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Changes in metabolic profiles of urine from rats following chronic exposure to anticholinesterase pesticides

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ABSTRACT

Previous studies have demonstrated that the anticholinesterase pesticides chlorpyrifos and carbaryl are neurotoxic to mammals. However, the toxicity of these pesticides to other organs and their potential interactive effects remain unclear. Our goal in this study was to assess the toxicities of ingestion of chlorpyrifos and carbaryl both separately, and in combination to non-nervous systems, especially the effect on urinary metabolic profiles, in rats. Chlorpyrifos, carbaryl and a mixture of these pesticides, were administered orally to Wistar rats for 90 consecutive days. Histopathological examination of liver and kidney and metabonomic analysis based on the urinary ^{1}H nuclear magnetic resonance spectra were used to investigate the toxic effects. The results showed that no histopathological changes were observed in the liver or kidney tissues, but metabonomic analysis revealed alternations in a number of urinary metabolites involving in the energy metabolism in liver mitochondria. Treatment of rats with chlorpyrifos alone led to an increase in creatine, glycine, dimethylglycine, dimethylamine, glutamine, succinate, alanine, lactate, and glucose. The categories of main differential urinary metabolites in carbaryl-treated rats were similar to those in chlorpyrifos-treated rats, whereas the changes were of varying degree. A combination of a low dose of chlorpyrifos and carbaryl resulted in an increase in the levels of main urinary metabolites compared to the controls, and the increase in signal intensity of the main metabolites was lower than that in the rats exposed to chlorpyrifos or carbaryl alone. All above results suggest that chronic exposure to chlorpyrifos and carbaryl alone, or in combination could cause disturbance of metabolic function in liver mitochondria and renal failure. Overall, we have shown that urine metabonomic analysis is non-invasive, sensitive, and relatively fast for assessing the individual or mutual effects following exposure to pesticides.

1. Introduction

Generally risk assessment in human toxicology has focused on acute or repeated exposure to single pollutants. However, environmental exposures to multiple pesticides for humans are usually at low levels and for long periods of time. It is generally considered that conventional toxicological methods are not suitable for toxicological evaluation and risk assessment of pesticide mixtures, especially at lower concentrations.

Metabonomics combines the techniques of high resolution nuclear magnetic resonance (NMR) and pattern recognition technology to rapidly evaluate the metabolic status of an animal. This allows the onset, duration, severity and target organ localization to be determined from peripheral samples such as urine, serum, and other body fluids. The information obtained from metabonomics is complementary to that from proteomics and genomics and is applicable to a wide range of problems in the preclinical toxicological, environmental, clinical, and biomedical areas. To date, metabonomics has had perhaps its greatest impact in the area of toxicology, particularly preclinical toxicology [1–4]. It is now recognized as an independent and widely used technique for identifying target organ toxicity [5–9] and evaluating the toxicities of candidate chemical agents [10–14].

Abbreviations: CAR, carbaryl; CPF, chlorpyrifos; D_2O, deuterium oxide; DMA, dimethylamine; DMG, dimethylglycine; FIDs, free induction decays; NMR, nuclear magnetic resonance; PCA, principal components analysis; SIMCA, soft independent modeling of class analogy; TCA, tricarboxylic acid; TMAO, trimethylamine oxide; TSP, 2,2′,3,3′-deuterotrimethylsilylpropionic acid.

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Chlorpyrifos, an organophosphorus pesticide, and carbaryl, an N-methyl carbamate pesticide, are widely used in indoor and outdoor applications for agricultural, commercial, medicinal, and veterinary purposes. Although chlorpyrifos and carbaryl are generally regarded as having high efficiency and low residue, they pose considerable risk to human health and ecosystems. Knowledge of the mechanisms through which these pesticides cause toxicity is essential to predict their damage to humans and the environment.

Until now, most studies on chlorpyrifos and carbaryl have emphasized their acute and developmental neurotoxicity. Recent studies indicated that chlorpyrifos can affect the brain development and the cognition ability of animals [15,16] and human children [17]. In addition, adverse effects on other systems, such as hepatotoxicity [18], immunotoxicity [19], reproductive toxicity [20] and cardiac dysfunction [21] have been established. Other studies indicate that chlorpyrifos and carbaryl may have carcinogenic effects [11]. There have been some in vitro studies on interactive effects between organophosphates and carbamates [22–25], but little in vivo research on this has been done [26,27].

Although the acute neurotoxicity of chlorpyrifos and carbaryl has been well established, the mechanisms through which chronic exposure to a combination of these pesticides causing toxicity have not been completely elucidated [28–33]. Our laboratory investigated metabolic profiles in serum from rats and found that an analysis of metabolic profiles can make exceptional contributions to the understanding of the individual or mutual effects following exposure to a low dose of pesticides [34]. In this study, we analyzed the metabolic profile of urine from rats following chronic exposure to chlorpyrifos, carbaryl, and a mixture of these pesticides by NMR-based metabonomic approach to evaluate toxicities of pesticides with no obvious neurological signs.

