Metabonomic analysis of quercetin against the toxicity of chronic exposure to a mixture of four organophosphate pesticides in rat plasma

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Abstract
1. A metabonomics approach was performed to investigate the effect of quercetin on the toxicity of chronic exposure to a mixture of four organophosphate pesticides (OPs) at their corresponding no-observed-adverse-effect level (NOAEL). The rats were divided into six groups (n = 10/group): control, two different doses of quercetin, OPs mixture and different doses of quercetin plus OPs mixture-treated groups.
2. Nine metabolites, including two quercetin metabolites and seven endogenous metabolites were identified in plasma. The intensities of metabolites significantly changed in the OP mixture-treated group compared with the control group (p < 0.01), such as lysoPE (16:0/0:0), lysoPC (17:0/0:0), lysoPC (15:0/0:0) and 4-pyridoxic acid, significantly increased; by contrast, the intensities of arachidonic acid and citric acid significantly decreased. Anomalous intensity changes in aforementioned metabolites were alleviated in the OP mixture plus 50 mg/kg bw/d quercetin-treated group compared with the OP mixture-treated group (p < 0.05).
3. The results indicated that quercetin elicited partial protective effects against the toxicity induced by a mixture of OPs, which include regulation of lipid metabolism, improvement of tricarboxylic acid (TCA) cycle disorders, enhancement of antioxidant defence system to protect the liver.

Keywords
Metabonomics, no observed adverse effect level, organophosphate pesticide mixture, quercetin, rat plasma, UPLC-MS

Introduction
Organophosphate pesticides (OPs) are widely used for agricultural, commercial, medicinal and veterinary purposes worldwide (Joshi & Rajini, 2009; Yarsan & Cakir, 2006). Given that different pesticides are simultaneously being used against pests and weeds is a possibility. The wide application of OPs causes varying degrees of environmental pollution, resulting in an inevitable exposure of human population to multiple pesticides from various sources. For nonprofessional populations, exposure to OPs occurs mainly through residues in food and water. As such, potential health risks of exposure to OPs have been extensively investigated in environmental and toxicological research.

OP compounds are primarily recognized because these substances induce toxic effects on mammals through acetylcholinesterase (AChE) inhibition: thus, acetylcholine accumulates and cholinergic, muscarinic and nicotinic receptors become activated. Over the past decade, considerable research has been carried out to conduct risk assessment of pesticide mixtures, which indicated that pesticide mixtures showed joint toxicity effects (Blanchard, 2002; Coffey et al., 2005; Du et al., 2014; Karanth et al., 2001, 2004; Moser et al., 2005). Therefore, studies to reduce toxicity induced by pesticide mixtures have been a public concern.

Quercetin is a member of a group of polyphenolic compounds known as flavonoids. This flavonoid is ubiquitously present in foods, including vegetables, fruit, tea and wine. Quercetin exhibits various biological actions, such as anti-oxidation, anti-inflammation, anti-anaphylaxis and DNA protection (Gonzalez-Gallego et al., 2010; Kelly, 2011). Some epidemiological studies have also found that an increase in the intake of dietary quercetin can reduce the risk of cardiovascular disease, some tumor, osteoporosis and aging (Bodewes et al., 2011; Kris-Etherton et al., 2002). Especially, the role of quercetin-scavenging free radicals play an important part in these beneficial health effects (Justino et al., 2004). Animal experiments have indicated that quercetin can protect organisms from toxic effects exogenous poisons, such as pesticides and hazardous metals (Barcelos et al., 2011; Janisch et al., 2004; Li et al., 2015). Studies have mainly focused on toxic effects of quercetin against a single kind of harmful substances and on a tissue or organ level; however, few studies have investigated the impacts of quercetin on global

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metabolism in rats exposed to multiple hazardous substances. In fact, the toxicity of OPs elicits negative effects on many organs and systems. Although the effect of quercetin on toxicity induced by a single species of dichlorvos has been studied using metabonomics technology in our previous study (Zeng et al., 2014), we speculate that its effect on toxicity may be different under the condition of exposure to multiple pesticides at the same time. As such, a metabonomics method should be performed to visualize changes in global circulating metabolites and investigate related mechanisms of quercetin on OP mixture-induced toxicity at an organism level.

Metabonomics is defined as the quantitative measurement of the dynamic, multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification (Nicholson et al., 1999). Endogenous metabolite profiles from a biological sample (typically urine, serum or biological tissue extract) can be determined and interpreted; these procedures have provided opportunities to investigate changes induced by external stimuli or enhance our knowledge of inherent biological variation within subpopulations. Thus, metabonomics has been widely applied to investigate the biochemical effects of toxins. This approach is considered as an independent and widely used technique to identify target organ toxicity via biomarkers and through systematic evaluation of chemical toxicity (Du et al., 2014; Wang et al., 2009).

The interaction between chemicals in a mixture can alter chemical metabolism (Wilkinson et al., 2000). Animals and humans are frequently exposed to multiple chemicals in the body. It is reported that flavonoids in human diets may have the similar pathway with pesticides and some other man-made chemicals (Safe, 1998). Life is a complete system; the regulation of biological molecules is interrelated and interdependent in complex organisms, and changes in each body part can affect alterations in life activities; as a result, metabolites in organisms also change. Considering that the intake of quercetin, along with low-level exposure to pesticides, can modify tissue antioxidants (Zeng et al., 2014), we infer that quercetin can possibly affect OP mixture-induced toxicity at an organism’s metabolism level. Dichlorvos, acephate, dimethoate and phorate are among the most widely used OPs in agriculture; public health programs in China pay more attention to the OPs residues in foods (Zhao et al., 2003). In a previous study, the combined toxic effects of these pesticides on rat plasma were evaluated through metabonomic technology at their corresponding no-observed-adverse-effect level (NOAEL); results show that the mixture of these pesticides elicits a combined toxic response (Du et al., 2014). Using the same method, the current study aims to investigate whether quercetin elicits a protective effect against OP mixture-induced toxicity and provide further insights into related mechanisms.

