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Metabolomic biomarkers in urine of rats following long-term low-dose exposure of cadmium and/or chlorpyrifos

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ABSTRACT

Heavy metals and pesticides can be easily enriched in food chains and accumulated in organisms, thus pose significant threat to human health. However, their combined effects for long-term exposure at low dose has not been thoroughly investigated; especially there was no biofluid biomarker available to noninvasively diagnose the toxicosis of the combined exposure of the two chemicals at their low levels. In this study, we investigated the change of urine metabolites of rats with 90-day exposure to heavy metal cadmium (Cd) and/or organophosphorus pesticide chlorpyrifos (CPF) using gas chromatography-mass spectrometry (GC-MS)-based metabolomics approach. Our results showed that the interaction of Cd and CPF mainly displayed an antagonistic effect. We identified the panels of metabolite biomarkers in urine: benzoic acid and mannose were unique biomarkers for Cd exposure; creatinine and N-phenylacetyl glycine were unique biomarkers for CPF exposure; anthranilic acid, ribitol, and glucose were unique biomarkers for Cd plus CPF exposure. Our results suggest that 90-day exposure to Cd and/or CPF could cause a disturbance in energy and amino acid metabolism. And urine metabolomics analysis can help understand the toxicity of low dose exposure to mixed environmental chemicals.

1. Introduction

Cadmium (Cd) is a toxic metal that are widely present in the environment (Schwarz et al., 2014). Cd can enter the food chain through water and soil and be accumulated in the organisms for a long time with half-life of 15–30 years (Jarup and Akesson, 2009). Chronic Cd exposure can induce toxicity in bones, liver, and kidneys (Brzoska and Moniuszko-Jakoniuk, 2005; Satarug, 2012; Siddiqui, 2013). Chlorpyrifos (CPF) is a broad-spectrum organophosphorus insecticide widely used for pest control by inhibiting acetylcholinesterase activity. In non-target species, CPF induces not only cholinergic neurotoxicity (Richardson, 1995; Marty et al., 2012; Wang et al., 2014) but also liver and kidney damage (Acker et al., 2012; Tripathi and Srivastav, 2010). Both Cd and CPF can accumulate in the environment and people are probably simultaneously exposed to Cd and CPF from food, water, air, and other environmental media or agricultural products (Forget et al., 1999; Harris et al., 2011). Since multiple toxicants can have complicated interaction and cause different toxicity, it is essential to study the combined toxicity of two or more toxicants. Some studies suggest that co-exposure to different environmental pollutants can potentiate the toxicity of individual chemicals (Corbel et al., 2006; Banni et al., 2011; Wang et al., 2014). At present, the acute and subchronic toxicity of individual Cd and CPF has been well characterized (Acker et al., 2012; Cardona et al., 2013; Elsharkawy et al., 2013; Edwards and Ackerman, 2016; Chandurvelan et al., 2017; Nasiadek et al., 2019), which includes the effect of the chemicals on metabolism (Gao et al., 2014; Lee et al., 2014; Zhao et al., 2016; Deng et al., 2016), but it is still unclear whether Cd and CPF may cause the expected combined toxicity at environmentally relevant low concentrations upon long-term exposure.

Metabolomics is a widely used approach to characterize the metabolic changes induced by toxicants and identify different sets of biomarkers for different xenobiotics exposure (Zheng et al., 2013; Beger et al., 2010; Robertson et al., 2011). Gas chromatography-mass

Abbreviations: AMDIS, automatic mass spectral deconvolution and identification system; ANOVA, analysis of variance; AUC, area under curve; BW, body weight; Cd, cadmium; CPF, chlorpyrifos; GC-MS, gas chromatography-mass spectrometry; LD50, half-lethal doses; MSTFA, methyl-trimethyl-silyl-trifluoroacetamide; PLS-DA, partial least squares discriminant analysis; ROC, receiver-operating characteristic; TCP, 3; 4, 5-trichloropyrindinol; TMCS, trimethylchlorosilane; VIP, variable importance in the project

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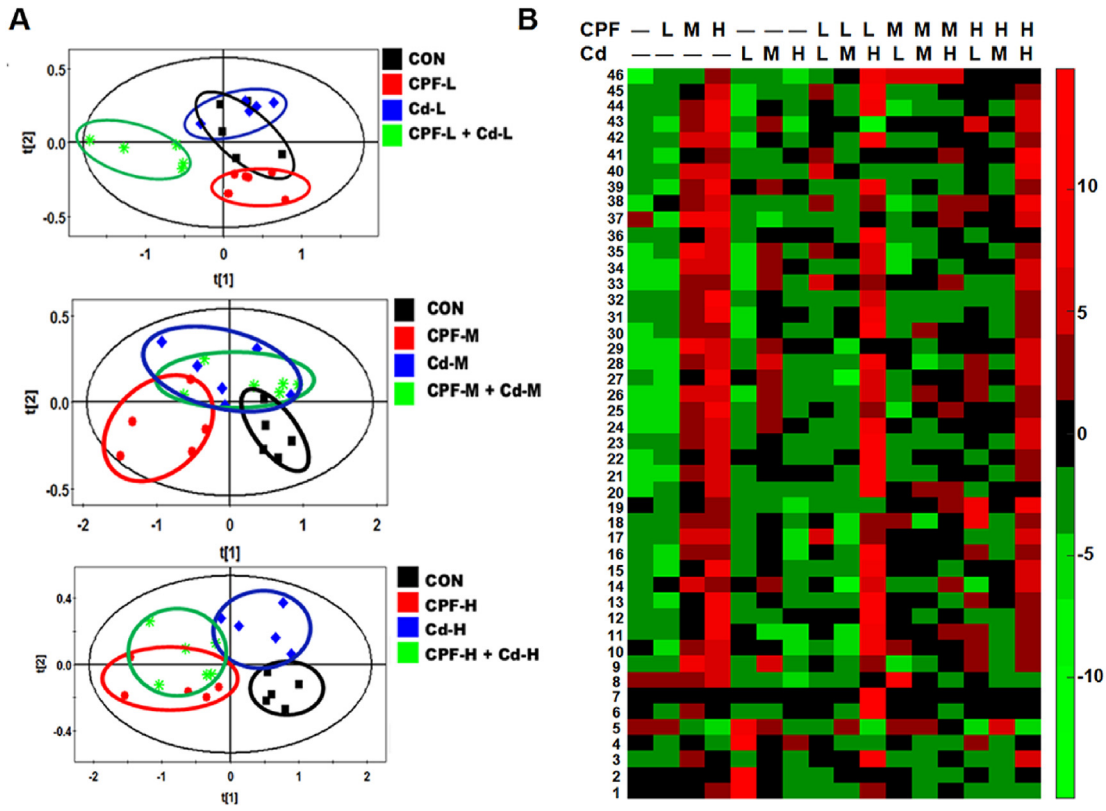