2. Materials and methods

2.1. Chemicals

Chlorpyrifos (CPF) (purity >95%) was obtained from Shuangma Chemical Co. Ltd. (Jiangsu, China). The carbamate carbaryl (CAR) (purity >99%) was obtained from HailiGuixi Chemical Pesticide Co. Ltd. (Jiangxi, China). 2,2′,3,3′-Deuterotrimethylsilylpropionic acid (TSP) and deuterium oxide (D_2O) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals and administration

Six to eight week old Wistar rats were obtained from WeitongLihua Laboratory Animal Technology Company (Beijing, China). They were individually housed in stainless steel, wire-mesh cages. Animals were acclimated for at least 1 week prior to the start of the experiment. During the experiment, rats were kept at 22 ± 2 °C and 50–60% humidity under a light/dark cycle of 12 h and had free access to water and food. All animal procedures were performed in accordance with the current China legislation and approved by the Institute of Zoology Animal and Medical Ethics Committee.

Fifty male and fifty female Wistar rats were randomly assigned to 10 groups, each group comprised of 5 male and 5 female rats. For each treatment, three groups were randomly assigned to one of the three levels of treatment (low, middle, and high doses) for each pesticide and a combination of the two pesticides, with one group assigned as the control. Based on data from previous studies that showed the acute oral half-lethal doses (LD_{50}) of chlorpyrifos and carbaryl were 163 mg/kg and 850 mg/kg for male [35], and 135 mg/kg and 500 mg/kg for female rats [36], respectively, we chose the doses of 1/125, 1/50, and 1/20 LD_{50} of each pesticides as low-, medium-, and high-dose for the pesticides treatment groups in this study. Therefore, male rats in the low, medium, and high chlorpyrifos treatment groups received 1.30, 3.26, and 8.15 mg/kg/day chlorpyrifos respectively, and females 1.08, 2.70, and 6.75 mg/kg/day respectively. Male rats in the low, medium, and high carbaryl treatment groups received 6.8, 17.0, and 42.5 mg/kg/day carbaryl respectively, and females 4.0, 10.0, and 25.0 mg/kg/day respectively. Rats in the combined chlorpyrifos and carbaryl (CPF/CAR) treatment groups of low, medium, and high doses were treated with a mixture of equivalent amounts of low, medium, and high doses of either chlorpyrifos or carbaryl respectively. All pesticides were dissolved in corn oil and were applied per os by gavage in a volume of 1 ml/kg. The control group received an equivalent volume of corn oil. Pesticides were given daily for 90 consecutive days.

2.3. Sample preparation

Individual urine samples of 24 h following the final dose of 90-days were collected into ice-cold vessels containing 1% sodium azide [37,38]. Supernatant liquor was obtained by centrifugation, and then stored at −80 °C until required for NMR spectroscopic analysis. Animals were sacrificed by anesthesia with barbitalum natricum. During the process, blood samples were collected. For each blood sample, serum samples were separated by centrifugation for biochemical measurement.

2.4. Cholinesterase activity assays

Serum cholinesterase (ChE) activity was assayed by standard spectrophotometric methods on an Autolab PM4000 Automatic Analyzer (AMS company, Rome, Italy). Data were presented as mean ± SE.

2.5. Histopathology

Liver and kidney tissue samples were fixed in 10% formalin, processed into 4 μm paraffin sections and then stained with hematoxylin and eosin for histopathological assessment.

2.6. ^1H NMR spectroscopy of urine samples

Urine samples were centrifuged for 10 min (1300*g*, 4 °C), and 500 μl of the resultant supernatant mixed with 400 μl buffer solution (0.2 M sodium phosphate, pH 7.4) in a microcontainer. The resulting solution was left to stand for 10 min before being centrifuged at 13,000*g* for 10 min to remove any precipitates. Aliquots of the resulting supernatant (600 μl) were placed in 5 mm NMR tubes to which a 50 μl solution of TSP in D_2O was added (final concentration, 1 mM). The D_2O provided the deuterium lock signal, and TSP the chemical shift reference (δ0.0), required by the NMR spectrometer.

^1H NMR spectra of all urine samples were obtained at 599.69 MHz on a Varian INOVA 600 NMR spectrometer (Palo Alto, CA, USA). A one dimensional spectrum was acquired using a standard NOESY pulse sequence with water suppression during a relaxation delay of 2 s and the mixing time of 150 ms. Sixty-four free induction decays (FIDs) were collected into 64K data points using a spectral width of 7002.8 Hz, an acquisition time of 4.68 s and a total pulse recycle delay of 6.68 s. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.5 Hz prior to Fourier transformation. All spectra were referenced to the CH_3 resonance of creatinine at δ3.05.

