and high-dose TCDD versus control group for inosine, respectively. Nevertheless, the enhanced levels of hypoxanthine and inosine as well as the disturbed amino acid metabolism might link to the neural development [18]. Meanwhile, amino acids could undergo the catabolism in the hippocampus of rats introduced by either the treatment of TCDD or senescence. Leucine (or isoleucine) represents the branched-chain α-amino acid whereas phenylalanine is a precursor of the neurotransmitters called catecholamines highly concentrated in the brain. Interestingly, similar phenomenon was also observed in our recent report on the metabolic traits in brain tissues related to high-fructose feeding in rats [19]. The perturbation of amino acids and hydroxy purine/nucleoside in the hippocampus might be associated with the imbalance energy metabolism [20]. To some extent, such metabolic traits were pinpointed to TCDD toxicity or ageing rather than a specific disease.

In conclusion, the notorious TCDD was administrated to male SD rats and caused the body weight loss, anorexia and the dysfunction of liver and kidney tissues. LC/QTOFMS-based metabolomic analysis was applied to uncover the relationship between TCDD exposure and ageing. Inosine, hypoxanthine, phenylalanine and leucine/isoleucine were highlighted to delineate the metabolic traits. PCA and box plots were performed for the improving visualization of the grouping. The obtained results underlined that metabolic signatures could reveal TCDD toxicity associated with ageing in rats. Again, metabolomics analysis broadens the understanding of the biochemical changes in neurotoxicity induced by TCDD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.05.007.

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