Metabolomics analysis of urine from rats administered with long-term, low-dose acrylamide by ultra-performance liquid chromatography-mass spectrometry

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Abstract

1. To study the toxic effect of chronic exposure to acrylamide (AA) at low-dose levels, we applied metabolomics approach based on ultra-performance liquid chromatography/mass spectrometry (UPLC–MS). A total of 40 male Wistar rats were randomly assigned to different groups: control, low-dose AA (0.2 mg/kg.bw), middle-dose AA (1 mg/kg.bw) and high-dose AA (5 mg/kg.bw). The rats continuously received AA via drinking water for 16 weeks. Rat urine samples were collected at different time points for measurement of metabolomic profiles.

2. Thirteen metabolites, including the biomarkers of AA exposure (AAMA, GAMA and iso-GAMA), were identified from the metabolomic profiles of rat urine. Compared with the control group, the treated groups showed significantly increased intensities of GAMA, AAMA, iso-GAMA, vinylacetylglycine, 1-salicylate glucuronide, PE (20:1(11Z)/14:0), cysteic acid, L-cysteine, p-cresol sulfate and 7-ketodeoxycholic acid, as well as decreased intensities of 3-acetamidobutanal, 2-indolecarboxylic acid and kynurenic acid in rat urine. Notably, three new candidate biomarkers (p-cresol sulfate, 7-ketodeoxycholic acid and 1-salicylate glucuronide) in rat urine exposed to AA have been found in this study.

3. The results indicate exposure to AA disrupts the metabolism of lipids and amino acids, induces oxidative stress.

Keywords
Acrylamide, low-dose chronic exposure, metabolomics, NOAEL, rat urine

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Introduction

For decades, acrylamide (AA) has been used in various industries, such as water purification, sewage treatment, paper production, cosmetics and fixing agents. For a long period, AA was considered only for occupational poisoning (Exon, 2006). However, in April 2002, the Swedish National Food Administration and researchers from Stockholm University informed that AA is found in baked or fried carbohydrate-rich food, particularly in plant-based products such as potato chips, French fries, bread, coffee and others (Tareke et al., 2000, 2002). In our daily life, exposure can originate from a variety of sources, and the primary route of AA exposure for nonprofessional people is through food and drinking water. This exposure is characterized by long-term, low-dose oral intake. Thus, the adverse effects of AA exposure on human health have raised concerns.

AA is known as a toxicant in humans and rodents (Pedreschi et al., 2014). In addition, the compound is well-documented to be a neurotoxicant in occupationally exposed human populations and laboratory animals that produce central and peripheral distal axonopathies (LoPachin, 2004; Spencer & Schaumburg, 1974; Spencer et al., 1975). To date, AA disrupts membrane fusion processes that mediate neurotransmission and membrane turnover in nerve terminals (LoPachin, 2005). In addition, genotoxicity (Besaratinia & Pfeifer, 2004; Dearfield et al., 1988), reproductive toxicity (Dearfield et al., 1988; Tyl & Friedman, 2003) and carcinogenicity (Rice, 2005) have been extensively investigated. Although the toxicity of AA described above has been well studied, the toxicity mechanism of AA has not been completely elucidated. Therefore, a new approach for studying the toxicity effects and mechanism of low-level, long-term AA exposure is necessary.

Metabolomics has been defined by Nicholson as the “quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification.” Metabolomics involves the detection, identification, quantification, and cataloging of the history of time-related metabolic changes in an integrated
biological system rather than the individual cell (Nicholson et al., 1999). With this approach, the biochemical changes in easily accessible biological fluids, such as blood or urine, are detected as biomarkers for toxicity-related pathogenesis. Three commonly applied techniques in metabolomics investigations are nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). Owing to its high-chromatographic resolution, high sensitivity and rapid separation, ultra-performance liquid chromatography (UPLC) coupled with mass spectrometry (MS) has been widely used in metabolic alterations in complex mixtures (Bansal et al., 2016; Du et al., 2013). The approach of metabolomics is currently recognized as an independent and widely applied technique through biomarkers and evaluating chemical toxicities (Celik & Tuluce, 2007; Sicilia et al., 2008).

To date, only a small number of researchers have studied AA-induced toxicity by metabolomics (Sun et al., 2010; Wang et al., 2015). However, the dose levels used were relatively high and administrated for a relatively short time, which is not consistent with how people are exposed to AA. The toxic effects of chemicals are closely related to its exposure dose and time. Therefore, study on toxicity caused by low-level, long-term exposure to AA using metabolomics technique has garnered considerable attention.