**Materials and methods**

**Chemicals and reagents**

Dichlorvos (purity > 95%), dimethoate (purity > 98%) and acephate (purity > 95%) were supplied by Hebei Century Insecticides, Ltd (Hebei, China), Hunan Haili Changde Pesticides Chemical Industry, Ltd (Hunan, China), and Nantong Weilike Chemical Industry, Ltd (Nantong, China), respectively. The analytical standard of phorate was obtained from Sigma–Aldrich (St Louis, MO). Quercetin (95% purity) was purchased from Sigma–Aldrich (Steinheim, Germany). HPLC-grade methanol and acetonitrile were obtained from Dikma Science and Technology, Co. Ltd. (California). The standard arachidonic acid (99% purity), glycocholic acid (97% purity), citric acid (99% purity), 4-pyridoxic acid (98% purity), isorhamnetin (98% purity) and quercetin-3-glucuronide (95% purity) were purchased from Sigma (Steinheim, Germany). The standard lysoPE (16:0/0:0) (99% purity), lysoPC (17:0/0:0) (99% purity), lysoPC (15:0/0:0) (99% purity) were purchased from Aladdin (Shanghai, China). The assay kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholinesterase (ChE), albumin (ALB), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG) and total cholesterol (TCHO) were purchased from BioSino Bio-technology and Science Inc. (Beijing, China). The kits for superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), malondialdehyde (MDA), phospholipase A2 (PLA2) and free fatty acids (FFA) were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Distilled water was filtered using a Milli-Q system (Millipore, Billerica, MA). Leucine enkephalin was obtained from Sigma-Aldrich. Carboxymethylated cellulose (CMC) and other chemicals were of analytical grade.

**Animal handling and test design**

For the quercetin treatment groups, two dose groups were designed as follows: low-dose (10 mg/kg-bw/d), high-dose (50 mg/kg-bw/d) groups and the low dose was equivalent to the Chinese exposure level (Zhang et al., 2010). Quercetin was dissolved in 0.5% CMC according to the dose of the treatment group and was administered to the rats via gavage once a day. OPs were administered to the rats at the dose equal to NOAEL: dichlorvos (2.4 mg/kg-bw/d), dimethoate (0.04 mg/kg-bw/d), acephate (0.5 mg/kg-bw/d) and phorate (0.05 mg/kg-bw/d). The NOAEL was obtained from International Program on Chemical Safety Information from Intergovernmental Organizations (IPCS INCHEM). OPs mixture dose was selected on the basis of our previous study, which indicated that exposure to a mixture of four OPs may cause hazardous effects at specific NOAEL (Du et al., 2013, 2014). OPs was diluted in drinking water according to the expected dose of the treatment groups.

A total of 60 male Wistar rats (180–200 g) were purchased from Vital Laboratory Animal Technology Co. Ltd. (Beijing, China). All rats were housed individually in metabolism cages for 7 days before treatment. Temperature and humidity were regulated at 22 ± 2 °C and 45 ± 15%, respectively. A cycle of 12-h light/12-h dark was established. Food and drinking water was provided ad libitum. After the adaptation, all rats were divided randomly into six groups (each consisting of 10 rats): control group (C), low dose of quercetin group (Q1), high dose of quercetin group (Q2), OPs mixture-treated group (P), low dose of quercetin plus OPs-treated group (PQ1) and high dose of quercetin plus OPs-treated group (PQ2). Each group had a similar average initial body weight. The treatment was administrated continually for 12 weeks. The body weight of each rat was measured every week and daily water
consumption of each rat was recorded during the test. The water consumption of all rats at different time points as seen in the Supplementary Table 1. Water consumption between the experiment groups and the control group showed no significant changes during the experiment ($p > 0.05$).

All experimental procedures were conducted in conformity with the institutional guidelines for the care and use of laboratory animals of Harbin Medical University (Heilongjiang, China), and conformed to the National Research Council’s Guide for the Care and Use of Laboratory Animals.

**Sample collection, storage and preparation**

After the rats were anesthetized with pentobarbital via intraperitoneal injection, blood samples were collected from the aorta abdominatis. Serum is obtained by centrifugation at 835 g for 15 min and allowing it to clot naturally. The serum supernate was immediately analyzed for biochemical analysis using Hitachi7100 automated biochemical analyzer (Hitachi Co., Shiga, Japan). Plasma is prepared by mixing blood with an anticoagulant (heparin) followed by centrifugation at 4 °C to separate the plasma from the formed components of the blood (red and white blood cells and platelets). Following the preparation of serum and plasma, aliquots should be rapidly frozen and stored at −80 °C until analyzed.

Before ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC/Q-TOF/MS) analysis, plasma samples of all the rats ($n=60$) were prepared. The plasma proteins were precipitated using methanol. Methanol (450 μL) was added to plasma (150 μL) and vortexed for 2 min, and then the supernatant was obtained. Following centrifugation (13,362 g, 10 min), the supernatant was removed and then dried using a Bath Nitrogen Blow Instrument (TTL-DCI, Beijing, China). Then, the extract was resuspended in 600 μL acetonitrile:water (2:1, v/v) for analysis. In parallel, a quality control (QC) sample was prepared by mixing equal volumes 100 μL from each of the 60 samples. An aliquot of 150 μL of this pooled plasma sample was prepared in the same way as the test samples.