Fig. 1. Metabolomics analysis of the metabolites in urine from rats. The SD rats were administrated orally with Cd and CPF at doses of 0.7 and 1.7 mg/kg BW/day (low dose, L), 2 and 5 mg/kg BW/day (middle dose, M), 6 and 15 mg/kg BW/day (high dose, H), respectively, and their combinations for 90 days. After the 90-day experimental treatment, 24-h urine samples were collected and the urine metabolites were measured by GC-MS. (**A**) The representative PLS-DA score plots of urine metabolite profiles of the rats, which were administrated orally with low-, middle-, and high-dose of Cd, CPF, or their combinations at the same dose levels, i.e. Cd-**L** plus CPF-**L**, Cd-**M** plus CPF-**M**, and Cd–**H** plus CPF-**H**. Blank ellipse: control group; blue ellipse: Cd-treated groups; red ellipse: CPF-treated groups; green ellipse: CPF plus Cd-treated groups. (**B**) Heat map of the levels of total 46 urine metabolites of rats, which changed significantly in the treated groups. The leftmost numbers represent the corresponding metabolites (for detailed names of the metabolites see Table 1). Each metabolite is represented by a single row of colored boxes, whereas columns represented different treatment. Different colors indicate different metabolite levels. Black (z scores of ≈ 0) indicates that the concentration of the metabolite is the same as the average. Red (z scores of > 0) indicates that the concentration of the metabolite is greater than the average, while green (z scores of < 0) indicates that the concentration of the metabolite is less than the average. The z score was defined by the value of the difference between the observed value and the average, which was divided by the standard deviation. Abbreviations: Cd, cadmium; CPF, chlorpyrifos; CPF + Cd, chlorpyrifos plus cadmium; L, low dose; M, middle dose; H, high dose. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
The urine metabolites that changed significantly in the treated rats.

No.	Retention time (min)	Metabolites	No.	Retention time (min)	Metabolites
1	9.118	Acetic acid	24	29.503	Ribonic acid
2	9.176	Propanoic acid	25	29.230	Glycerophosphate
3	11.996	Butanoic	26	30.226	Ornithine
4	13.690	Benzoic acid	27	32.106	N-Phenylacetyl glycine
5	14.048	Phosphate acid	28	32.205	N-Acetylglycine
6	14.355	Leucine	29	32.551	Ascorbic acid
7	14.850	Butanedioic acid	30	32.513	Fructose
8	15.293	Glycine	31	33.818	Glucose
9	16.211	Dihydroxybutanoic acid	32	33.763	Octyl aniline
10	18.256	Alanine	33	33.942	Gluconic acid
11	18.970	Aminomalonic acid	34	34.380	Lysine
12	19.982	Malic acid	35	34.456	Glucitol
13	20.477	Pyrimidinamine	36	34.907	Tyrosine
14	20.996	Aspartic acid	37	36.669	Mannose
15	21.417	5-Methyl-4-pyrimidinamine	38	38.727	Indole
16	22.281	Creatinine	39	38.866	Hydrocinnamic acid
17	23.278	Anthranilic acid	40	39.592	Uric acid
18	25.550	Hexanedioic acid	41	40.143	Inositol
19	25.567	Galacturonic acid	42	46.463	Glucaric acid
20	25.898	Isoleucine	43	46.959	Glucopyranosiduronic acid
21	26.440	Ribose	44	47.793	Arabinofuranose
22	27.901	Ribitol	45	47.868	Uridine
23	27.956	Galactose	46	48.868	Eicosanoic acid