No observed adverse effect level (NOAEL) is defined as “the highest level of continual exposure to a chemical that causes no significant adverse effect on the morphology, biochemicals, functional capacity, growth, development, or life span of the target species, as determined by traditional toxicology.” NOAEL is obtained using traditional toxicological methods. Considering that the characteristic advantages of metabolomics and AA-induced toxicity appear to be dependent on dose and time, in the current study, we carried out a urinary metabolomics study by using UPLC–MS coupled with multivariate statistics, including principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), to investigate if chronic exposure to acrylamide at NOAEL could induce toxicity in rats at the body metabolism level. Furthermore, the potential exposure biomarkers, metabolic pathways, and mechanisms of AA toxicity were investigated.

Materials and methods

Chemicals and reagents

Acrylamide (99.8% purity) and Leucine enkephalin using in HPLC were obtained from Sigma-Aldrich (St. Louis, MO). HPLC-grade methanol and acetonitrile were supplied by Dikma Science and Technology Co. Ltd (Lake Forest, CA). HPLC-grade formic acid was purchased from Beijing Reagent Ltd (Beijing, China). Acetylcysteine (99% purity), 2-indolecarboxylic acid (98% purity), cysteic acid (98% purity), and L-cysteine (99% purity) were purchased from J&K Scientific Ltd (Beijing, China). p-Cresol sulfate (98% purity) was purchased from Alschim Chemical Co. (Illkirch Graffenstaden, FRA). Kynurenic acid (97% purity) was purchased from Flurochem Ltd. (Old Glossop, UK).

Animal treatment

A total of 40 male Wistar rats weighing 180–220 g were obtained from Vital Laboratory Animal Technology Co. Ltd (Beijing, Chain) and acclimated for 1 week prior to the commencement of the experiment. AIN-93M diets and drinking water were available ad libitum. The rats were housed individually in metabolic cages under controlled humidity (50–60%) and temperature ($22 \pm 2^\circ C$) with a 12-h light–dark cycle. All aspects of this study were in accordance with the Institute of Zoology Animal and Medical Ethics Committee of Harbin Medical University.

After acclimatization, the rats were allocated with body weight-basis randomization to four groups (10 animals per group): the low-dose group (0.2 mg/kg, bw, NOAEL), the middle-dose group (1 mg/kg, bw, five times the NOAEL), the high-dose group (5 mg/kg, bw, 25 times the NOAEL) and the control group. The NOAEL of AA for a non-carcinogenic end-point was 0.2 mg/kg, bw per day was noted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its sixty-fourth meeting. This end-point was based on the induction of morphological nerve changes in rats following administration of AA in drinking-water (World Health Organization, 2006). AA was diluted in water according to the dose of each treatment group, and administered to the rats continually for 16 weeks via drinking water ad libitum. The control rats were given drinking water. Each rat’s body weight and food consumption were measured weekly during the study. Daily water volume given to each rat was its average amount of water consumption last week plus 5 ml, and daily water consumption of every rat was recorded during the test. For the body weight gain of rats grow fast in the preceding period, the amount of AA in the water was adjusted twice a week for the first 8 weeks, and then once a week after 8 weeks until the end of the experiment. Water consumption increased from 24 ml to 48 ml in the first 8 weeks after dosing and maintained at about 47 ml until the end of the treatment (Table S1). Water consumption every week showed no significant changes in the treated groups compared with the time-matched control group ($p > 0.05$).

Methods for neurological testing

AA-induced neurological were quantified using three different indices, which were tested at 8, 12 and 16weeks. Gait score, which converted the observations to numerical values ranging from 1 to 4 (LoPachin et al., 2002). Hind limb splay, every rat was dropped onto the paper and the distance...
between the center of the right and left heels was measured (LoPachin et al., 2002). Tail-flick test, which placed every rat tail quickly in a water bath (52 ± 0.5°C) to mark record rat tail flick time (Xiongxiong et al., 2011).

Sample collection and preparation
Urine samples were collected for 24 h in metabolic cages on ice packs at each time point (24 h pre-dose as well as 4, 8, 12, 16 weeks post-dose). Urine sample of each rat was then centrifuged at 3000 rpm (936 g) for 15 min, and the supernatants were stored at −80°C until analysis. Prior to analysis, urine sample of each rat was thawed at 4°C and centrifuged at 12,000 rpm (13201 g) for 10 min. The supernatants were diluted with distilled water in a 1:3 (vol/vol) ratio and vortexed for ultra-performance liquid chromatography/mass spectrometry (UPLC–MS) analysis. Quality control (QC) sample was prepared by mixing equal volumes of different individual urine samples (A total of 40), and was analyzed with every ten urine samples, in order to evaluate the stability of the analysis.