2.7. Data reduction and principal components analysis

All NMR spectra were phased and baseline corrected and then data-reduced to 225 integrated regions of equal width (0.04 ppm) corresponding to the region of δ9.4 to δ0.4 using the VNMR 6.1C software package (Varian Inc., Palo Alto, CA, USA). The region of δ6.2–4.6 was excluded from the recognition analysis to remove the possibility of including signals from residual water or urea. The area for each segmented region of chemical shift was calculated, and the integral values contributed to an intensity distribution of the whole spectrum. All regions of the spectra were then scaled to the total integrated area of the spectra to reduce any significant concentration differences from individual animals. The values of all NMR data were mean-centered and Pareto-scaled prior to the principal components analysis (PCA) using the soft independent modeling of class analogy (SIMCA) software package (Version 10, Umetrics AB, Umea, Sweden). Pareto scaling gives each variable a variance numerically equal to its standard deviation. Scores plots based on NMR spectra were used to visualize the separation between pesticide treatment groups and control group, and the loadings plots identified the NMR spectral regions that contribute most to the separation of samples in the scores plots. Each spectral region corresponded to a particular metabolite. An increase or a decrease of the assigned metabolites presented the changes of these metabolites in rats treated with chlorpyrifos and carbaryl alone, or in combination compared to the controls.

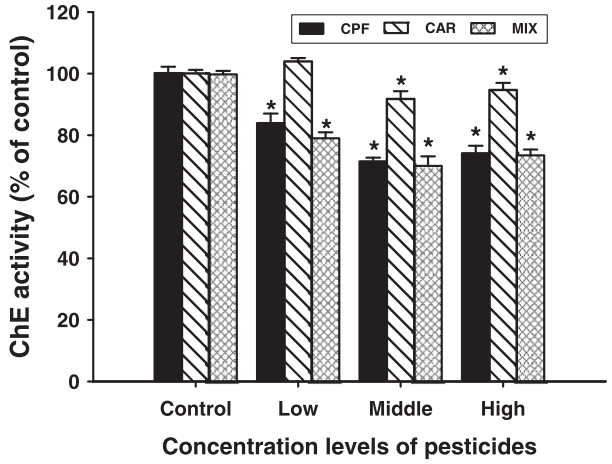

Fig. 1. The effect of anticholinesterase pesticides on the serum ChE activity in rats. Rats were administered orally with chlorpyrifos (CPF) and carbaryl (CAR) at respective doses of 0 mg/kg/day (control), 1.3 and 6.8 mg/kg/day (low), 3.26 and 17 mg/kg/day (middle), 8.15 and 42.5 mg/kg/day (high) and their mixtures for 90 consecutive days. Data were presented as mean ± SE. Statistical analysis was performed by ANOVA followed by Dunnett's test. $^{*}p < 0.05$ for significant difference from control group.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was used to assess for statistical significance of the integral values of assigned spectral peaks from metabonomic analysis. If significant effects were identified ($p < 0.05$), post hoc multiple analyses were performed using Dunnett's test.