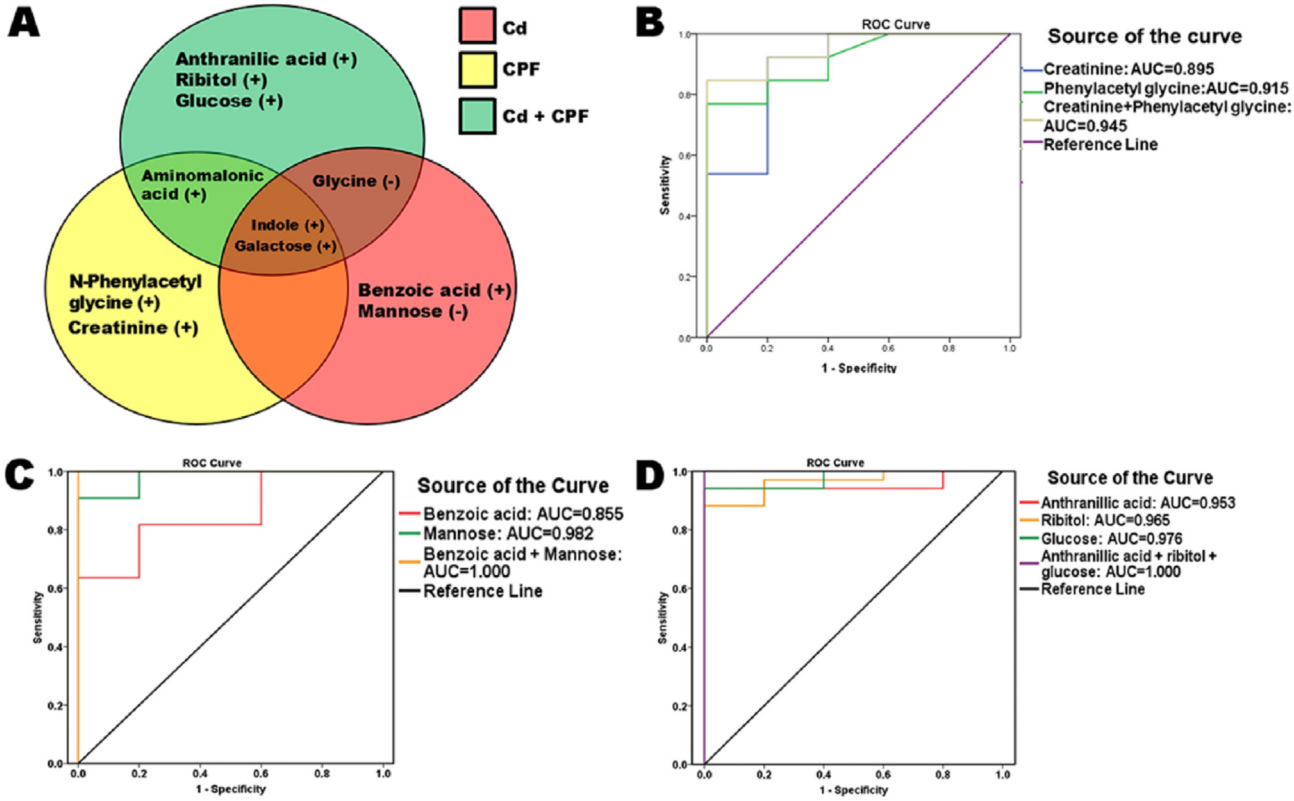

Fig. 2. Identification of urine metabolite biomarkers. (**A**) The summary diagram of potential urine biomarkers for Cd-, CPF- and Cd plus CPF-treated groups. The metabolites could distinguish CPF- (yellow), Cd- (red) and CPF plus Cd- (green) treated groups from control group; **(B–D)** The ROC curves and AUC values for creatinine, N-phenylacetyl glycine and their combination (**B**); benzoic acid, mannose, and their combination (**C**); and ribitol, glucose, anthranilic acid, and their combination (**D**). '+' indicated the increased level of metabolites in the treated groups; '-' indicated decreased level of metabolites in the treated groups. Abbreviations: AUC: area under curve; Cd, cadmium; CPF, chlorpyrifos; Cd + CPF, cadmium plus chlorpyrifos. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

spectrometer (GC-MS) is among the most common approaches used in metabolomics, which can detect very low levels of metabolites in urine, serum samples, and tissue extracts (A et al., 2005; Wu et al., 2009). Our previous studies revealed that the exposure of Cd and CPF at low doses can induce metabolomic changes in the organs including brain, liver, and kidney from the rats exposed to the chemicals (Xu et al., 2015, 2017; 2019); however, their combined metabolism toxicity and biomarkers in urine are not clear. In the present study, we used GC-MS-based metabolomics approach to study the combined toxicity of Cd and CPF and identified potential biomarkers in urine.

2. Materials and methods

2.1. Chemicals

Methyl-trimethyl-silyl-trifluoroacetamide (MSTFA), guanidine hydrochloride, methoxyamine, trimethylchlorosilane (TMCS), and thiobarbituric acid were obtained from J & K Chemical Ltd (Beijing, China). Cadmium chloride ($CdCl_2$) was obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Hexane and methanol were chromatographic grade and obtained from Dikma Technologies Inc. (Beijing, China). Chlorpyrifos (CPF, purity > 96%) was obtained from Nantong Shuangma Fine Chemical Co., Ltd (Jiangsu, China). All other reagents were of analytical grade that obtained from commercial providers.

2.2. Animals and treatment

Eighty 6 ~ 8-week-old male SD rats were obtained from Beijing HFK Bioscience Co., Ltd (Beijing, China) and were housed under a specific-pathogen-free (SPF) condition with 22 ± 2 °C, 50%–60% humidity and a light/dark cycle of 12 h. Animals had free access to water and the commercially prepared laboratory animal diet.

The rats were randomly grouped and treated with $CdCl_2$ ("Cd^{2+}"; it is hereinafter referred to simply as "Cd"), CPF, alone or in combination in the same way as reported in our early study (Xu et al., 2017) with five rats in each group. Previous studies showed the acute oral half-lethal doses (LD_{50}) of Cd and CPF were 88 mg/kg (Siddiqui, 2013) and 229 mg/kg (FAO, 2008) for rats, respectively. So in this study we chose the doses of 1/135 LD_{50}, 1/45 LD_{50}, and 1/15 LD_{50} of each chemical as their corresponding low-, middle-, and high-dose for the treatment groups, respectively. The doses used were Cd: 0.7 (low dose), 2 (middle dose), and 6 (high dose) mg/kg body weight/day; CPF: 1.7 (low dose), 5 (middle dose), and 15 (high dose) mg/kg body weight/day. Experimental design for the combined effects of the two chemicals was summarized in Suppl. Table S1.