After 16 weeks of experiment, all the rats were fasted about 12 h. The rats were anesthetized with chloral hydrate via intraperitoneal injection, the blood samples were obtained from the aorta abdominalis before the rats were sacrificed. The blood samples were drawn and allowed to clot. Serum was obtained by centrifugation at 3000 rpm (936 g) for 15 min. The serum samples were analyzed by clinical chemistry methods using a Hitachi 7100 automated biochemical analyzer (Hitachi Co., Tokyo, Japan) to test for CR, BUN, UA. Simultaneously, the kidney was rapidly removed and was divided into two parts. One part was rapidly frozen in liquid nitrogen, and kept at −80°C until use for analyze the levels of GSH and MDA, and the antioxidant enzyme activities (SOD, CAT). The antioxidant enzyme activities of SOD, CAT, and the levels of GSH and MDA in the kidney tissues were analyzed by using the assay kit methods. The other part was fixed in 10% formalin and processed in wax blocks, after which serial transverse sections were prepared. Kidneys were stained with hematoxylin and eosin and then examined by light microscopy.

Chromatography
Chromatographic separation was performed on an HSS T3 column (100 × 2.1 mm, 1.8 μm i.d.; Waters Corporation, Milford, MA) using a Waters’ ACQUITY UPLC System (Waters Corporation). The temperatures of the column and autosampler were maintained at 35°C and 4°C, respectively. The flow rate was 0.45 ml/min, and a 2 μl aliquot of each sample was injected onto the column. The mobile phase consisted of 0.1% aqueous formic acid (solution A) and acetonitrile (solution B). The metabolites were eluted using a linear gradient of 0–2% B for 0–0.5 min, 2–5% B for 0.5–1 min, 5–12% B for 1–2 min, 12–20% B for 2–5 min, 20–32% B for 5–6.5 min, 32–45% B for 6.5–8.5 min, 45–65% B for 8.5–10 min, 65–98% B for 10–11 min, 98% B for 11–12 min, 98–30% B for 12–13 min, 30–2% B for 13–14 min and 2% B for 14–16 min in the positive ion mode. 0–2% B for 0–0.5 min, 2–20% B for 0.5–7 min, 20–35% B for 7–8 min, 35–70% B for 8–9.5 min, 70–98% B for 9.5–10 min, 98% B for 10–12 min, 98–2% B for 12–14 min and 2% B for 14–16 min in the negative ion mode.

Mass spectrometry
Mass spectrometry was performed on a Xevo G2 Q-TOF mass spectrometry equipped with electrospray ionization (ESI) source (Waters Corp., Milford, MA), operating in positive and negative modes and in full scan mode from m/z 50–1200 for 0–16 min. The analytical parameters were as follows: desolvation gas (nitrogen, 900 L/h), cone gas (nitrogen, 50 L/h); desolvation temperature (450°C), source temperature (120°C), capillary voltage (positive and negative ion modes were both 0.5 kV) and cone voltage (30V). For accurate mass acquisition, a lock mass of leucine enkephalin was used through a lock spray interface at a flow rate of 10μL/min monitoring for positive ([M + H]+= 556.2771) and negative ([M – H]=554.2615) ion modes. The lock spray frequency was set at 10 s in the positive ion mode and 15 s in the negative ion mode.

Data processing and metabolite identification
Data processing was conducted by the Progenesis QI software (version 2.1; Waters Corporation, Milford, MA), which is able to perform alignment, peak-picking and mining of metabolomics data to quantify and then identify significant molecular alterations between groups of samples. Metabolites of interest were filtered according to the ANOVA p value <0.05 and fold change >2.

Subsequently, the selected compounds were exported into EZinfo statistical analysis software (version 2.0; Umetrics AB, Umeå, Sweden) for analysis. The data were mean-centered and Pareto-scaled before multivariate statistical analysis. Principal component analysis (PCA), including QC samples, to visualize the data, assess its quality and reveal similarities between observations, trends and deviating samples (the corresponding PCA loading plots were used for the selection of biomarkers). After an initial overview of the data, the partial least-squares discriminant analysis (PLS-DA) was applied. A default seven-fold (Leave-1/7th Samples-Out) cross-validation procedure and testing with 100 random permutations were performed to avoid the over-fitting of supervised PLS-DA models, using SIMCA-P software (version 12.0; Umetrics AB, Umeå, Sweden). The Variable Importance in Projection (VIP) values of each variable in the model has been calculated in order to indicate its contribution to the classification of samples. The metabolites with a VIP value > 1.0 would be considered important in discriminating between different groups.