The collected liver tissues were washed in 0.9% NaCl and immediately weighed. A portion of the fresh tissues was immediately used in the single-cell gel electrophoresis (SCGE) assay, whereas a second portion of the fresh samples were fixed in 10% neutral formalin for histopathological examination. Simultaneously, remaining part of liver tissues was stored at −80 °C until use for enzyme assays.

**SCGE assay**

Immediately after euthanasia, each piece of liver tissues (0.1 g) was placed in 2 mL of cold phosphate-buffered saline (PBS), minced using small dissecting scissors, grinded by glass homogenizer and to obtain the cell suspension after filtered by 300 stainless steel mesh. The cell suspension was left undisturbed for 5 min before it was centrifuged at 2599 g for 5 min. The cell pellets were resuspended in PBS buffer at 4 °C, with a density of $10^6$ cells/mL. Up to 25 μL liver cell suspensions were mixed with 75 μL of 0.8% low-melting-point agarose and placed on microscope slides covered with 0.8% normal melting agarose that were subsequently coverslipped and cooled at 4 °C for 30 min. The prepared slides were immersed in a fresh chilled lysis solution (2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Trizma, 10% DMSO and 1% Triton X-100, pH 10) in the dark for 1.5 h at 4 °C. The slides were subsequently transferred to an electrophoresis chamber with a chilled alkaline solution (300 mM NaOH and 1 mM Na2-EDTA, pH 13) and incubated in the dark for another 20 min at 0 °C. Electrophoresis was conducted in the dark at 0 °C for 30 min using 21 V and approximately 200 mA. Subsequently, the slides were neutralized and stained with 70 μL 250 μg/mL ethidium bromide for 30 s. The slides were evaluated under a fluorescence microscope at 20 × magnification. Two slides were made from each sample. Fifty cells were counted per animal.

**Chromatography and mass spectrometry**

Chromatographic separation was carried out at 35 °C on a UPLC BEH C18 column (100 × 2.1 mm, i.d.1.7 μm, Waters Corporation, Milford, MA) using a Waters ACQUITY UPLC System (Waters Corporation, Wexford, Ireland). A 2 μL aliquot of the sample was injected into the column. The pooled QC samples were injected every 10 samples to further monitor the stability of the analysis. The acquired QC data were used to investigate the analytical variability in the whole run. This was necessary in order to evaluate whether the analytical system had changed (and to what extent) over the time course of the analysis. This is critical for evaluating the variation in the analytical results, and therefore the reliability of the metabolite profiling data (Gika et al., 2007). The UPLC mobile phase consisted of water (A) and acetonitrile (B), each containing 0.1% formic acid. The optimized UPLC elution conditions were: 0–0.5 min, 2% B; 0.5–1.5 min, 2–20% B; 1.5–6 min, 20–70% B; 6–10.0 min, 70–98% B; 10–12 min, 98% B; 12–14 min, 98–70% B; and 14–16 min, 70–2% B. The gradient duration was 16 min at a constant flow rate of 0.35 mL/min. A needle wash cycle was performed after every sample injection to remove sample remnants and to prepare the equipment for the next injection. In addition, the eluent was directly transferred to the MS in a split mode. A blank was analyzed between every five samples to wash the column.

Mass spectrometry was performed on a Waters Micromass Q-ToF Micro Mass Spectrometer (Manchester, UK) with electrospray ionization (ESI) in positive and negative modes. The scan range was from 50 to 1000 m/z. The desolvation gas was set to 650 L/h at a temperature of 300 °C, the cone gas was set to 50 L/h and the source temperature was set to 100 °C. The capillary voltage was set to 3.0 kV in positive-ion mode and at 2.8 kV in negative-ion mode, and the cone voltage was set to 35 V. A lock spray was used to ensure the accuracy and reproducibility of all the analyses. A lock mass of leucine encephalin was used via a lock spray interface at a flow rate of 10 μL/min for monitoring in positive-ion mode ($[M+H]^+ = 556.2771$) and negative mode ($[M−H]^− = 554.2615$). The lock spray frequency was set at 0.48 s, and the lock mass data were averaged over 10 scans for correction. All the acquisition and analysis of data were controlled by Waters MassLynx v4.1 software.
Candidate biomarker detection and metabolite identification

Potential markers extracted from S-plots constructed after data were subjected to orthogonal partial least squares discriminant analysis (OPLS-DA). These markers were selected on the basis of their corresponding contribution to variation and correlation within the data set. The detected ions were arranged in a descending order according to variable importance in the projection (VIP) values, which indicate the effect of each metabolite on the two groups. A variable that deviates from the origin corresponds to a high VIP value. At VIP > 1.0, the variable can be considered as a contributor to the classification of control and treatment groups. Therefore, potential biomarkers were selected according to VIP values, S plots and further loading plots. Metabolites were identified using the following procedures. First, compounds were searched on the basis of probable elementary composition, structural message and properties, including calculated mass, mass deviation formula, molecular weight, mass and positive- and negative-ion modes, in a local database or ChemSpider with Mass Fragment (Waters Corp.) to acquire a mol file of the compounds. The mol file was imported into the corresponding MS2 spectra to evaluate and compare the possibility of group fragment and structural information; one or several probable compounds were preliminarily screened and removed. The identification of potential biomarkers was achieved by comparison with free online databases, such as Scripps Center for Metabolomics (http://metlin.scripps.edu/), MassBank (http://www.massbank.jp/) and LIPID MAPS (http://www.lipidmaps.org/), using exact mass and MS/MS spectra. Finally, the biomarkers were further confirmed by authentic standard compounds based on both retention times and MS/MS spectra (Sumner et al., 2007). The mass spectra of biomarkers were interpreted using relevant biochemical databases, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Data analysis

The UPLC/Q-TOF/MS data files were processed using the Markerlynx XS (Waters, Milford, MA) within Masslynx software (version 4.1) for peak detection and alignment. The parameters of the processing method were as follows: mass window, 0.05 Da; RT window, 0.2 min; and mass tolerance, 10 mDa. The high- and low-mass ranges were set at 1000 and 50 Da, respectively, and the initial and final retention times to 0.5 and 16 min, respectively. The data matrix processed containing peak numbers (retention time – m/z pairs), sample names and ion intensities were introduced to the EZinfo 2.0 software (Umeå, Sweden) for principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and OPLS-DA, which were used to obtain the biomarkers. Pareto scaling was used in all the models to avoid chemical noise. A default sevenfold (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 data of SCGE assay were processed using the CASP (version 1.2.1; Krzysztof Konca, Poland) software. Tail length (TL) and the percentage of tail DNA (TDNA %) were determined as parameters of DNA damage.