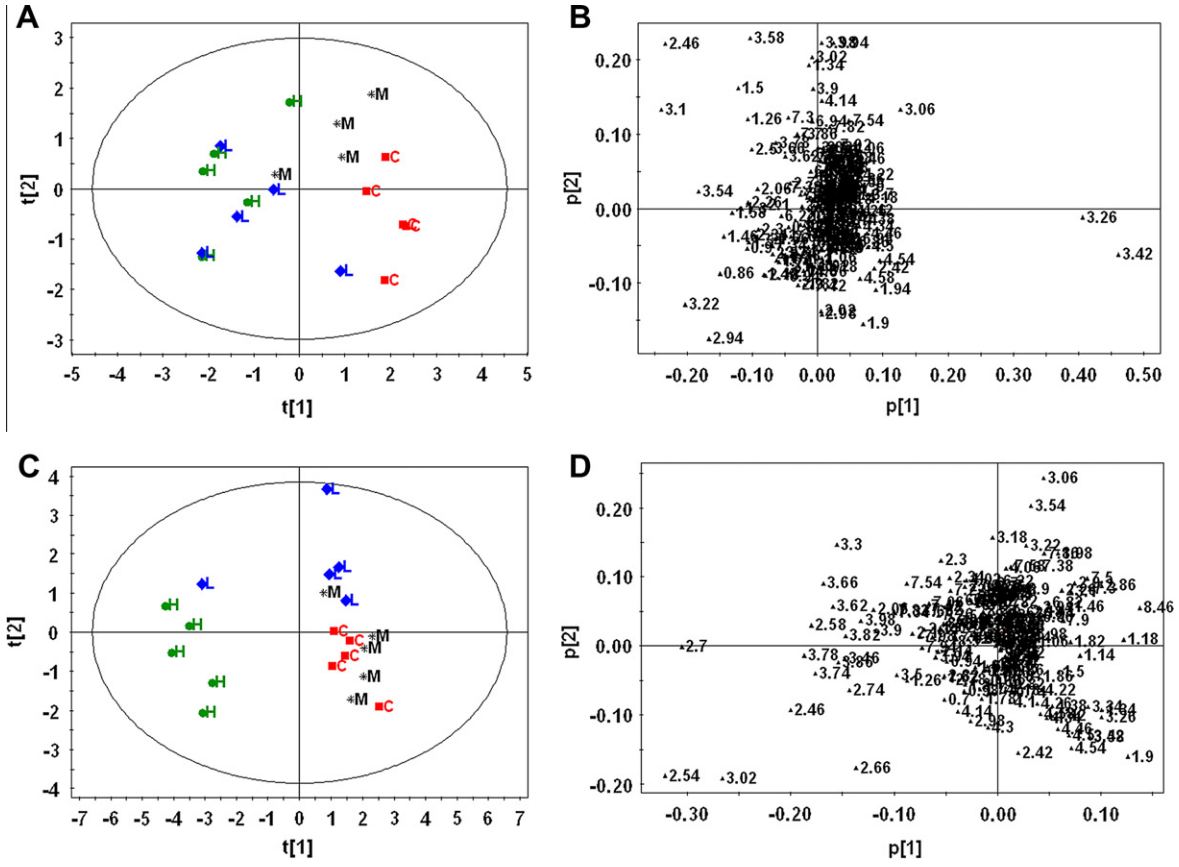

Fig. 2. PCA scores plots (A, C) and loadings plots (B, D) based on the ^{1}H NMR spectra of urine from male (A, B) and female (C, D) rats treated with chlorpyrifos (CPF) for 90 days. The male rats were given chlorpyrifos at a dosage of 0 mg/kg/day (C), 1.3 mg/kg/day (L), 3.26 mg/kg/day (M), and 8.15 mg/kg/day (H), and the females at 0 mg/kg/day (C), 1.08 mg/kg/day (L), 2.7 mg/kg/day (M), and 6.75 mg/kg/day (H).

3. Results

3.1. Serum ChE activity

Wistar rats treated with pesticides for 90 consecutive days did not demonstrate overt signs of toxicity. However, rats dosed with chlorpyrifos, carbaryl, and a mixture of these two pesticides demonstrated significantly lower serum ChE activity compared to controls, with the exception of rats receiving the low dose of carbaryl (Fig. 1). These rats displayed no significant changes in ChE activity compared to control rats.

3.2. Histopathology

No significant histopathological changes were found in liver and kidney tissues of rats treated with chlorpyrifos and carbaryl alone, or in combination for 90 consecutive days (data not shown).

3.3. Changes in urinary metabolites induced by chlorpyrifos or carbaryl

The biochemical effects of chlorpyrifos or carbaryl were investigated by applying PCA to the ^{1}H NMR data sets from the controls and the treatment rats. The scores plots revealed clear separation between the controls and all the treatment groups (Figs. 2A and 3A, C). Data points of low- and middle dose groups overlapped (Fig. 3C), which suggested that these groups had similar metabolic profiles. The loadings plots (Figs. 2B, D and 3B, D) identified the spectral regions that contribute most to the separation of samples in the scores plots. Biomarkers of pesticide induced toxicity were identified on this basis.

Levels of creatine (δ3.06), glycine (δ3.58), dimethylglycine (DMG, δ2.94), dimethylamine (DMA, δ2.74), glutamine (δ2.46), succinate (δ2.42), alanine (δ1.5), lactate (δ1.34, δ4.14), choline (δ3.22), N-acetyl group (δ3.1), glycoprotein (δ2.06), valine (δ2.26), and glucose (δ3.54, δ3.62, δ3.66, δ3.74, δ3.78, δ3.82, δ3.86, δ3.9) increased in chlorpyrifos treatment groups relative to the controls (Fig. 2B and D).

The categories of main differential urinary metabolites in carbaryl-treated rats were similar to those in chlorpyrifos-treated rats. In addition, the levels of phenylalanine (δ7.38, δ7.42) and methylguanidine (δ2.82) increased dramatically in the urine of male rats (Figs. 2B and 3B). Signals from male rats were stronger in the low-dose group whereas signals from female rats were stronger in low- and middle dose groups.

3.4. Dose-dependent changes in urinary metabolites induced by equitoxic mixtures of chlorpyrifos and carbaryl

Fig. 4 shows the NMR spectra of urine metabolites from male rats after treatment with a mixture of chlorpyrifos and carbaryl. The urine of low- and middle dose groups displayed relatively low signal intensity and the high-dose group had relatively high signal intensity. There was clear separation between the control and all treatment groups. Signal intensity of the low- and middle-dose groups overlapped in male rats (Fig. 5A) and the medium- and high-dose groups overlapped in female rats (Fig. 5C), suggesting they had similar metabolic profiles. The