The differentiated compounds which found in EZinfo were reimported into Progenesis QI software for metabolite identification. Initial identification of metabolites was performed using the Human Metabolome Database (HMDB), leading to multiple ambiguous identifications for each compound of interest. The mass tolerance for the HMDB database search was set at 10 ppm or 5 mDa. The chromatographic retention behavior and fragmentation information were also considered to reduce the false-positive matches. Finally, metabolites were confirmed by comparison of retention time and fragmentation pattern with authentic
standards. The implicated pathways of biomarkers were interpreted using databases, including HMDB (http://www.hmdb.ca/) and KEGG (http://www.kegg.com/).

Statistical analysis
The changes of the metabolite intensities were performed by covariance (ANCOVA) using SPSS (version 21.0; Beijing Stats Data Mining Co. Ltd., China) and $p$ < 0.05 was considered to be statistically significant. Receiver operator characteristic (ROC) curve analysis, based on a logistic regression model, was performed using SPSS version 21.0 to determine the area under the curve (AUC) as a measure for comparing the predictive ability of important metabolites.

Other data were expressed as the mean ± SD. The one-way analysis of variance (ANOVA) was performed using SPSS version 21.0, and significant differences were considered significant when test $p$ values were less than 0.05. The data of gait score was conducted using the Mann–Whitney U-test by SPSS version 21.0.

Results
Body weight and kidney viscera coefficients
The body weight (BW) of the rats at each time point and group are shown in (Figure S1). Except for the BW significantly decreased in the high-dose group on the 16th week compared with the time-matched control group ($p$ < 0.05), no significant changes were observed in the three AA-treated groups at any time point compared with the time-matched control group ($p$ > 0.05).

After 16 weeks of experiment, all rat kidneys were weighed, and the ratio of kidney weight to the total body weight of rats (namely, kidney viscera coefficients) in each group was calculated. As shown in Figure S2, no significant changes were observed between all treatment groups compared with the control group ($p$ > 0.05).

Clinical chemistry
Various clinical parameters were measured in the serum of the control and treatment rats at the end of the experiment (Table 1). The contents of CR, BUN and UA were significantly increased in the middle-dose group (A2; $p$ < 0.05) and the high-dose group (A3; $p$ < 0.01) compared with the control group (C). No significant changes were observed in the low-dose group (A1) compared with the control group ($p$ > 0.05).

Antioxidant indices
The levels of GSH and MDA, as well as the activities of antioxidant enzymes (SOD, CAT) in rat kidney, are summarized in Table 2. The activities of SOD and CAT and the level of GSH are significantly decreased in groups A2 and A3 in comparison with those in the control group ($p$ < 0.05 or $p$ < 0.01). By contrast, MDA was significantly increased in groups A2 and A3 compared with those of the control group ($p$ < 0.01). No significant changes were observed in group A1 compared with group C ($p$ > 0.05).

Neurological testing
AA-induced neurological effects were detected and quantified at 8, 12 and 16 weeks (Figure S3). No statistically significant changes were observed for all treatment groups at the 8th and 12th week in comparison with the time-matched control group ($p$ > 0.05). Compared with the time-matched control group, the landing hind limb foot splay and gait score of group A3 were significantly increased ($p$ < 0.01) on the 16th week, and the tail flick time in groups A2 and A3 was both significantly decreased ($p$ < 0.05 and $p$ < 0.01).

Histopathology
Observational histopathological changes in the kidney were characterized by vacuole degeneration of renal tubular epithelial cells, vascular dilatation and hyperemia (red cell tube type), which were observed in group A3. In group A2, the lumen is not evident in most renal tubular epithelial degeneration. No significant histopathological changes were found in the kidney tissues of groups A1 and C rats (Figure 1).