Other data were expressed as the mean ± SD of the means. The one-way analysis of variance (ANOVA) was performed using SPSS (version 16.0; Beijing Stats Data Mining Co. Ltd., Beijing, China), and significant differences were considered significant when test p values were less than 0.05.

Results

Liver viscera coefficients

After 12 weeks of experiment, rat livers of control and different experimental groups were weighed; the ratio of liver weight to total body weight of rats (namely, liver viscera coefficients) in each group was calculated. No significant changes were observed between the experimental groups and the control group (p > 0.05).

Serum biochemical indices

Various serum biochemical indices were measured in the serum of control and experimental rats at the end of the experiment. Several serum biochemical indices in the treatment groups significantly altered compared with those in group C and/or P (Table 1). ChE, ALB and HDL contents significantly decreased in groups P (p < 0.01), PQ1 (p < 0.01) and PQ2 (p < 0.05) compared with those in group C; by contrast, ChE, ALB and HDL contents significantly increased by 8.52%, 7.29% and 4.71% in group PQ2, respectively, compared with group P (p < 0.05). ALT, AST, TCHO, TG, LDL and FFA contents significantly increased in groups P (p < 0.01), PQ1 (p < 0.01) and PQ2 (p < 0.05) compared with group C. By contrast, ALT, AST, TCHO, TG, LDL and FFA contents significantly decreased by 12.44%, 9.30%, 15.54%, 7.06%, 8.33% and 15.37% in group PQ2, respectively, compared with group P (p < 0.05). PLA2 activities significantly increased in groups P (p < 0.01), PQ1 (p < 0.01) and PQ2 (p < 0.05) compared with group C (Figure 1); conversely, PLA2 activities significantly decreased in group PQ2 compared with group P (p < 0.05). No significant differences in all of the selected clinical parameters were observed among groups Q1, Q2 and C (p > 0.05).

Histopathological characteristics of liver tissues

No histopathological changes were detected in the liver tissues of groups Q1, Q2 and C (Figure 2). Histopathological changes were observed in groups P, PQ1 and PQ2; these changes exhibited fat drops, empty lipocytes, apoptosis cells and Kupffer cells. Histopathological changes were evident in group P and characterized by increased number of fat drops, apoptosis cells and Kupffer cells. The number of fat drops was lower in group PQ2 than in group P.

Antioxidant enzyme activities and MDA level

Antioxidant enzyme activity and MDA level in rat liver are summarized in Table 2. SOD, CAT and GSH activities significantly decreased in groups P (p < 0.01), PQ1 (p < 0.01) and PQ2 (p < 0.05) compared with the control group. By contrast, SOD, CAT and GSH activities significantly
### Table 1. The changes of serum biochemical indices in control and experiment groups at the end of the treatment.

| Groups | ChE (U/L) | ALT (U/L) | AST (U/L) | ALB (g/L) | TCHO (mmol/L) | TG (mmol/L) | HDL (mmol/L) | LDL (mmol/L) | FFA (μmol/L) |
|--------|-----------|-----------|-----------|-----------|---------------|-------------|--------------|--------------|-------------|
| C      | 245.30 ± 15.12 | 31.10 ± 4.75 | 102.70 ± 10.85 | 39.20 ± 1.14 | 1.74 ± 0.44 | 0.73 ± 0.04 | 0.93 ± 0.02 | 0.40 ± 0.03 | 1124.90 ± 209.74 |
| Q1     | 253.40 ± 13.66 | 32.00 ± 2.99 | 92.90 ± 9.51 | 40.00 ± 2.87 | 1.79 ± 0.3 | 0.74 ± 0.04 | 0.91 ± 0.02 | 0.39 ± 0.03 | 1096.55 ± 202.76 |
| Q2     | 249.40 ± 19.39 | 30.70 ± 3.95 | 103.50 ± 6.59 | 39.30 ± 1.70 | 1.87 ± 0.22 | 0.77 ± 0.07 | 0.90 ± 0.04 | 0.37 ± 0.02 | 1134.87 ± 219.21 |
| P      | 210.10 ± 19.97 | 41.00 ± 4.99 | 126.90 ± 15.31 | 34.30 ± 2.50 | 2.51 ± 0.35 | 0.85 ± 0.06 | 0.85 ± 0.05 | 0.48 ± 0.02 | 1634.75 ± 205.57 |
| PQ1    | 213.10 ± 18.84 | 37.90 ± 4.80 | 115.10 ± 10.90 | 36.80 ± 1.99 | 2.41 ± 0.37 | 0.79 ± 0.03 | 0.89 ± 0.05 | 0.44 ± 0.03 | 1383.49 ± 222.81 |
| PQ2    | 228.00 ± 11.95 | 35.90 ± 4.25 | 1124.90 ± 209.74 |

C, control group; Q1, low-dose quercetin-treated group; Q2, high-dose quercetin-treated group; P, OP mixture-treated group; PQ1, low-dose quercetin plus OP mixture treated-group; PQ2, high-dose quercetin plus OP mixture-treated group. Values are expressed as mean ± SD (n = 10).

