Aquatic Toxicology 188 (2017) 159–169

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Metabolic profiling of goldfish (*Carassius auratis*) after long-term glyphosate-based herbicide exposure

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ARTICLE INFO

Keywords:
Glyphosate
Toxicity
NMR-based metabolomics
OSC-PLS-DA
Correlation network

ABSTRACT

Glyphosate is an efficient herbicide widely used worldwide. However, its toxicity to non-targeted organisms has not been fully elucidated. In this study, the toxicity of glyphosate-based herbicide was evaluated on goldfish (*Carassius auratus*) after long-term exposure. Tissues of brains, kidneys and livers were collected and submitted to NMR-based metabolomics analysis and histopathological inspection. Plasma was collected and the blood biochemical indexes of AST, ALT, BUN, CRE, LDH, SOD, GSH-Px, GR and MDA were measured. Long-term glyphosate exposure caused disorders of blood biochemical indexes and renal tissue injury in goldfish. Metabolomics analysis combined with correlation network analysis uncovered significant perturbations in oxidative stress, energy metabolism, amino acids metabolism and nucleosides metabolism in glyphosate dosed fish, which provide new clues to the toxicity of glyphosate. This integrated metabolomics approach showed its applicability in discovering the toxic mechanisms of pesticides, which provided new strategy for the assessment of the environmental risk of herbicides to non-target organisms.

1. Introduction

Among the most widely used broad-spectrum herbicides in the world, herbicides containing glyphosate as an active component are of great concern mostly due to their excessive use on corn and soybean crops which are modified by genetic engineering to tolerate it. Glyphosate can be released into the surrounding environment by agriculture, industry and other human activities, commonly detected in overland runoff and bed sediment in rural suburban, and urban areas due to the close proximity or frequency of their use or their association with the pour of production sewage and domestic sewage, causing the risks of human and environmental accumulations. Potential toxic effects and the ecotoxicological risks of glyphosate have been studied previously (Li et al., 2016). Glyphosate-based herbicides could cause hepatorenal, teratogenic and tumorigenic effects by endocrine disruption and oxidative stress (Mesnage et al., 2015). These evidences raised great concerns and called for further studies on its toxicities.

Metabolomics has been grown to be a useful tool utilized in a variety of research and industries, which focus on the complete set of low molecular weight metabolites that varying according to the heredity, development and pathophysiology of the organism and environment state (Oliver, 2002). Metabolomics has proven to be highly sensitive in detecting effects that associated with both drugs and environmental toxicants, where metabolic perturbations often happened much earlier than histopathological changes (Griffin et al., 2000). NMR (nuclear magnetic resonance) have several advantages for metabolomics study, such as minimal sample preparation, nondestructive and noninvasive manner. NMR-based metabonomics approach has become more and more useful in toxicology, for the reason that it can potentially yield novel biomarkers, and provide a holistically molecular basis underlying the toxicities.

In this study, integration of metabolomics analysis with traditional toxicity evaluation was carried out on goldfish (*Carassius auratus*) after a long-term exposure to glyphosate-based herbicide, to reveal the toxicity mechanism of glyphosate.

2. Materials and methods

2.1. Chemicals

The commercial formulation, containing 30% (w/v) glyphosate, named "Nongteshi®" (Longbang Chemical Industry Co., Ltd, Wuxi, China) was used in the experiments. All other reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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http://dx.doi.org/10.1016/j.aquatox.2017.05.004
Received 20 March 2017; Received in revised form 3 May 2017; Accepted 6 May 2017
Available online 13 May 2017
0166-445X/

2.2. Experimental design

Male goldfish (*Carassius auratus*), 13 ± 2 cm in length, weighting 23 ± 3 g, were purchased from Fuzimiao Flower and Bird Market in Nanjing. Fish were kept undisturbed and were acclimated to laboratory conditions for one month in 60 L polycarbonate tanks under natural photoperiod in aerated and dechlorinated tap water with constant temperature 20 ± 2 °C, dissolved oxygen 8.8 ± 0.3 mg/L, pH 7.4 and hardness 29 mg/L (determined as $CaCO_3$). Fish were fed once a day to apparent satiation with commercial pellets for cyprinids (crude protein ≥40%, crude fat ≥4%, crude fiber ≤ 3%, crude ash ≤12%, moisture ≤10%, phosphorus ≥1%, calcium ≥1.8%). After acclimation, fish were randomly categorized into a control group and a glyphosate group. Concentrations of glyphosate were determined by reference (Ayoola, 2008) and by our previous experiments (Li et al., 2016). Glyphosate of 0.2 mmol/L were selected for exposure in this study. The exposure dosage of glyphosate in water was analyzed by high performance liquid chromatography (HPLC). The details for the HPLC analysis were provided in the supporting information.

Fish in the control group were maintained in consistent with the conditions of the domestication stage. A static renewal toxicity exposure method were employed in polycarbonate tanks (10 L) for fish in the glyphosate group. Water was renewed every 24 h to maintain its quality and to regulate the glyphosate concentration. The exposure duration was 90 days. At the end of the exposure experiment, fish were sacrificed in accordance with the Animal Ethics Committee of Nanjing University of Science and Technology and the National Institutes of Health Guidelines for Animal Research. Tissues of brains, livers and kidneys were removed rapidly from the fish body, immediately immersed in liquid nitrogen and then stored at −80 °C until use for metabolomics analysis.