Cd and CPF were dissolved in deionized water and corn oil, respectively, and administered via oral gavage (0.5 mL/kg body weight) for 5 consecutive days every week. Rats were given these chemicals for 90 days. The rats received an equivalent volume of corn oil and water served as control.

All animal procedures were performed in accordance with current China legislation and approved by the Animal and Medical Ethics Committee from Institute of Zoology, Chinese Academy of Sciences.

2.3. Sample preparation

After the 90-day experimental treatment, 24-h urine samples from the animals were collected by regular metabolic cage for rats and 1% sodium azide was added. Then urine samples were centrifuged at 4, 000 × *g* for 10 min to remove insoluble residues. Then the prepared

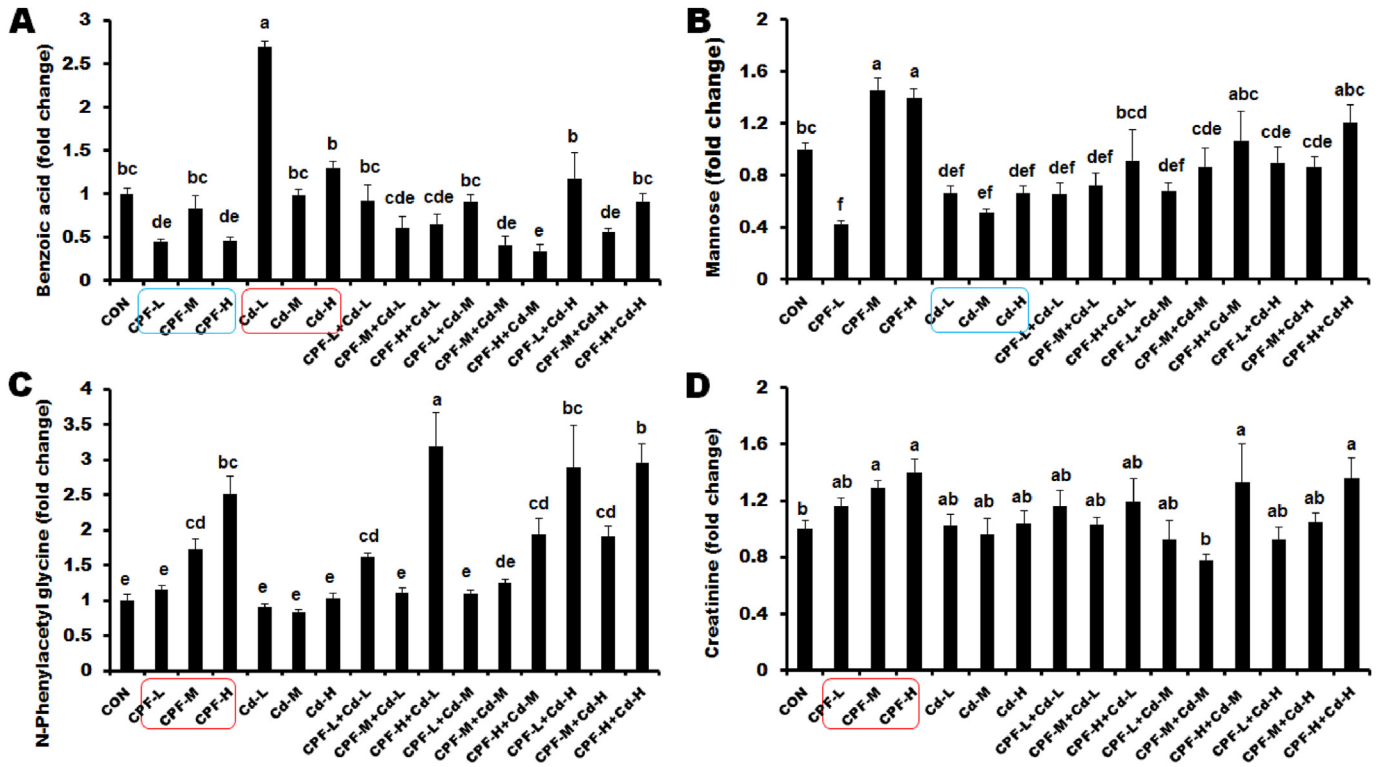

Fig. 3. The levels of benzoic acid (**A**), mannose (**B**), N-phenylacetyl glycine (**C**), and creatinine (**D**) in urine of rats administrated orally with Cd and CPF at respective doses of 0.7 and 1.7 mg/kg BW/day (low dose), 2 and 5 mg/kg BW/day (middle dose), 6 and 15 mg/kg BW/day (high dose) and their mixtures for 90 days. Different letters above the columns indicate a significant difference among groups ($P < 0.05$), while the same letters indicate no significant difference among groups ($P > 0.05$). Abbreviations: BW, body weight; Cd, cadmium; CPF, chlorpyrifos; CPF + Cd, chlorpyrifos plus cadmium; L, low dose; M, middle dose; H, high dose.

urine samples were stored at −80 °C until use.

2.4. Sample preparation for GC-MS analysis

The urine samples to be tested were dissolved at 37 °C and swirled before use. Thirty units of urease were added to 200 μl of urine. The sample was incubated at 37 °C for 15 min. Then 800 μl of methanol and 10 μl of heptadecanoic acid (6 mg/mL) were added to the urine and vigorously extracted for 10 min and centrifuged at 15, 000 × *g* at 4 °C for 10 min. The supernatant was transferred to a GC vial, and dried in a vacuum concentrator.