*Significantly different from the control group at p < 0.05 (ANOVA).
**Significantly different from the control group at p < 0.01 (ANOVA).
***Significantly different from the mixture of OPs-treated group at p < 0.05 (ANOVA).

Metabolic profiling

Metabolic profiling, which indicates physiological states and pathological conditions, was analyzed on plasma samples from control and treatment groups. Totally 1130 variables in negative model and 1867 variables in positive model were used for multivariate statistical analysis. An initial overview of the quality of the analytical run was obtained by PCA of the sample data set that included the QC injections. As can be seen, the QCIs were tightly clustered in the middle of the plot in figures (Supplementary Figure 1, red starts). This type of result thus provides some evidence that the UPLC-MS system was stable and therefore providing reliable data suitable for further statistical analysis. PLS-DA is a common approach to analyze multivariate metabonomics data and maximize the variance matrix product of measured variables and the correlation of measured data with properties of interests. To assess the risk that the current PLS-DA model was spurious, the permutation tests for PLS-DA models were applied. And the results strongly indicated the validity of the PLS-DA models, since the Q2 regression line in blue had a negative intercept and all permuted R2-values in green on the left were lower than the original point of the R2-value on the right (Supplementary Figure 2). Furthermore, the results of CV-ANOVA suggest that the PLS-DA models are

SCGE assay

SCGE assay is a simple, rapid, visual and sensitive method used to determine DNA single- and double-strand breakage in individual cells. Several parameters, such as TDNA% and TL, are among the most commonly reported because these parameters indicate the appearance of comets and is linearly related to DNA breakage frequency in a wide range of damage levels. The results of SCGE assay of rat hepatocytes are summarized in Table 3. TDNA% and TL significantly increased in groups P (p < 0.01), PQ1 (p < 0.01) and PQ2 (p < 0.05) compared with the control group. However, TDNA% and TL significantly decreased in group PQ2 compared with group P (p < 0.05). TDNA% and TL did not significantly differ among groups Q1, Q2 and C (p > 0.05).
highly significant, having a $p$ value of $6.10 \times 10^{-13}$ (negative model, Supplementary Table 2) and $8.52 \times 10^{-34}$ (positive model, Supplementary Table 3). The PLS-DA scores in negative and positive modes are shown in Figure 3. The samples of all of the experimental groups completely deviated from the control group. No clear distinction was observed between groups P and PQ1; by contrast, group PQ2 clearly deviated from group P. Using metabolite identification methods described in the Materials and methods section, nine metabolites were identified in plasma (8 from the negative mode and 1 from the positive mode; Table 4). Table 5 shows the changes of the metabolite intensities in negative and positive modes after treatments were administered. Several metabolites significantly changed in treatment groups compared with group C or P. The intensities of lysoPE (16:0/0:0), lysoPC (17:0/0:0), lysoPC (15:0/0:0), glycocholic acid and 4-pyridoxic acid significantly increased in groups P ($p < 0.01$), PQ1 ($p < 0.01$) and PQ2 ($p < 0.05$) compared with group C; by contrast, the intensities of these parameters significantly decreased in group PQ2 compared with group P ($p < 0.05$). The intensities of arachidonic acid (AA) and citric acid significantly decreased in group P ($p < 0.01$), PQ1 ($p < 0.01$) and PQ2 ($p < 0.05$) compared with group C; conversely, the intensities of these parameters significantly increased in group PQ2 compared with group P ($p < 0.05$). The intensities of quercetin metabolites, namely isorhamnetin and quercetin-3-glucuronide, significantly increased in groups Q1, Q2, PQ1 and PQ2 compared with group C or P ($p < 0.01$). In addition, we also detected other unknown metabolites in positive mode. According to the relevant references and databases (such as Scripps Center for Metabolomics, MassBank, and LIPID MAPS), the indefinite metabolites were speculated as some other kinds of lysoPCs. Currently, the structure of these metabolites has yet to be determined due to the lack of standard substances.

![Figure 2](image-url)

**Figure 2.** Histopathological characteristics of liver tissues collected from rats treated with OP mixture and/or quercetin for 12 weeks. (A) control group; (B) low-dose quercetin-treated group; (C) high-dose quercetin-treated group; (D) OP mixture-treated group; (E) low-dose quercetin plus OP mixture-treated group; (F) high-dose quercetin plus OP mixture-treated group. HE stains, magnification, 200 x.

| Groups | SOD (U/mgprot) | CAT (U/mgprot) | GSH (mg/gprot) | MDA (U/mgprot) |
|--------|----------------|----------------|----------------|----------------|
| C      | 105.35 ± 9.07  | 52.88 ± 6.95   | 101.72 ± 10.81 | 0.85 ± 0.22    |
| Q1     | 114.61 ± 14.69 | 57.22 ± 4.66   | 111.84 ± 11.14 | 0.69 ± 0.16    |
| Q2     | 122.58 ± 12.81 | 60.84 ± 7.08   | 106.32 ± 13.73 | 0.84 ± 0.35    |
| P      | 79.04 ± 8.67   | 37.80 ± 4.99   | 76.61 ± 10.66  | 1.52 ± 0.37    |
| P + Q1 | 80.52 ± 7.41   | 39.63 ± 6.10   | 83.96 ± 10.71  | 1.27 ± 0.34    |
| P + Q2 | 93.44 ± 7.96   | 45.74 ± 6.14   | 88.60 ± 6.98   | 1.19 ± 0.37    |