2.3. Hematoxylin and eosin (H & E) staining

Tissues were quickly removed from the body, rinsed with cold phosphate buffered saline and then immersed in 10% neutral-buffered formaldehyde for 24 h, embedded in paraffin, and sliced into 5 μm thickness along the largest truncation surface (N = 4). The sliced sections were stained with hematoxylin and eosin (H & E), and examined under light microscopy.

2.4. Biochemical assays

Blood samples of 0.4 mL were collected from the caudal vessels using a 1 mL heparinized and ice-cooled syringe (N = 6). Blood were then centrifuged at 3000 rpm, 4 °C for 10 min to obtain plasma. Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (CRE), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reducase (GR) and methanedicarboxylic aldehyde (MDA) were measured using the commercially available kits (Nanjing Jiancheng Biotech Inc., Nanjing, China) according to the manufacturers' instructions.

2.5. Sample preparation and ^{1}H NMR analysis

Samples were weighed, homogenized with pre-cooled 50% acetonitrile at the ratio of 1 mg of tissues to 5 mL of extraction solvents (N = 11 and N = 15 for control and glyphosate dosed group, respectively). Homogenates were vortexed and centrifuged for 10 min at 12,000*g* and 4 °C. The supernatants were then transferred into fresh tube, frozen and lyophilized to dryness on a vacuum concentrator. The dried samples were stored at −80 °C prior to analysis. For NMR measurements, samples were dissolved in 550 μL 99.8% D_2O phosphate buffer (0.2 M, pH = 7.0) containing 0.05% (w/v) sodium 3-(trimethylsilyl) propionate-2, 2, 3, 3-d4 (TSP). After vortexing and centrifugation, the supernatants were transferred to 5 mm NMR tubes for ^{1}H NMR analysis.

^{1}H NMR spectra of samples were recorded on a Bruker AVANCE III 500 MHz NMR spectrometer at 298 K. Deuteroxide (D_2O) and TSP were used for field frequency locking and for chemical shift reference (^{1}H, 0.00 ppm), respectively. A transverse relaxation-edited Carr-Purcell-Meiboom-Gill (CPMG) sequence (90(τ-180-τ) n-acquisition) with a total spin-echo delay (2 nτ) of 40 ms was used to suppress the signals of macromolecules and proteins. ^{1}H NMR spectra were measured with 128 scans into 32 K data points over a spectral width of 10,000 Hz. The spectra were Fourier transformed after multiplication the FIDs (free induction decay) by an exponential weighting function corresponding to a line-broadening of 0.5 Hz.

2.6. Spectra pre-processing and multivariate data analysis

All ^{1}H NMR spectra were phase and baseline corrected, and aligned to TSP (0 ppm) manually in the Topspin software (version 2.1, Bruker), and then were exported to ASCII files using MestReNova (version 8.0.1, Mestrelab Research SL), which were then imported into R software (Team, 2013) for further phase and baseline correction, and peak alignment. Spectra were binned using an adaptive, intelligent algorithm (De Meyer et al., 2008) between 0.7 and 9.0 ppm. Regions containing the residual water signals were removed. The total areas of the remaining bins were normalized using the probabilistic quotient normalization method (Dieterle et al., 2006), which has been proposed as a more robust method for metabolomics analysis. Data were pareto scaled before multivariate data analysis.

Orthogonal signal correction partial least squares discriminant analysis (OSC-PLS-DA), a supervised pattern recognition method, was performed using house made R scripts to maximize covariance between the measured data and the predictive classifications. The scores plots showed the clusters between groups. S-plot were used to identify differential metabolites between groups, which were far away from the point of origin in the upper right and lower left quadrant. The models were validated by repeated two-fold cross validation and a permutation test. The quality of the model could be evaluated by R^2, which indicates the total explained variation, and Q^2, which represents the model predictability.

2.7. Metabolites identification

Metabolites were assigned by querying the public metabolome databases such as Human Metabolome Database (HMDB) (Wishart et al., 2012) and Madison-Qingdao Metabolomics Consortium Database (MMCD) (Cui et al., 2008). The commercial available software Chenomx NMR suite v.8.1 (Chenomx Inc., Edmonton, Canada) and the statistical total correlation spectroscopy techniques (STOCSY) (Cloarec et al., 2005) were also employed to help metabolites identification. The two-dimensional NMR techniques such as total correlation spectroscopy (TOCSY) and heteronuclear single quantum correlation (HSQC) were used for confirmation.

2.8. Univariate statistical analyses of metabolites identification

A parametric Student's *t*-test or a nonparametric Mann-Whitney test (according to the conformity to normal distribution of the integral area) was performed on the integrated areas to evaluate the significance of the fold changes of metabolites between groups. The fold change (FC) values of metabolites and their associated *p*-values corrected by Benjamini-Hochberg adjusted method (Benjamini and Hochberg, 1995; Hochberg and Benjamini, 1990) were calculated and visualized as colored tables. Fold change values were calculated by formula: FC equals glyphosate group divided by control group, color coded after logarithmic ($\log_2$) transformation. The red colors represent metabolites with increased concentrations in poisoned fish, and blue colors