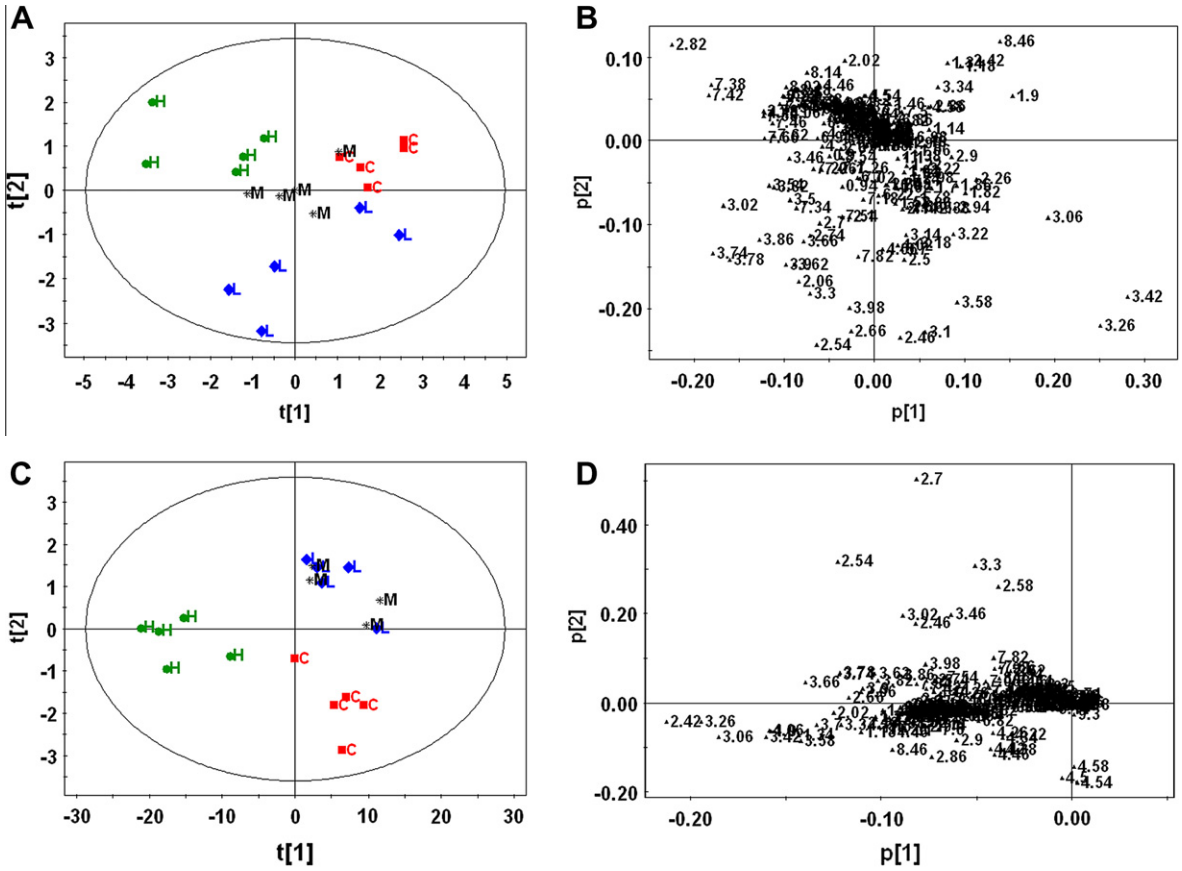

Fig. 3. PCA scores plots (A, C) and loadings plots (B, D) based on the ^{1}H NMR spectra of urine from male (A, B) and female rats (C, D) treated with carbaryl (CAR) for 90 days. The males were given at a dosage of 0 mg/kg/day (C), 6.8 mg/kg/day (L), 17 mg/kg/day (M), and 42.5 mg/kg/day (H), and the females at 0 mg/kg/day (C), 4 mg/kg/day (L), 10 mg/kg/day (M), and 25 mg/kg/day (H).

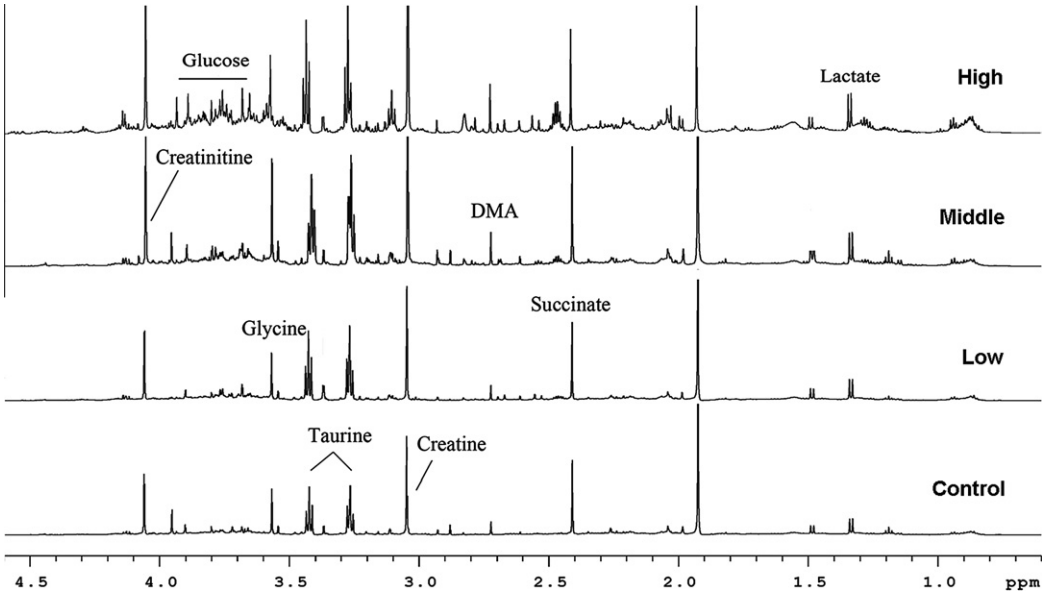

Fig. 4. Representative 600 MHz ^{1}H NMR spectra of urine from male rats treated for 90 days with the combination of chlorpyrifos (CPF) and carbaryl (CAR). Rats were administered orally with CPF and CAR at respective doses of 0 mg/kg/day (control), 1.3 and 6.8 mg/kg/day (low), 3.26 and 17 mg/kg/day (middle), 8.15 and 42.5 mg/kg/day (high).

