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Hepatic Metabolomics Profiling of Cyprinus Carpio after Acute Cypermethrin Toxicity

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Abstract

In the current study, The GC-MS metabolomics approach was used to evaluate the effect of cypermethrin on the metabolic profile in liver tissue of common carp fish. Fish were exposed to low and high concentrations of cypermethrin (0.1 and 1 µg/L respectively) for 24 and 96 h with subsequent PCA analysis to illustrate the response of the metabolic system after exposure to the pyrethroid. Cypermethrin induced obvious alteration in the intermediates of TCA cycle and encouraged consumption of non-carbohydrate sources in liver at all-time points. Additionally, cypermethrin exposure reduced the level of several amino acids and induced damage to the nucleotides reflecting the effect of cypermethrin on purine metabolism. GC-MS based metabolomics approach is a new and powerful tool to understand the toxicological effects and the underlying mechanism of cypermethrin on fish.

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Introduction

Pyrethroids are synthetic organic insecticides that are extensively used worldwide due to their low toxicity to mammals and effective insecticidal action comparing to organophosphorus compounds and other insecticides [1]. On the other side, these chemicals have high aquatic toxicity because the ability of fish to metabolize and eliminate such xenobiotics is limited [2]. Pyrethroids are classified into two types; type I and type II depending on their chemical structure. Cypermethrin is a common use highly active type II synthetic pyrethroid insecticide that is chemically modified via the addition of α-cyano group at the phenoxybenzyl alcohol moiety resulting in improving of its photo-stability and potentiating of its toxicity than that of type I. Cypermethrin caused behavioural changes together with alterations in nitrogen, nucleic acid and acetylcholine esterase activity [3-7]. Previous studies illustrated the toxic effects of cypermethrin in fish, but there are only few reports demonstrated the metabolic disorders in the whole body.

Recently, metabolomics have been used for comprehensively understand the response of fish to various environmental pollutants such as butachlor [8], 2,4 dichlorophenol [9], fluoxetine [10], epoxiconazole [11], diclofenac [12] and short-chain chlorinated paraffins [13]. Metabolomics techniques have been providing new possibilities to unravel the modes of action of chemicals [14], besides discovery of new biomarkers [15]. Metabolomics based toxicity evaluations are very effective to detect the effect of environmental toxicants even at very low concentrations. The metabolic stress is the first response to physical and chemical stressors; therefore, metabolomics is powerful tool to determine the perturbations of the metabolome that often take place much earlier than other biological changes such as histopathological changes, growth retardation and mortality [16].

The present study, for the first time, was designed to assess the effects of cypermethrin on the metabolic profile in hepatopancreas of Japanese carp under laboratory conditions using metabolomics strategy with the gas chromatography/mass spectrometry.

Materials and Methods

Chemicals

Cypermethrin standard, Chloroform, acetone, hexane with pesticide-analytical grade, methanol with HPLC analytical grade, *O*-Methyl-hydroxylammonium chloride (methoxylamine hydrochloride) and dehydrated pyridine were purchased from Wako Pure Chemicals (Osaka, Japan). *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1%TMCS) was purchased from Thermo Fisher Scientific Inc. (PA, USA).

Exposure of Fish to Cypermethrin

Japanese carp with average weight 25-30 g were purchased from a private fish farm in Kagoshima Prefecture, Japan. The fish were acclimatized for two months under the laboratory conditions before starting the experiment. During adaptation period, fish were fed a commercial fish meal (Nippon Formula Feed Manufacturing, Yokohama, Japan) several times at a feeding rate of 1% fish weight per day [17].