C, control group; Q1, low-dose quercetin-treated group; Q2, high-dose quercetin-treated group; P, OP mixture-treated group; PQ1, low-dose quercetin plus OP mixture-treated group; PQ2, high-dose quercetin plus OP mixture-treated group. Values are expressed as mean ± SD ($n = 10$). SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; MDA, malondialdehyde.

| Groups | TDNA% | TL       |
|--------|-------|----------|
| C      | 3.59 ± 0.69 | 157.26 ± 14.41 |
| Q1     | 3.72 ± 0.56 | 145.85 ± 15.99 |
| Q2     | 3.44 ± 0.41 | 151.08 ± 16.26 |
| P      | 4.11 ± 0.47b | 173.61 ± 15.12b |
| P + Q1 | 3.98 ± 0.56b | 172.07 ± 18.66b |
| P + Q2 | 3.85 ± 0.48a,c | 164.68 ± 19.33a,c |

C, control group; Q1, low-dose quercetin-treated group; Q2, high-dose quercetin-treated group; P, OP mixture-treated group; PQ1, low-dose quercetin plus OP mixture-treated group; PQ2, high-dose quercetin plus OP mixture-treated group. Values are expressed as mean ± SD ($n = 10$). TDNA %, the percentage of tail DNA; TL, tail length.

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

Significantly different from the control group at $p < 0.01$ (ANOVA).

Significantly different from the mixture of OPs-treated group at $p < 0.05$ (ANOVA).
Applying UPLC/Q-TOF/MS in conjunction with multivariate data analysis methods, the global metabolite profile of plasma samples were measured, which obtained from the rats treated with the mixture of OPs and quercetin. The supervised PLS-DA model was established to characterize the plasma metabolite profile of the treated animals and to examine systemic metabolic changes in response to the treatment. Figure 3 shows the PLS-DA scores of the plasma from control and experimental groups in negative and positive modes. The treatment groups deviated from the control group, and data points of low-dose quercetin plus OP mixture treated group were not clearly separated from those of the mixture of OPs treatment group. By contrast, the difference between high-dose quercetin plus the mixture of OPs treated group and the mixture of OPs treated group was observed. Therefore, high-dose quercetin (50 mg/kg bw/d) affects toxicity induced by the mixture of OPs.

Based on metabolite identification methods described in the "Materials and methods" section, nine endogenous metabolites in plasma were identified (Table 4). Statistical changes in the intensities of these metabolites were detected in several treatment groups compared with the control group or the mixture of OP-treated group (Table 5). Individual biomarkers may provide information to understand the toxicity induced by the mixture of OPs.

Table 4. Potential biomarkers between control and treated groups.

| Retention time (min) | Measured mass (Da) | Calculated mass (Da) | Mass error (ppm) | Elemental composition | Scan mode | Metabolites                        |
|----------------------|--------------------|----------------------|------------------|----------------------|-----------|-----------------------------------|
| 8.36                 | 303.2318           | 303.2324             | 0.0006           | C_20H_32O_2          | –         | Arachidonic acid                  |
| 4.80                 | 452.2789           | 452.2777             | 0.0012           | C_21H_44NO_7P_2      | –         | LysoPE (16:0/0:0)                 |
| 6.30                 | 508.3454           | 508.3481             | 0.0027           | C_22H_45NO_7P_2      | –         | LysoPC (17:0/0:0)                 |
| 3.43                 | 464.3045           | 464.3091             | 0.0018           | C_25H_52NO_7P_2      | –         | Glycocholic acid                  |
| 2.34                 | 182.0554           | 182.0532             | 0.0022           | C_8H_9NO_4           | –         | 4-Pyridoxic acid                  |
| 0.97                 | 191.0261           | 191.0270             | 0.0009           | C_9H_11O_5           | –         | Citric acid                       |
| 3.03                 | 315.0530           | 315.0505             | 0.0025           | C_7H_11O_5           | –         | Isorhamnetin                      |
| 3.12                 | 477.0771           | 477.0747             | 0.0024           | C_21H_18O_13         | –         | Quercetin-3-glucuronide           |
| 6.67                 | 482.3209           | 482.3168             | 0.0041           | C_20H_40NO_7P_2      | +         | LysoPC (15:0/0:0)                 |

**Discussion**

Applying UPLC/Q-TOF/MS in conjunction with multivariate analysis methods, the global metabolite profile of plasma samples were measured, which obtained from the rats treated with the mixture of OPs and quercetin. The supervised PLS-DA model was established to characterize the plasma metabolite profile of the treated animals and to examine systemic metabolic changes in response to the treatment. Figure 3 shows the PLS-DA scores of the plasma from control and experimental groups in negative and positive modes. The treatment groups deviated from the control group, and data points of low-dose quercetin plus the mixture of the OP-treated group were not clearly separated from those of the mixture of OPs treatment group. By contrast, the difference between high-dose quercetin plus the mixture of OP-treated group and the mixture of OP-treated group was observed. Therefore, high-dose quercetin (50 mg/kg bw/d) affects toxicity induced by the mixture of OPs.

Based on metabolite identification methods described in the "Materials and methods" section, nine endogenous metabolites in plasma were identified (Table 4). Statistical changes in the intensities of these metabolites were detected in several treatment groups compared with the control group or the mixture of OP-treated group (Table 5). Individual biomarkers may provide information to understand the toxicity induced by the mixture of OPs.
response of organisms to exogenous stimulus. In this study, biological relationships between potential biomarkers and toxic effects induced by the OP mixture were described; the mechanism by which quercetin affects this toxicity was also discussed on the basis of the three pathways (Figure 4). Furthermore, serum biochemical indicators were detected to support the metabonomic results.