Fifty microliters (50 μl) of methoxyamine in pyridine were added to the dried metabolites extract and vigorously vortexed. After incubated for 16 h at room temperature, the samples were trimethylsilylated for another 1 h by adding 100 μl of MSTFA with 1% TMCS. Finally, 200 μl of hexane was added, and the samples were vigorously vortexed again, centrifuged at 15, 000 × *g* for 15 min and the supernatant was transferred to the GC sample vials for GC/MS analysis.

2.5. GC-MS analysis

The GC-MS analysis was carried out as reported in our early study (Xu et al., 2017). GC was performed on a 6890N gas chromatograph system (Agilent Co., Palo Alto, CA, USA) equipped with a HP-5 MS capillary column (60 m × 0.25 mm × 0.25 μm). The injection temperature was 250 °C. The carrier gas flow rate was 1 mL/min. The column temperature was held at 90 °C for 1 min and then raised to 175 °C at the rate of 5 °C/min and held for 3 min. The temperature was subsequently raised to 270 °C at the rate of 3 °C/min and then to 310 °C at the rate of 20 °C/min. And then the temperature was maintained at 310 °C for 15 min. The temperatures of the transfer interface and the ion source were 250 and 200 °C, respectively. Ions were generated by a 70 eV electron source at the full scan mode (m/z 40–600), with the acquisition rate of 20 spectra/s.

2.6. Metabolomics data analysis

The metabolomics data analysis was carried out in a similar way as reported in our early study (Xu et al., 2017). Automatic mass spectral deconvolution & identification system (AMDIS) was used to address spectral convolution of the urine samples, and peaks with signal-to-noise ratio higher than 3 were selected for further analysis. The differential metabolites of the samples were identified by using NIST library 2005 and the peaks were matched to Metabolon's library with an accuracy of > 90. Data pretreatment procedures for metabolites were performed by Matlab 7.1 (The MathWorks, Inc., Natick, MA, USA). Partial least squares discriminant analysis (PLS-DA) was used for multivariate statistical analysis with SIMCA-P 11.5 software (Umetrics, Umeå, Sweden). In order to select the candidate biomarkers, the variable importance in the project (VIP) values were calculated by using SIMCA-P software. The metabolites data were checked to have a normal distribution by using SPSS18.0 software (SPSS, Inc., Chicago, MI, USA). Thus, the significance among groups for these candidate biomarkers was calculated by using analysis of variance (ANOVA) with F-test and only those with $P < 0.05$ were selected. Finally, to determine the accuracy of the biomarkers to discriminate the treated groups with the control group, the area under the curve (AUC) value for receiver-operating characteristic (ROC) curve was calculated using SPSS 18.0 software. The detailed meanings of AUC values are: AUC = 0.5: not accurate, $0.5 < AUC \leq 0.7$ or $0.3 \leq AUC < 0.5$: less accurate, $0.7 < AUC \leq 0.9$ or $0.1 \leq AUC < 0.3$: moderately accurate, $0.9 < AUC < 1$ or $0 < AUC < 0.1$: highly accurate; AUC = 1 or 0: super accurate (Hosseininejad et al., 2009). Thus, in this study, metabolites with AUC > 0.9 or AUC < 0.1 were selected as potential biomarkers.