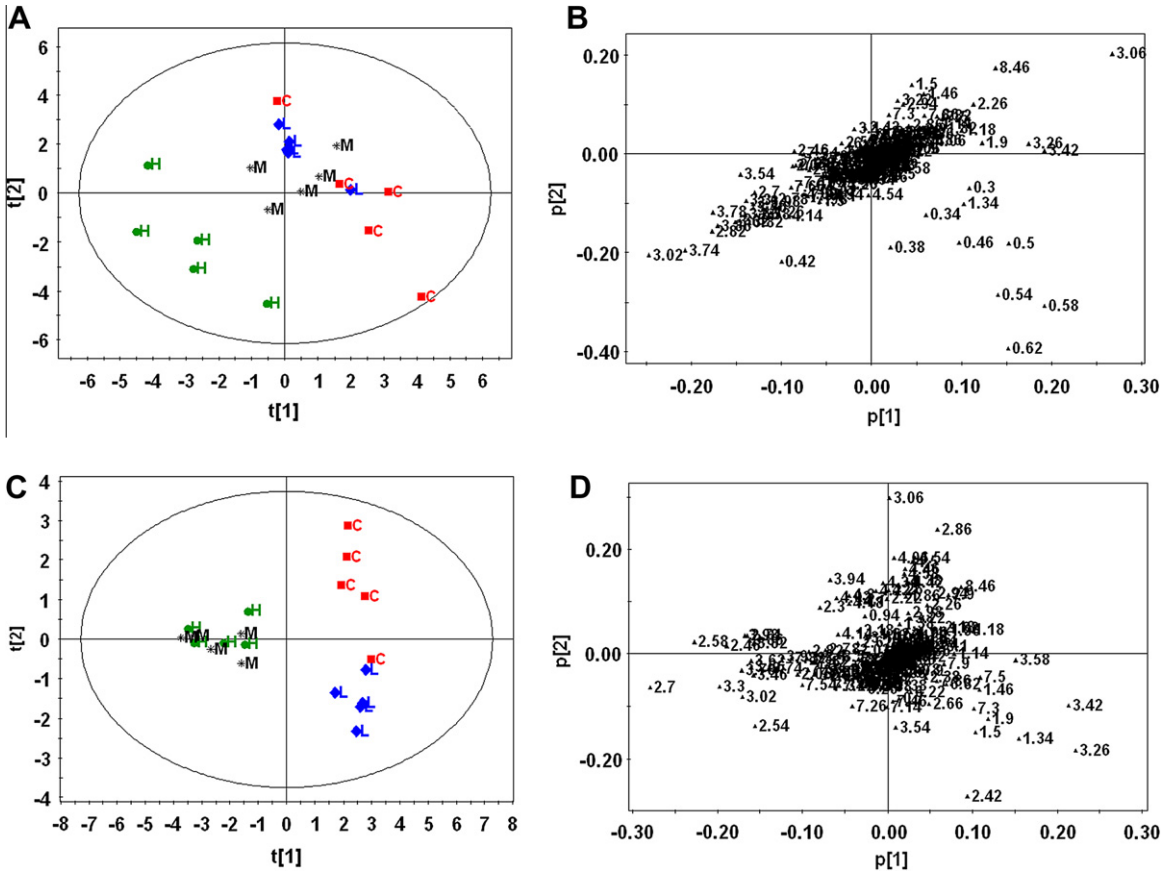

Fig. 5. PCA scores plots (A, C) and loadings plots (B, D) based on the ^{1}H NMR spectra of urine from male (A, B) and female (C, D) Wistar rats treated with chlorpyrifos (CPF) and carbaryl (CAR) in combination for 90 days. The male rats were administered orally with chlorpyrifos and carbaryl at respective doses of 0 mg/kg/day (C), 1.3 and 6.18 mg/kg/day (L), 3.26 and 17 mg/kg/day (M), 8.15 and 42.5 mg/kg/day (H), and the females at 0 mg/kg/day (C), 1.08 and 4.00 mg/kg/day (L), 2.7 and 10 mg/kg/day (M), 6.75 and 25.00 mg/kg/day (H) respectively.

PCA loading plots (Fig. 5B and D) show the spectral regions that contribute the most to the separation of samples in the scores plots.

Levels of endogenous urinary metabolites increased in all treatment groups compared to the controls. The major metabolites including creatine, glycine, DMA, citrate, succinate, glucose, lactate

and N-acetyl group markedly increased in high dose group of male rats. Other metabolites, such as DMG, alanine, and glycoprotein, were detected in the urine of female rats. Levels of alanine and lactate in female rats were markedly higher in the low-dose group compared to males of the low-dose group (Table 1). Fig. 6 shows the main differential data points from the rats treated with low dose of chlorpyrifos and carbaryl, or in equitoxic combination. Most changed urinary metabolites such as citrate, glucose, glycoprotein, 2-oxoglutarate, and alanine in the low dose mixture group increased compared to the controls, and the alterations were lower than those of rats exposed to chlorpyrifos or carbaryl alone (see Table 1).