In this study, thirty six carp fish was kept in three aquariums. The feeding was stopped 24 hours before exposure. Fish were divided into three groups (n = 3); first group was the control one (cypermethrin free) and the other two groups were exposed to two concentrations of 0.1 (L-group) and 1 µg/L (H-group) of waterborne cypermethrin solved by for 24 and 96 hours. The concentration of L-group was established similar with that in the aquatic environment. The reported water concentrations of CYP vary from area to another; it was less than 3.5 µg/L in Parana River water in Argentina [18], about 3.55 µg/L Mekong Delta of Vietnam [19]. Meanwhile, the concentration of H-group was based from reported 96 hours LC_{50} of cypermethrin under acetone solvent condition in *Cyprinus carpio* as 1.7 µg/L [20]. Fish were kept in glass tanks (40 L) with mild aeration. The water temperature was maintained at 24±1 ºC, pH was 6±1 and dissolved oxygen concentration was 5±1 mg/L. Photoperiod was 12 h light/12 h dark cycles. The control and test water was completely replaced once per day with dechlorinated tap water left for one day before use.

During sampling, 2-phenoxyethanol of 500 mL/L was used as anaesthetic agent. Hepatopancrease tissues were collected at 24 and 96 h from each group (n = 6).

Immediately after sampling, the tissues were frozen by liquid nitrogen to stop the metabolic and enzyme actions and stored at – 80 ºC until metabolomics analysis.

Samples Preparation for Metabolomics Analysis

Hepatopancrease tissue samples (ranging from 10-15 mg for each) were placed in a 2-mL PP microtube and 1 mL of a mixture of chloroform, methanol, and Milli-Q water (1:2:0.8, v/v/v) was added, together with a zirconia ball (diameter 5 mm). The microtube was vigorously shaken for 6 min by a sample disruption system (Tissue Lyser II, QIAGEN, and Hilden, Germany). Then, 500 µl of an equal mixture of chloroform and Milli-Q water (1:1, v/v) was added to the tube, which was centrifuged at 12,000×g (4°C) for 10 min. The upper layer from each sample was collected, placed in another 2 mL PP microtube and dried completely in a vacuum centrifuge drier (TAITEC,vc-15sp, Japan).

Derivatization of liver metabolites was performed in two steps. The first, oximation was done by adding 10 µL of a methoxylamine hydrochloride solution prepared by dissolving methoxylamine hydrochloride in pyridine (40 mg/mL) to the dried residue by means of continuous shaking in a water bath at 30°C for 90 min. Secondly, the silylation was done by addition of 90 µL of MSTFA + 1% TMCS to the mixture at water bath at 37°C for 30 min. After derivatization, 100 µL of hexane was added to each sample. The resulting solution was diluted 10-folds and used for analysis.

GC/MS Analysis

Metabolites were analysed on an Agilent Technologies 6890 Series gas chromatograph equipped with a 5973 MSD mass selective detector and a DB 5-ms capillary column (i.d.0.25 mm×30 m, 0.25-µm film thickness; J&W Scientific, USA). One microliter of each sample was injected into the column. The temperatures of the injector and detector were 250 and 290°C, respectively. The oven temperature program was as follows: 60°C for 1 min, increase to 325°C at 10°C/min, and then fixed at 325°C for 12 min.

Statistical Analysis

After getting the peaks on the chromatogram, the baseline was drawn to obtain peak area, deconvoluted, and aligned using MetAlign™ (ver. 080311, Wageningen University, Wageningen, the Netherlands). The area obtained from each peak was divided by the weight of each fish and normalized to the internal standard [16]. The resultant value was multiplied by 10^5 and analysed by principal component analysis (PCA) that was performed for metabolites to evaluate the differences of the toxic effects among the groups. PCA score plots and component loadings were used to evaluate the clustering of metabolites along the plot and their contribution to the difference after exposure to cypermethrin. Statistical analysis was performed with the R programming language (http:// www.rproject.org/). For all results, p value < 0.05 was considered significant [16].

Results and discussion

GC/MS Analysis

More than 300 individual metabolite peaks can be detected by GC/MS. The current work focused on the endogenous metabolites related to amino acids, intermediates of kerbs cycle and purines. The statistical analyses were done only using the identified metabolites.