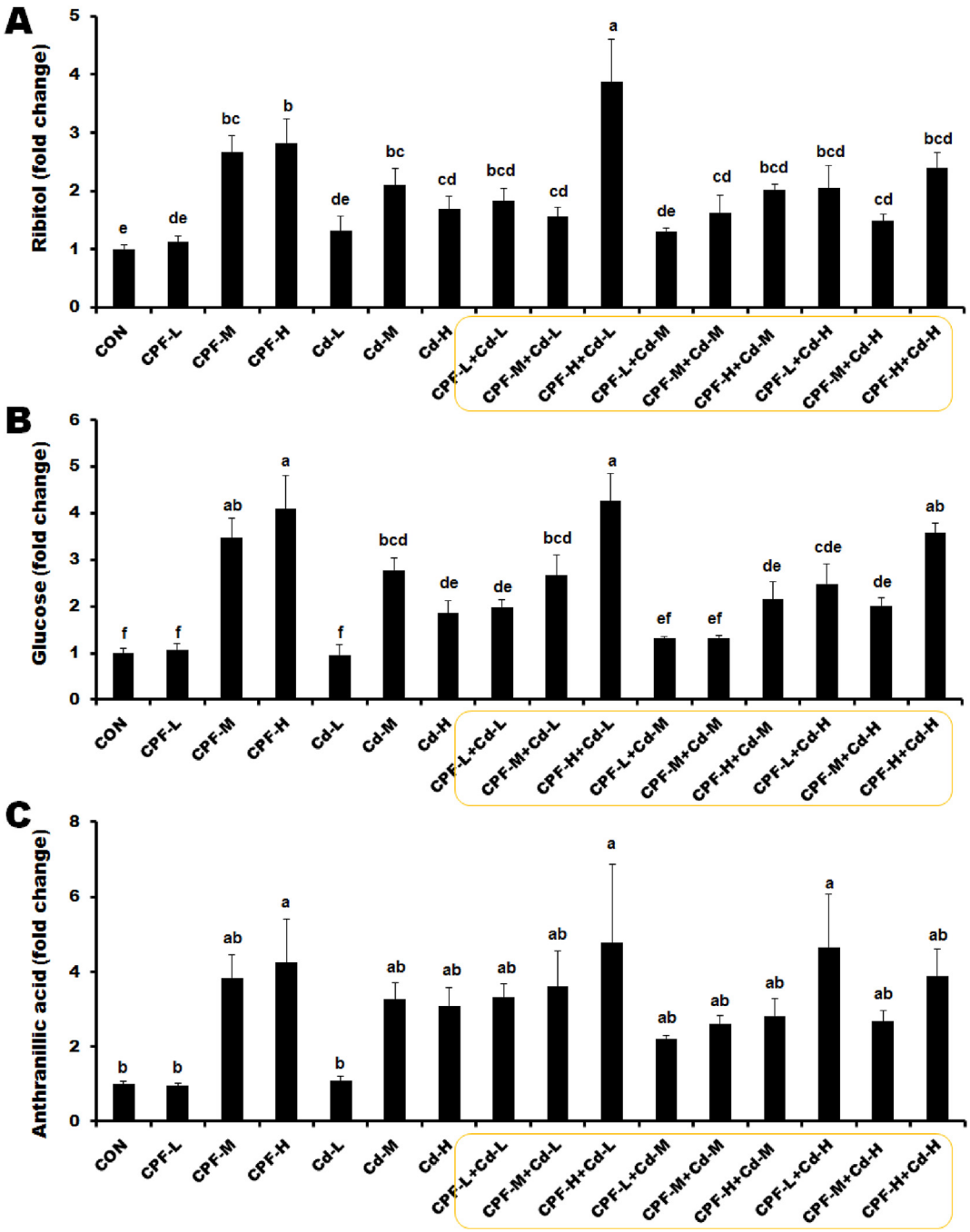

Fig. 4. The levels of ribitol (**A**), glucose (**B**), and anthranillic acid (**C**) in urine of rats administrated orally with Cd and CPF at respective doses of 0.7 and 1.7 mg/kg BW/day (low dose), 2 and 5 mg/kg BW/day (middle dose), 6 and 15 mg/kg BW/day (high dose) and their mixtures for 90 days. Different letters above the columns indicate a significant difference among groups ($P < 0.05$), while the same letters indicate no significant difference among groups ($P > 0.05$). Abbreviations: BW, body weight; Cd, cadmium; CPF, chlorpyrifos; CPF + Cd, chlorpyrifos plus cadmium; L, low dose; M, middle dose; H, high dose.

2.7. Measurement of chlorpyrifos and its metabolite level

The assay was carried out by the method reported by the previous study (Abu-Qare and Abou-Donia, 2001). Briefly, the urine samples were mixed with methanol and then centrifuged. The supernatants were collected, dried under nitrogen and then dissolved in 150 μl of methanol. The samples were analyzed by the Agilent 1100 series HPLC system (Santa Clara, CA, USA) with UV detector. Calibration standards of CPF and its toxic metabolite 3, 4, 5-trichloropyrindinol (TCP) were prepared in acetonitrile with concentrations ranging from 0.1 to 10 mg/mL. Linear calibration curves were obtained by plotting the peak areas of CPF or TCP as a function of the concentration.

2.8. Measurement of Cd level in urine

The levels of Cd in the urine of rats were determined by the inductively coupled plasma mass spectrometry (ICP-MS) according to the method reported in a previous study (Yang et al., 2007). Briefly, the urine samples were digested with a mixture of nitric acid and hydrogen peroxide (3/2, v/v) for 20 min at 180 °C by microwave. Then the concentrations of Cd were quantified by Agilent 7500 ICP-MS (USA).

2.9. Statistical analysis

SPSS 18.0 software (SPSS, Inc., Chicago, MI, USA) was employed for

Table 2
The change of urine biomarkers of low dose chemicals-treated rats compared with the control rats.

No.	Retention time (min)	Metabolites	Cd	CPF	Cd + CPF
1	13.690	Benzoic acid	↑↑*	↓↓	↓
2	15.293	Glycine	↓↓*	↓	↓*
3	18.970	Aminomalonic acid	↑	↑*	↑*
4	22.281	Creatinine	–	↑↑*	–
5	23.278	Anthranilic acid	–	–	↑*
6	27.901	Ribitol	–	↑	↑*
7	27.956	Galactose	↑↑*	↑↑*	↑↑*
8	32.106	N-Phenylacetyl glycine	–	↑↑*	↑↑
9	33.818	Glucose	–	–	↑↑*
10	36.669	Mannose	↓↓*	↓*	↓*
11	38.727	Indole	↑*	↑*	↑*

Rats were administrated orally with Cd and CPF at doses of 0.7 and 1.7 mg/kg BW/day, respectively, and their combinations for 90 days. After the 90-day experimental treatment, 24-h urine samples were collected and the urine metabolites were measured by GC-MS. Changes are relative to control samples: - , no change; ↓, decrease; ↓↓, more decrease; ↑, increase; ↑↑, more increase. *P < 0.05, compared with the control. Abbreviations: BW, body weight; Cd, cadmium; CPF, chlorpyrifos; Cd + CPF, cadmium plus chlorpyrifos.

the statistical evaluation. The raw data were checked to have a normal distribution by using SPSS. Analysis of variance (ANOVA) test was performed to access significant difference among the groups, followed by post hoc Dunnett's test. The difference was considered significant with the value of $P < 0.05$.

3. Results

3.1. Changes of body weight and organ weight of the rats exposed to the chemicals

To investigate the general toxicity after the exposure of Cd, CPF, and Cd plus CPF, we measured toxic signs, body weights, and relative kidney weight. No deaths were found during the whole course of the study. Rats developed significant tremors and other signs of CPF toxicity such as piloerection and diarrhea at high dose of CPF. No obvious toxic signs of Cd were detected in the rats. The rats in all groups progressively gained weight over the 90-day period. High-dose of CPF exposure caused a slower increase of body weight in rats, while relative kidney weight was not altered in the Cd-treated animals compared with the controls (Suppl. Table S2).