Metabolic profiling
Urine samples from the control and treated rats were analyzed by using UPLC–MS in both positive and negative ionization modes to ensure the widest possible detection coverage. An initial overview of the analytical run quality was obtained by

| Groups | CR (µmol/l) | BUN (mmol/l) | UA (µmol/l) |
|--------|-------------|--------------|-------------|
| C      | 34.00 ± 2.05 | 7.16 ± 0.58  | 94.77 ± 14.51 |
| A1     | 35.10 ± 3.00 | 7.65 ± 0.76  | 108.41 ± 12.47 |
| A2     | 37.50 ± 2.64 | 8.04 ± 0.93  | 116.40 ± 19.74 |
| A3     | 39.30 ± 3.83 | 8.57 ± 0.69  | 127.22 ± 31.16 |

C: control group; A1: low-dose group; A2: middle-dose group; A3: high-dose group. Values expressed as mean ± SD ($n$ = 10).

| Groups | SOD (U/mgprot) | GSH (mgGSH/gprot) | CAT (U/mgprot) | MDA (nmol/mgprot) |
|--------|----------------|-------------------|---------------|------------------|
| C      | 72.06 ± 10.45  | 5.35 ± 1.79       | 15.59 ± 2.74  | 0.80 ± 0.26      |
| A1     | 63.92 ± 13.73  | 4.39 ± 1.45       | 13.82 ± 2.69  | 1.28 ± 0.40      |
| A2     | 53.93 ± 9.46   | 3.88 ± 0.90       | 12.74 ± 3.08  | 1.50 ± 0.67      |
| A3     | 46.85 ± 8.01   | 3.45 ± 1.22       | 12.09 ± 2.72  | 1.78 ± 0.76      |

C: control group; A1: low-dose group; A2: middle-dose group; A3: high-dose group. Values expressed as mean ± SD ($n$ = 10).

Significantly different from control group at $p$ < 0.05 (ANOVA).

Significantly different from control group at $p$ < 0.01 (ANOVA).
unsupervised PCA of the sample dataset that included the QC samples (Figure 2). The reproducibility of the method was determined with principal component analysis (PCA), and reliability was assessed by extracting six ions from chromatographic peaks of the QC samples. QC samples were clustered tightly in the middle of the score plot. Six ions extracted from the chromatographic peaks \((m/z) 202.0170, 230.1023, 309.0838, 329.0283, 436.6906 \text{ and } 508.0724\) in the negative ion mode) were selected for method reliability. The relative standard deviations (CV) of peak intensity, retention times, and \(m/z\) were estimated as 1.30–1.68%, 0.54–0.67% and 0.15–0.35%, respectively.

Supervised PLS-DA models were constructed based on the four groups to understand the metabolic changes and characterize the metabolite profile of the control and treated samples. The parameters of PLS-DA model on the 16th week including the values of \(R^2Y\) and \(Q^2\) (0.957, 0.914 in positive mode and 0.901, 0.826 in negative mode) were all more than 0.5, which indicates that these models were suitable for these recognition analysis. To assess the risk that the current PLS-DA models were spurious, the permutation test for PLS-DA in the negative and positive modes was applied. All \(R^2X\) and \(Q^2\) values to the left were lower than the original points to the right (Figure S4). Furthermore, the results of CV-ANOVA suggest that the PLS-DA models are highly significant (Tables S2 and S3). The PLS-DA scores in the negative mode are shown in Figure 3, whereas those in the positive mode are shown in Figure S5. The PLS-DA scores were collected from four different time points and demonstrated the time- and dose-dependent alterations.

Figure 3(a) (0 weeks before administration) shows that the data points did not separate from one another. Figure 3(b) (4 weeks after treatment) shows that the data points of the high- and middle-dose groups were separated from those of the control group; however, overlaps were observed between the low-dose and control groups. In Figure 3(c) (12 weeks after treatment), the data points of the treated groups were separated from those of the control group, particularly in the high-dose group, but overlaps still existed between the low-dose and control groups. In Figure 3(d) (16 weeks after treatment), the data points of the treatment groups were clearly separated from those of the control group, and the cluster of each group was extremely close. Moreover, a distinct separation was observed among the three treatment groups, but overlaps between the low-dose and control groups were observed.

In total, 13 metabolites (Table 3 and Supplementary Table 4) were identified based on the aforementioned methods (Data processing and metabolite identification). These metabolites were also found in the PCA loading plot of positive and negative modes in the 16th week (Figure S6). The changes of the metabolite intensities after AA treatment are presented in Tables S5 and S6. Statistically significant changes in several of the metabolites were found in the treatment groups compared with the control group. In the positive mode, GAMMA, AAMA, vinylacetylglycine, 1-salicylate glucuronide and PE (20:1(11Z)/14:0) were increased; by contrast, 3-acetamidobutanal, 2-indolecarboxylic acid, and kynurenic acid were decreased.
decreased in the treated group. In the negative mode, GAMA, acetylcysteine, cysteic acid, L-cysteine, p-cresol sulfate (PCS) and 7-ketodeoxycholic acid were all increased in the treated group. Notably, a metabolite in the low-dose group also showed significant changes compared with that in the control group. PCS was significantly increased after the rats were treated for 8 weeks in the low-dose AA-treated group compared with the control group ($p < 0.05$).