4. Discussion

In this study, after treatment with chlorpyrifos and carbaryl alone, or in combination for 90 consecutive days, the rats displayed

Table 1
^{1}H chemical shifts and assignments for endogenous urinary metabolites from rats treated with CPF, CAR and their equivalent low toxicity mixture.

Major metabolites	^{1}H Chemical shifts (ppm)	Male			Female		
		CPF	CAR	MIX	CPF	CAR	MIX
Citrate	2.54, 2.66 (d)	−/↑	↑↑	↑	−/↑	↑↑*	↑
Glucose	3.62, 3.66, 3.74, 3.78, 3.86 (m)	↑	↑↑*	−/↑	↑	↑↑*	−/↑
Glycoprotein	2.06 (s)	↑	↑↑*	↑	↑	↑↑*	−/↑
2-Oxoglutarate	2.46, 3.02 (t)	↑	↑↑	−/↑	−/↑	↑↑*	↑
Taurine	3.26, 3.42 (t)	↓↓	−/↑	↓	↓↓	↓	↑
Glycine	3.58 (s)	↑↑	↑	−/↓	↓↓	↓	−/↑
Lactate	1.34 (d)	↓	↓	↓↓*	↓	↓↓*	↑↑*
Alanine	1.5 (d)	↑↑	−/↓	↑	−/↓	↓*	↑
N-acetyl group	3.1 (m)	↑↑*	↑	−/↑	−/↑	↓*	−/↓
Choline	3.22 (s)	↑↑	−/↑	↑	↑	−/↓	−/↓

Note: s, singlet; d, doublet; t, triplet; m, multiplet. Changes are relative to control samples: –, no change; ↓, decrease; ↑, increase; ↑↑, more increase. The integral values for each segmented region of chemical shift were represented for each metabolite resonance. One-way ANOVA was used to assess for statistical significance of the integral values of assigned spectral peaks among the groups.
* $p < 0.05$, compared with the control.