3.2. Cd and CPF treatment altered the metabolomic profiles in urine

Multivariate analysis on the metabolic data was performed to assess the metabolomic differences between the urine samples treated with Cd, CPF, or Cd plus CPF and control (Fig. 1A; Suppl. Fig. S1). The PLS-DA model (R^2X = 0.721, 0.764, and 0.649; R^2Y = 0.302, 0.609, and

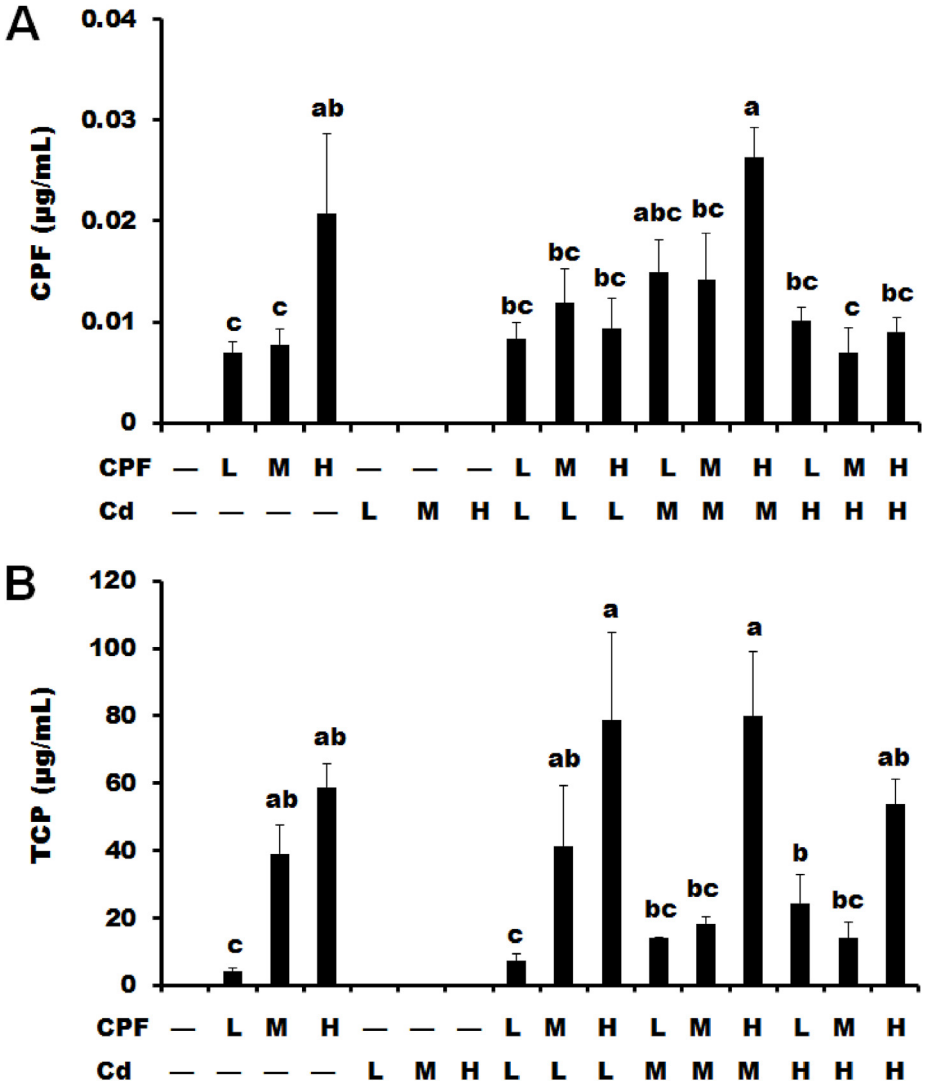

Fig. 5. The levels of CPF and its metabolite TCP in urine of rats. The rats were administrated orally with Cd, CPF at doses of 0.7 and 1.7 mg/kg BW/day (low dose), 2 and 5 mg/kg BW/day (middle dose), 6 and 15 mg/kg BW/day (high dose), respectively, and their mixtures for 90 days. After the 90-day administration, the levels of CPF (**A**) and its metabolite TCP (**B**) in urine of the rats were measured by using HPLC method. Data were expressed as mean ± SE and the differences among different groups evaluated by ANOVA. Different letters above the columns indicate a significant difference among groups ($P < 0.05$), while the same letters indicate no significant difference among groups ($P > 0.05$). Abbreviations: BW, body weight; Cd, cadmium; CPF, chlorpyrifos; TCP, 3, 4, 5-trichloropyrindinol; L, low dose; M, middle dose; H, high dose.

0.423; Q^2 = 0.518, 0.423, and 0.544) showed clear distinctions among the groups control, Cd, CPF, and Cd plus CPF (Fig. 1A; Suppl. Fig. S1). These metabolites were displayed by the heat map, which also showed that the trends of concentration change of urine metabolites after Cd and CPF individual treatment was opposite to the one after Cd plus CPF treatment (Fig. 1B). There were 46 differential metabolites in the urine with VIP > 1 and significant differences ($P < 0.05$) (Fig. 1B; Table 1). Furthermore, the levels of most metabolites in urine increased after the treatment of Cd, CPF, and Cd plus CPF compared with the control, although we noticed previously that the contents of many metabolites decreased in the kidney after treatments in comparison with the control (Xu et al., 2019). And, in the urine, the metabolism of amino acids was also influenced by the treatment of Cd, CPF, and Cd plus CPF. It should be noticed that the concentration of most urine differential metabolites with medium cadmium concentration alone is higher or lower than both the low and high-exposure groups (Fig. 1B), which implies that cadmium probably has a particular dose-response pattern for the urine metabolomics. However, further investigations are needed to explain this phenomenon.