To evaluate the sensitivity and specificity of the metabolites ROC curve was performed, AUC values for the 13 metabolites were all above 0.7 at a 95% confidence interval (Figure S7). These results increased sensitivity and specificity for all identified potential biomarkers.

**Discussion**

In this study, UPLC/Q-TOF/MS-based metabolomics analysis was conducted to detect candidate metabolites in rat urine samples treated with AA. PCA, which was conducted on QC and other groups, revealed that QC samples were clustered in the PCA score plot (Figure 2). As well as the relative standard deviations of extracting six ions showed that the reproducibility and stability of the metabolomics platform were excellent throughout the run and sufficient to ensure the data quality for further global metabolomics analysis. For facilitating better characterization of the urine metabolite profile of the treated animals and examine the systemic metabolic changes with the time and dose, a supervised PLS-DA model was built based on the three dose groups and the control group. PLS-DA is a common approach in multivariate metabolomics data analysis, maximizing the product of the variance matrix of measured variables and the correlation of measured data with properties of interest. The permutation test for PLS-DA (Figure S4) showed that all R2X and Q2 values to the left were lower than the original points to the
Figure 3. PLS-DA score plots of urine in the negative mode. PLS-DA score $t_1$ versus $t_2$ of data obtained from mean variables of urine collected from the control and treated groups. *C: control group; ▲ A1: low-dose group; ■ A2: middle-dose group; ● A3: high-dose group. (a–d) 0, 4, 12 and 16 weeks after treatment.

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Metabolomics analysis of toxicity of acrylamide
Table 3. Potential biomarkers of toxicity induced by acrylamide.

| Retention time (min) | Measured mass (Da) | Calculated mass (Da) | Da ppm | Molecule composition | Scan mode | Metabolites | Trend |
|----------------------|--------------------|----------------------|--------|----------------------|-----------|-------------|-------|
| 1.02                 | 144.0663           | 144.0655             | 0.0008 | 5.42                 | +         | C6H9N03     | ↑     |
| 1.12                 | 250.0622           | 250.0623             | 0.0001 | 0.57                 | +         | C8H14N2O5S  | ↑     |
| 1.26                 | 129.1656           | 129.1652             | 0.0004 | 1.42                 | +         | C6H11N02     | ↑     |
| 1.73                 | 234.0682           | 234.0674             | 0.0008 | 3.30                 | +         | C8H14N2O4S  | ↑     |
| 2.45                 | 314.1344           | 314.1348             | 0.0004 | 0.70                 | +         | C13H14O9     | ↑     |
| 3.12                 | 162.0555           | 162.0555             | 0.0005 | 3.37                 | +         | C9H7N02      | ↑     |
| 3.12                 | 190.0506           | 190.0499             | 0.0007 | 3.85                 | +         | C10H7N03     | ↑     |
| 3.52                 | 717.5249           | 717.5201             | 0.0048 | 6.52                 | +         | C39H76NO8P   | ↑     |
| 0.9                  | 120.0114           | 120.0125             | 0.0011 | 8.94                 | -         | C3H7NO5S     | ↑     |
| 1.13                 | 249.0534           | 249.0551             | 0.0017 | 6.69                 | -         | C8H14N2O5S  | ↑     |
| 1.77                 | 162.0225           | 162.0232             | 0.0005 | 3.32                 | -         | C5H9NO3S     | ↑     |
| 6.37                 | 169.0063           | 169.0072             | 0.0009 | 2.32                 | -         | C3H7NO5S     | ↑     |
| 6.4                  | 187.0061           | 187.0071             | 0.0011 | 5.63                 | -         | C7H8O4S      | ↑     |
| 9.29                 | 406.2712           | 406.2717             | 0.0012 | 2.95                 | -         | C24H38O5     | ↑     |

*The intensity of the metabolite was significantly increased in the treated groups compared with the control group. †The intensity of the metabolite was significantly decreased in the treated groups compared with the control group.

The 13 metabolites that significantly contributed to this discrimination were identified through MS analysis. These metabolites were considered as potential biomarkers of AA-induced toxicity. Moreover, several indexes were detected to provide additional support to the metabolomics results. The contents of CR, BUN and UA in the serum, as well as the antioxidant indices of SOD, CAT, GSH and MDA in kidney tissues, were significantly changed in the middle- and high-dose groups in comparison with those in the control group (p < 0.05 or p < 0.01). The histopathology of kidney tissues was consistent with the above results. In this study, the biological relationships between potential biomarkers and toxic effects induced by AA are discussed according to the four pathways shown in Figure 4.