The first pathway is involved in metabolism, which involves lipid metabolism and tricarboxylic acid (TCA) cycle (Figure 4). AA is a polyunsaturated and essential fatty acid. AA belongs to a complex family of lipid mediators that regulate various physiological responses and pathological processes. AA can be oxidized by free-radical chain mechanism (Fridovich & Porter 1981). Furthermore, OPs can induce the generation of free radicals and oxidative stress (Lukaszewicz-Hussain, 2010). In this study, the exacerbated free radicals induced by the mixture of OPs promoted AA oxidation; as a result, the plasma level of AA in the OP mixture-treated group decreased. However, AA significantly increased in high-dose quercetin plus OP mixture-treated group compared with the OP mixture-treated group; this result may be due to the antioxidant activity of quercetin. The results of the current study are consistent with those of Baumannet et al. (Baumann & Wurm, 1980).

Two kinds of lysophosphatidylcholines (lysoPC) were identified in plasma. LysoPC is a major product of LDL oxidation. Approximately, half of the fatty acids in LDL are polyunsaturated fatty acids that can be oxidized by free radicals (Mertens & Holvoet, 2001), which may have caused a significant increase in lysoPCs in the OP mixture-treated group compared with the control group. In addition, PLA2 hydrolyzes phosphatidylcholine from the sn2 position to generate lysoPCs and FFA. In the current study, lysoPCs (Table 5) and FFA (Table 1) significantly increased in the OP mixture-treated group compared with the control group. Free radicals can activate PLA2 (Wereszczyńska-Siemia et al., 1998). Therefore, PLA2 was probably activated by OP mixture-induced free radicals. PLA2 activity significantly

### Table 5. Summary of intensity values of biomarkers detected in negative and positive ESI mode in rat plasma.

| Groups          | Arachidonic acid | LysoPE (16:0/0:0) | LysoPC (17:0/0:0) | Glycocholic acid | 4-Pyridoxic acid | Citric acid | Isorhamnetin Quercetin-3-glucuronide | LysoPC (15:0/0:0) |
|-----------------|------------------|-------------------|-------------------|-----------------|-----------------|-------------|------------------------------------|-------------------|
| C               | 51.1 ± 6.5       | 51.1 ± 7.9        | 199.2 ± 12.2      | 102.7 ± 9.7     | 10.9 ± 5.4      | 116.9 ± 12.4 | 0                                  | 43.3 ± 4.7        |
| Q1              | 45.2 ± 7.4       | 55.7 ± 8.4        | 208.2 ± 9.7       | 109.0 ± 12.9    | 10.1 ± 5.0      | 126.5 ± 10.4 | 9.2 ± 3.1b                         | 16.1 ± 2.7b       |
| Q2              | 46.9 ± 5.6       | 50.3 ± 7.1        | 200.1 ± 16.7      | 103.4 ± 14.9    | 14.0 ± 5.1      | 129.3 ± 18.6 | 15.7 ± 3.6b                        | 11.1 ± 2.2b       |
| P               | 33.9 ± 6.6c      | 68.7 ± 7.0b       | 227.7 ± 11.3b     | 128.33 ± 9.6b   | 21.6 ± 5.3b     | 94.1 ± 8.2b  | 0                                  | 55.8 ± 4.9b       |
| PQ1             | 38.6 ± 6.4ab     | 67.6 ± 9.4b       | 222.7 ± 17.1b     | 129.7 ± 14.0b   | 18.9 ± 3.4b     | 99.1 ± 7.6b  | 8.9 ± 1.4bd                         | 14.0 ± 2.6bd      |
| PQ2             | 42.8 ± 9.9ab     | 60.2 ± 8.5ce      | 213.2 ± 10.7abc   | 116.0 ± 8.6ce   | 16.4 ± 3.9abc   | 104.4 ± 8.9abc| 12.7 ± 2.9bd                        | 49.1 ± 3.4ab      |

C, control group; Q1, low-dose quercetin-treated group; Q2, high-dose quercetin-treated group; P, OP mixture-treated group; PQ1, low-dose quercetin plus OP mixture-treated group; PQ2, high-dose quercetin plus OP mixture-treated group. Values expressed as mean ± SD (n = 10).

aSignificantly different from the control group at \(p < 0.05\) (ANOVA).
bSignificantly different from the control group at \(p < 0.01\) (ANOVA).
cSignificantly different from the mixture of OPs-treated group at \(p < 0.05\) (ANOVA).
dSignificantly different from the mixture of OPs treated group at \(p < 0.01\) (ANOVA).

Figure 4. The mechanism pathways of quercetin affect OP mixture-induced toxicity. Upwards arrow or downwards arrow represent the intensities of metabolites were significantly increased or decreased in OP mixture-treated group compared with control group; upwards dashed arrow or downwards dashed arrow represent the intensities of metabolites were significantly increased or decreased in high-dose quercetin plus OP mixture-treated group compared with OP mixture-treated group.
increased in the OP mixture-treated group compared with the control group (Figure 1); these results are consistent with our speculation. Two kinds of lysoPC significantly decreased in high-dose quercetin plus OP mixture-treated group compared with the OP mixture-treated group; this decrease occurred possibly because quercetin exhibits free radical scavenging activity and protects LDL from free radical-induced oxidation (Janisch et al., 2004) and inhibits phospholipase A2 activity (Figure 1). Furthermore, FFA significantly decreased in high-dose quercetin plus OP mixture-treated group compared with the OP mixture-treated group, which may enhance hepatic lipid metabolism because of quercetin, particularly omega (o)-oxidation, and may reduce circulating lipid levels (Hoek-van den Hil et al., 2013).