3.3. Identification and validation of the urine metabolites biomarkers

Then, we performed a stepwise optimization algorithm based on receiver operating characteristic (ROC) to identify biomarkers with $0 < AUC < 0.1$ or $0.9 < AUC < 1$ (Fig. 2). We found that different groups had unique biomarkers. Increased concentrations of benzoic acid and decreased concentrations of mannose could discriminate Cd-treated groups from other groups (Fig. 2A and B; Fig. 3A and B). Increased concentrations of N-phenylacetyl glycine and creatinine could describe the significant difference between CPF-treated group and other groups (Fig. 2A and C; Fig. 3C and D). Increased concentrations of anthranilic acid, ribitol, and glucose could diagnose the toxicity of Cd plus CPF (Fig. 2A and D; Fig. 4). The changes of these biomarkers in the low dose of Cd, CPF or Cd plus CPF were shown in Table 2. The result showed that these biomarkers had different changes in the low-dose Cd plus CPF group compared with the low dose of individual Cd or CPF group (Table 2).

3.4. Cd affected the metabolism of CPF in urine

To investigate the mechanisms underlying the interaction of Cd and CPF, we detected the levels of CPF (Fig. 5A) or its metabolite TCP (Fig. 5B) and Cd in urine. However, due to the low dosage of Cd used in the study, Cd could not be detected in urine samples even from the high dose of Cd-treated rats. We found that the urine CPF level in the rats treated with high dose of CPF was significant higher than that in the rats treated with high dose of Cd plus CPF (Fig. 5A), and TCP, the metabolic product of CPF changed accordingly (Fig. 5B). This result suggested that Cd could accelerate the degradation of CPF, which may be one of the reasons for the lower toxicity of the Cd plus CPF groups, compared with the sum of the individual effects. However, the mechanism of the CPF metabolism affected by Cd merits further investigation.

4. Discussion

We all know that heavy metals including Cd and organophosphorus pesticides including CPF are widely distributed in the environment and commonly found together in water, soil, agricultural products, and even foods (Forget et al., 1999; Morgan et al., 2005; Harris et al., 2011; Schwarz et al., 2014). We need to pay more attention to the combined toxic effect of Cd and CPF. It was known that the combined effect of chemicals can usually be defined as additive, synergistic, or antagonistic effect (Crain et al., 2008). In the current study, additive effect is defined when the combined effect equals to the sum of the individual effects (Moser et al., 2003), and the synergistic or antagonistic effect means that the combined effect is greater or less than the sum of their individual effects, respectively, according to early reports (Crain et al., 2008; Folt et al., 1999; Piggott et al., 2015). Interestingly, Cd and CPF had very different modes of action in the organism. CPF mainly depressed the activity of acetylcholinesterase (Cardona et al., 2013; Reiss et al., 2012). Cd mainly caused nephrotoxicity in the body (Johri et al., 2010). Thus, the additive responses would be expected. However, our results indicate that the combinations of Cd and CPF likely have not more effect than the chemicals individually. In our previous studies, we have found that Cd, CPF, alone or in combination at middle- and high-doses could induce histopathological damage of organs and disturbance of amino acids metabolism. All of the results indicated that the response of the combinations of Cd and CPF were less than the additive effect of the individual Cd and CPF treatments (Xu et al., 2015, 2017; 2019). The change of the levels of biomarkers in the urine also showed that the metabolic response of the combinations of Cd and CPF were less than the additive effect. Thus, there was mainly an antagonistic effect between Cd and CPF.

Our results demonstrated that Cd and CPF significantly altered the urine metabolomic profiles. Forty-six metabolites in urine were significantly changed after administrations and could distinguish the chemical treatments from control. In the urine from the Cd-, CPF-, and Cd plus CPF-treated rats, we detected the evident alteration in a series of metabolites, which mainly derived from metabolism of the amino acids. There were also a few changes in energy metabolism and lipid metabolism. The altered metabolic pathways are generally consistent in the liver and kidney (Xu et al., 2017, 2019). The exposure of Cd, CPF, alone or in combination induced the alteration of similar metabolic pathways in the liver and kidneys.

In the present study, we found that many metabolites in urine changed after exposure of Cd and CPF in individual or in combinations. As far as we know, no biofluid biomarkers are now available to diagnose the toxicosis of Cd and CPF individually or in combinations at their low doses after long-term exposure. However, in practice, it is not feasible to measure a large number of metabolites to diagnose the poisoning. Therefore, the ROC analysis is suitable for the identification of the biomarkers to discriminate the exposure of Cd, CPF, and Cd plus CPF from control. The urine biomarkers (benzoic acid and mannose for Cd exposure; phenylacetyl glycine and creatinine for CPF exposure; anthranllic acid, ribitol, and glucose for Cd plus CPF exposure) could identify the toxicosis of Cd, CPF, and combination of the two chemicals with the high predictive power.

The observation of body weight, relative kidney weight in all of the treated rats suggests that Cd and CPF caused unobvious poisoning in the treated rats, except at high dose of CPF. However, many metabolites had significant changes at low dose of the chemicals before the toxic signs and pathological tissue damage could be observed (Xu et al., 2019). Thus, the changes of metabolites were more sensitive for the detection of the toxicity of Cd and CPF, in particular their combined toxicity.

In conclusion, we identified the urine biomarkers with high predictive accuracy for the exposures of Cd, CPF, either alone or in combination by using GC-MS-based metabolomics. These metabolites were independently validated in different treatment groups. These results lay the groundwork for the development of urine-based diagnostic test for the toxicity of Cd and CPF in individual and combination exposures.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

Ming-Yuan Xu: Investigation, Methodology, Writing - original draft. **Ying-Jian Sun:** Conceptualization, Writing - review & editing. **Pan Wang:** Writing - review & editing. **Lin Yang:** Methodology. **Yi-Jun**

Wu: Conceptualization, Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110467.

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