The first pathway concerns the metabolism of fatty acids and amino acids (Figure 4). PE (20:1(11Z)/14:0) is a glycerophospholipid. Phospholipids are ubiquitous in nature and are key components of the lipid bilayer of cells. In addition, phospholipids are involved in metabolism and signaling (Bochkov et al., 2010; Usatyuk & Natarajan, 2012). In this study, PE (20:1(11Z)/14:0) was significantly increased in middle- and high-dose groups in comparison with the control group, suggesting that exposure to AA could affect the fatty acid metabolism of rats. Vinlylacetylglycine is an acyl glycine. Acyl glycines are normally minor metabolites of fatty acids. Increased amounts of this metabolite in the urine have been reportedly linked to mitochondrial fatty acid beta-oxidation (Tanaka et al., 1980). In this study, vinylacetylglycine was significantly increased in middle- and high-dose groups compared with the control group. These results suggest that AA could affect the fatty acid metabolism of rats. Amino acids are the primary components of proteins and are involved in major metabolic pathways, including protein synthesis and catabolism. Cysteic acid is a crystalline amino acid that is formed in the oxidation of cysteine. Kynurenic acid is a tryptophan metabolite, and tryptophan is an essential amino acid. In this study, cysteic acid was significantly increased, whereas kynurenic acid was significantly decreased in middle- and high-dose groups in comparison with the control group, indicating that exposure to AA could disturb the amino acid metabolism.

The second pathway involves the antioxidant defense system (Figure 4). The imbalance between the production and the removal of free radicals increased oxidative stress. The origin of numerous diseases is closely related to oxidative damage caused by free radicals (Sayre et al., 2008). GSH performs important functions in antioxidant defense. Glutathione deficiency contributes to oxidative stress, playing a key function in aging and the pathogenesis of numerous diseases (Harris et al., 2015; Narainsamy et al., 2015; Raffa et al., 2011; Wu et al., 2004). The metabolites of AAMA, GAMA and iso-GAMA were detected in rat urine, indicating that the metabolites can be considered as sensitive biomarkers for rats exposed to AA. AA can be conjugated by GSH, degraded to form AAMA, or oxidized to form GA. GA formed in the latter reaction could further react with GSH, resulting in its biotransformation into GAMA and iso-GAMA (Awad et al., 1998; Fennell & Friedman, 2005; Friedman, 2003; Tareke et al., 2006; Tong et al., 2004). In this study, the detection of AAMA, GAMA and iso-GAMA suggested the possibility for disruption of glutathione synthesis pathways. Several researchers suggested that AA-induced toxicity was associated with oxidative stress (Chen et al., 2016; Lakshmi et al., 2012; Mehri et al., 2014; Yousef & El-Demerdash, 2006; Zhu et al., 2008), and the current results were consistent...
with this finding. 2-Indolecarboxylic acid is a strong inhibitor of lipid peroxidation; lipid peroxidation with tert-butyl hydroperoxide is a source of free radicals (Human Metabolome Database, 2007a). 3-Acetamidobutanal is part of the peroxisome pathways, and L-cysteine is also part of the antioxidant GSH, cysteine has antioxidant properties. As L-cysteine contains a sulphydryl group, it can undergo redox reactions. (Wu et al., 2004). Kynurenine is a central compound of the tryptophan metabolism pathway; tryptophan is primarily metabolized along the oxidative kynurenine pathway. Activation of the kynurenine pathway in tryptophan metabolism can induce oxidative stress (Blanco Ayala et al., 2015). In this study, significant changes in 2-indolecarboxylic acid, 3-acetamidobutanal, L-cysteine and kynurenic acid in rat urine indicated that exposure to AA could affect the antioxidant defense system of rats. In this study, the decreased activities of antioxidant enzymes (SOD and CAT) and GSH level as well as the increased MDA level further supported the aforementioned metabolomics results.

The third pathway is related to the nervous system (Figure 4). Kynurenic acid is formed in a ‘‘dead end’’ side-arm of the tryptophan metabolite pathway. Tryptophan can only be transported across the blood brain barrier in its free form by the competitive and nonspecific L-type amino acid transporter (Stone, 1993). Once in the central nervous system, tryptophan acts as a precursor to various metabolic pathways. This versatility results in different end-products, such as protein, serotonin, and kynurenines, followed by conversion to quinolinic acid, kynurenic acid, and so on (Ruddick et al., 2006, Schrocksnadel et al., 2006). Kynurenic acid is an endogenous neuroprotectant that is usually present in the brain at nanomolar concentrations. Increases in brain kynurenic acid were observed to exert sedative and anticonvulsant effects, and the compound was found to be protective against brain ischemia (Robotka et al., 2008). In this study, kynurenic acid in rat urine were significantly decreased, suggesting that exposure to AA causes tryptophan metabolism disorder and affects the nervous system of rats.