LysoPE (16:0/0:0) is the degradation product of phosphatidylethanolamine. LysoPEs increase after membranes are disrupted; this increase is highly correlated with the degree of cell death in the liver, as assessed by histological examination (Gonzalez et al., 2012). In the present study, lysoPE (16:0/0:0) significantly increased in the OP mixture-treated group compared with the control group because the mixture of OPs damaged the membranes. However, quercetin can impede the penetration of OP mixtures and protect membrane structure and function (Oteiza et al., 2005); this phenomenon may be accounted for a significant decrease in lysoPE (16:0/0:0) in high-dose quercetin plus OP mixture-treated group compared with the OP mixture-treated group. Therefore, changes in AA, lysoPCs and lysoPEs indicated that quercetin elicits a protective effect against the toxicity induced by a mixture of OPs. In the current study, changes in HDL, LDL, TG and TCHO (Table 1) in rat serum further confirmed the above conclusion.

In the present study, citric acid, an important intermediate of the TCA cycle, significantly decreased in the OP mixture-treated group compared with the control group. The results indicated that the OP mixtures affects the TCA cycle in rat liver. Chronic exposure to quercetin can stimulate TCA cycle by regulating important enzymes and can normalize the TCA cycle by reducing lipid peroxidation (Kar et al., 2011; Niklas et al., 2012). In the current study, citric acid significantly increased in high-dose quercetin plus OP mixture-treated group compared with the OP mixture-treated group; this result may be linked to the above-described effects of quercetin.

The second pathway is involved in oxidative stress (Figure 4). The two kinds of lysoPC not only disrupted lipid metabolism, but also closely related to the oxidative stress. LysoPCs can induce plasma membrane destabilization; as a result, intracellular reactive oxygen species are produced (Colles & Chisolm, 2000; Kim et al., 2009). The increase in lysoPC in the OP mixture-treated group indicated that exposure to OPs could induce oxidative stress. In addition, 4-pyridoxic acid is the final catabolite of B6 vitamins in the liver (Merrill & Henderson, 1990). Vitamin B6 deficiency can cause a decrease in the antioxidant defense system and an increase in oxidative stress in liver tissue (Taysi, 2005). In the current study, 4-pyridoxic acid significantly increased in the OP mixture-treated group compared with the control group; this result indicated that OPs could affect the antioxidant defense system. This result is also consistent with that in our previously published article, which indicated that exposure to the mixtures of OPs can induce oxidative damage in rats (Yang et al., 2012). It is reported that oxidative attack can induce different types of DNA lesions (Ayed-Boussem et al., 2012). Liver SCGE assay results (Table 3) further confirmed the above conclusion. However, TDNA% and TL significantly decreased in high-dose quercetin plus OP mixture-treated group compared with the OP mixture-treated group. These results may be associated with the following activities of quercetin. On the one hand, the antioxidant defense system can be enhanced after rats chronically exposed to quercetin (Ostrowska et al., 2004; Rodrigo et al., 2002). On the other hand, quercetin can inhibit oxidative damage to cellular DNA by scavenging free radicals (Johnson & Loo, 2000). In the present study, the results of liver antioxidant enzyme activities (SOD, CAT and GSH) and MDA level (Table 2) further support our conclusions.

The third pathway is involved in liver function (Figure 4). The liver performs a vital function in lipid metabolism and TCA cycle. Thus, abnormal changes in lipids (AA, lysoPCs and lysoPEs) and citric acid in the current experiment indicated that the OP mixtures could induce liver dysfunction. Furthermore, OP mixture elicited toxicity through the depletion of antioxidant defense systems; thus, the liver is exposed to oxidative attacks. In addition, glycocholic acid is a secondary bile acid and increased serum concentrations of bile acids are indicate hepatocyte damage (Korman et al., 1974). In the current study, glycocholic acid significantly increased in the OP mixture-treated group; this result also indicated that the mixtures of OPs could induce liver damage. The abnormal changes in these metabolites corresponded to liver dysfunction attributed to treatment with OP mixtures. However, abnormal changes in the above metabolites were ameliorated in the high-dose quercetin plus the mixtures of OP-treated group compared with the OP mixture-treated group. This result showed that quercetin can regulate lipid and amino acid metabolism and enhance antioxidant defense system by which quercetin protects rat liver against oxidative stress induced by OP mixtures. The histopathological examination results of liver tissues (Figure 2) and the results of serum clinical chemical parameters in Table 1 (ALT, AST and ALB) further supported the above conclusion.

In our previous study, a metabonomic analysis of the effect of quercetin on toxicity induced by chronic exposure to low-level dichlorvos was studied in rat plasma (Zeng et al., 2014). Compared to the previous results, the similar finding in the present study is that quercetin shows a regulating effect on lipid metabolism disorder under the condition of exposure to the mixture of four OP pesticides as well, which could be contributed by the antioxidant effect of quercetin. However, the other protective functions of quercetin, such as the regulation of amino acid metabolism and protection rat kidney against toxic effects induced by dichlorvos (Zeng et al., 2014), were not observed in the present study, which might be due to the different toxic effects between a single kind of OP pesticides and the mixture of four OP pesticides.

What deserves our attention is that the intensities of the identified metabolites were significantly ameliorated in the high-dose quercetin plus OP mixture-treated groups compared with the OP mixture-treated group ($p < 0.05$). However, the
intensities of identified metabolites in the high-dose quercetin plus the OP mixture-treated groups were still significantly different from those in the control group (p < 0.05). Changes in plasma metabolites indicated that quercetin elicits a partial protective effect on the toxicity induced by a mixture of Ops.

Conclusions

A metabonomic method was used to investigate the effects of quercetin on toxicity induced by the mixtures of four OPs at their respective NOAEL. The results indicated that quercetin (50 mg/kg bw/d) elicits a partial protective effect on the toxicity induced by a mixture of Ops through regulating lipid metabolism, improving TCA cycle disorder, enhancing anti-oxidant defence system.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online

Supplementary Figures 1 and 2
Supplementary Tables 1–3