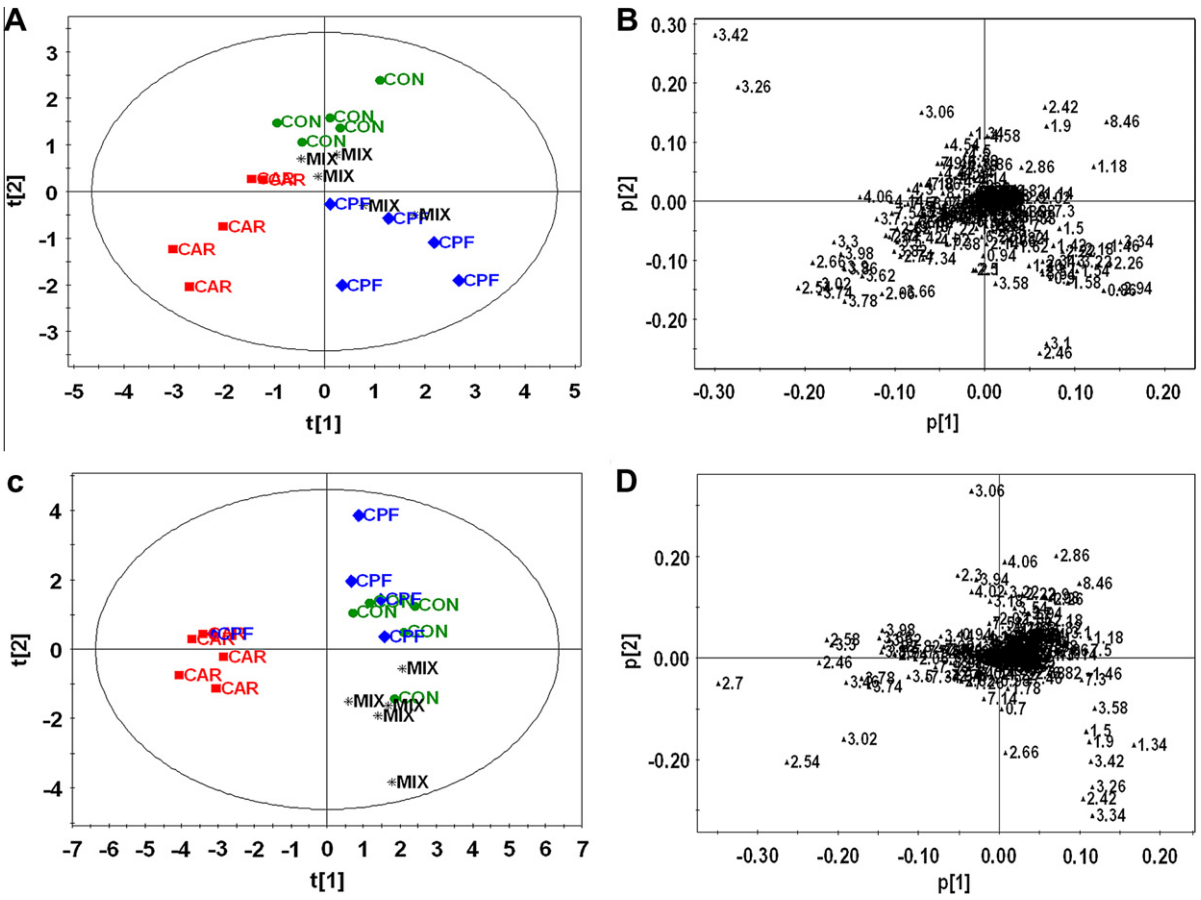

Fig. 6. PCA scores plots (A, C) and loadings plots (B, D) based on the ^{1}H NMR spectra of urine from male (A, B) and female (C, D) Wistar rats treated with a low dose of chlorpyrifos, carbaryl alone and in combination for 90 days. The male rats were dosed at 0 mg/kg (CON), 1.3 mg/kg chlorpyrifos (CPF), 6.8 mg/kg carbaryl (CAR), and 1.3 mg/kg CPF plus 6.8 mg/kg CAR (MIX); and the females at 0 mg/kg (CON), 1.08 mg/kg chlorpyrifos (CPF), 4.00 mg/kg carbaryl (CAR), and 1.08 mg/kg CPF plus 4.00 mg/kg CAR (MIX), respectively.

no significant histopathological changes in the liver or kidney tissues. This suggested that the subchronic toxicity induced by the pesticides was not enough to cause observed pathological changes in the low-dose group. In addition, the result of serum ChE activity assay showed that the rats treated with low-dose of carbaryl did not exhibit any significant change in ChE activity although the activity of the enzyme from other dosed rats was significantly inhibited. However, obvious changes of metabolites levels in urine were observed in the low dose of the pesticide-treated rats. We proposed that such changes in the urine metabolic profiles occurred earlier than those of the serum biochemical parameters after pesticide exposure. We may be able to detect anticholinesterase pesticides toxicity by determining the urine metabolic profiles of exposed animals with even no obvious change of blood parameters found by regular blood test including ChE activity assay.

The metabonomic analysis of urine showed that a number of metabolites increased in rats treated with pesticides, including glycine, succinate, citrate, 2-oxoglutarate, glutamine, alanine, lactate, DMG, DMA, and glucose. Succinate, citrate, and 2-oxoglutarate are intermediates in the tricarboxylic acid (TCA) cycle process which is localized mainly in liver mitochondria. The increased amount of these metabolites in the urine of rats suggested that the activities of mitochondrial enzymes involved in the TCA cycle were affected by these pesticides. Elevated levels of choline in the urine indicated that chlorpyrifos or carbaryl might induce hepatocyte injury by disturbing the amino acid metabolism and fatty acid metabolism. Yet, the alterations of urine metabolites reflecting liver injury in chlorpyrifos treatment group may not only be due to the toxic effect of CPF alone, but probably due to the effect of its metabolic product, because it has been demonstrated that CPF is metabolically oxidized to chlorpyrifos oxon by P450 isozymes in liver microsomes, which may produce higher systemic oxon levels and increased toxicity [39]. The increase of urinary glucose might mean a decrease in reabsorption of low molecular weight compounds, which was a typical manifestation of non-specific proximal tubular damage [40], and trimethylamine oxide (TMAO), dimethylamine (DMA), dimethylglycine (DMG), and acetate were important NMR markers of renal papilla lesions [41]. From the previous study, chlorpyrifos treatment is effective in altering the functional and structural integrity of kidney in a dose-related manner [42,43]. It is therefore possible that these pesticides may induce oxidative stress in kidney tissues and cause nephrotoxicity, particularly upon chronic exposures. Therefore, the increase in the urinary metabolites DMG, DMA, and glucose in rats could indicate renal dysfunction induced by these pesticides [44,45].

Previous studies indicated that a combination of chlorpyrifos and carbaryl has similar physiological and biochemical effects to chlorpyrifos and carbaryl alone but the mechanism for these combined effects is not identical to that of either chlorpyrifos or carbaryl. In this study, we found that treatment with a combination of chlorpyrifos and carbaryl caused levels of the main endogenous urinary metabolites to increase markedly in the high dose group. An in vitro study has shown that chlorpyrifos interferes with the metabolism of carbaryl by P450 [23]. In our study, the urinalysis of rats in the low-dose mixture group indicated that an interaction between chlorpyrifos and carbaryl might be due to lack of neither additive nor synergistic effect on the metabolism of liver or kidney.

In conclusion, our results show that energy metabolism and amino acid metabolism could be affected by these pesticide exposures before pathological injury occurs. Additionally, compared to the traditional histopathology and serum biochemistry tests, urinary metabonomic analysis is non-invasive and relatively fast for assessing the individual or mutual effects on rats following exposure to pesticides. This method may have the potential to evaluate the risk of residual pesticides in the environment to human health.

Acknowledgments

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