The fourth pathway is related to liver and kidney function (Figure 4). The liver performs a vital function in lipid metabolism and contributes both in exogenous and endogenous cycles of lipid metabolism. Patients with severe liver disease experience a disturbance in lipid metabolism (Blasie et al., 2007). PE (20:1(11Z)/14:0) increases after massive destruction of membranes, as indicated by cell death observed during the histological examination of the liver (Gonzalez et al., 2012). The increase in PE (20:1(11Z)/14:0) in this study indicates that exposure to AA could induce liver damage. 7-Ketodeoxycholic acid is a bile acid. Bile acids possess potent toxic properties (such as membrane disruption). The concentration of bile acids is always significantly elevated in chronic active liver and can be used as a more sensitive indication of active hepatitis (Korman et al., 1974). The increase in 7-ketodeoxycholic acid in the present study indicates that AA could induce liver damage. The increase in 1-salicylate glucuronide in the present study indicated that AA could affect liver function (Human Metabolome Database, 2007b). PCS is a uremic toxin that causes kidney dysfunction by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inducing ROS production. PCS aggravated hypoxia of the kidney via enhancement of tubular oxygen consumption (Watanabe et al., 2013). Accumulation of PCS may lead to irreversible binding to albumin. There is a increasing amount of evidence showing that accumulation of PCS can cause renal inflammation and fibrosis (Palm et al., 2010; Watanabe et al., 2015). Thus, the accumulation of PCS is considered to be a risk factor for chronic kidney disease. In this study, PCS was significantly increased in middle- and high-dose groups in comparison with the control group, suggesting that exposure to AA could induce renal injury. In addition, significant changes in routine
kidney function parameters (CR, BUN and UA; Table 1) in rat serum, antioxidant indices (SOD, CAT, GSH and MDA; Table 2) in the kidney, and the kidney histopathology further supported that AA can cause renal toxicity via induction of oxidative stress.

The toxic impact of chemicals usually appears to be dependent on dose and time. Several previously published articles investigated the toxic effects of AA by using metabolomics technology; however, the dose levels used were relatively high and administrated for a relatively short time (Sun et al., 2010; Wang et al., 2015). Compared with metabolites identified from aforementioned studies, three new candidate biomarkers in rat urine exposed to AA were found in this study. Among these biomarkers, PCS is related to kidney dysfunction, and the remaining two metabolites (7-ketodeoxycholic acid and 1-salicylate glucuronide) are related to liver function. These findings suggest that the toxic effect of chronic low-dose exposure to AA is different from high-level, short-term exposure. Histopathological examination results of the kidney (Figure 2) of the rats in the low-dose group did not show any change. Notably, the intensities of PCS were significantly increased in the low-dose (NOAEL level) AA-treated group compared with the control group ($p < 0.05$). These results indicate that the obtained NOAEL of AA through traditional toxicological methods could induce significant changes in the metabolites in the body metabolic level. This finding suggests that the aforementioned factor should be considered for the revision of the acceptable daily intake of AA in the future. In addition, biomarkers for rats exposed to AA (AAMA, GAMA, iso-GAMA) combined with the other 10 metabolites can serve as potentially sensitive biomarkers in urine samples from AA-treated rats.

Conclusions

In conclusion, a metabolomics approach was applied to study the metabolite profile of rats after chronic low-dose exposure to AA. The results indicate that chronic exposure to AA at NOAEL does not exert a toxic effect on rats at the body metabolism level. Results of this study show that AA could disrupt the rat metabolisms (such as lipid and amino acid metabolisms) and induce oxidative stress. Further studies will be conducted to measure the metabolomics profiles of the tissues, which is conducive for the comprehensive understanding of the toxic effects of AA.

Declaration of interest

The authors declared that they have no conflicts of interest in this work. Financial support from the National Natural Science Foundation of China (81573157) and the Key Laboratory of Public Health Safety (Fudan University), Ministry of Education, China (GW2015-2) are gratefully acknowledged. The laboratory of nutrition and food hygiene in Harbin Medical University is the key laboratory of Heilongjiang Province and Heilongjiang Higher Education Institutions.

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Supplementary material available online

Supplementary Tables 1–6 and Figures 1–7