Pattern Recognition in Liver Tissue Metabolites

The principal components analysis (PCA) was performed to comprehensively illustrate the variations in the metabolite levels among the groups in response to cypermethrin exposure. At 24 h, the control group clustered on the left side of the plot related to PC1 and the (L & H) groups clustered on the right side of the plot (Figure 1-A). The separation along the PC1 indicated the effects common to the exposure while the separation along PC2 indicated the effects related mainly to the high group. PC1 and PC2 represented 58.44 % and 14.31 % for the variance.

At 96 h, the control group clustered in the left side of the plot while the exposure groups clustered on the right side of the plot along the PC1 which accounted for 65.91 % of the variance (Figure 1-B). Adversely, there is little clustering of the data along PC2 which accounted for 14.77 % for the variance. That strong clustering along the PC1 indicated the intense metabolic perturbation by cypermethrin exposure according to its concentration. (Table 1)

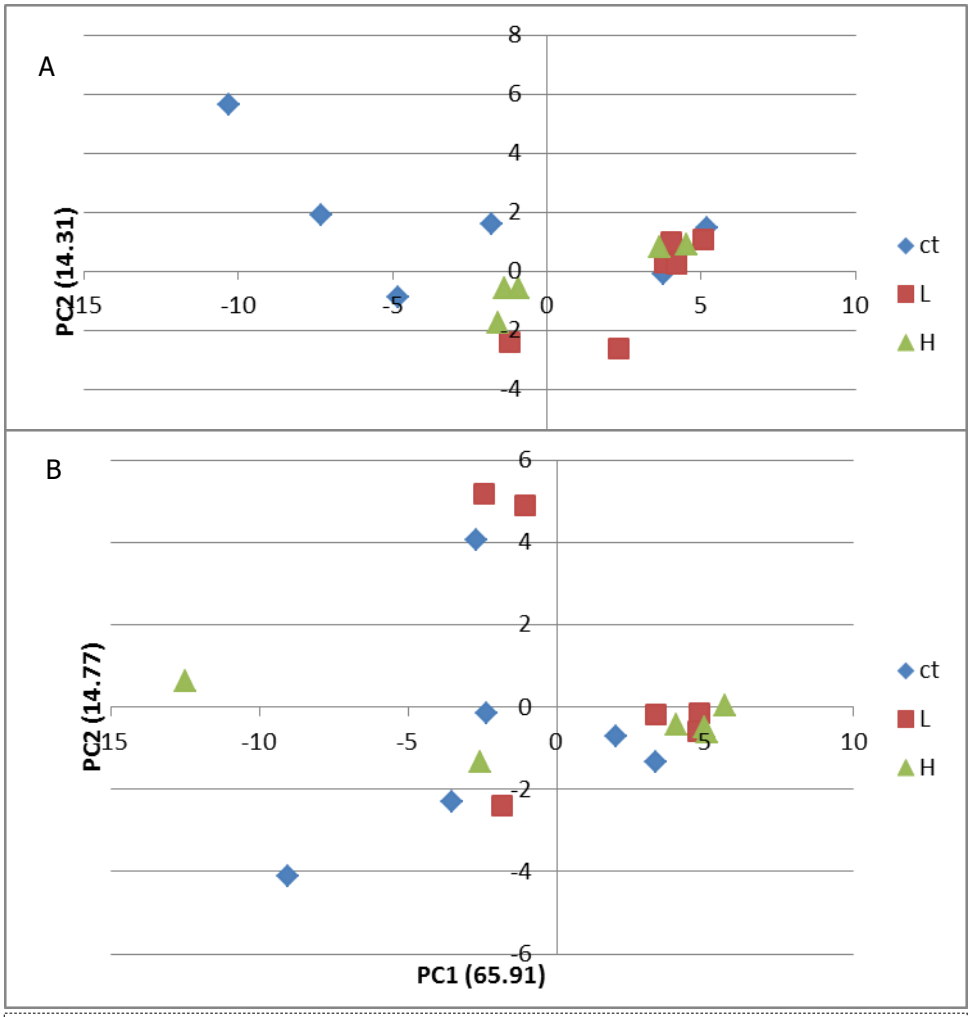

Figure 1. Principle component analyses (PCA) score plots of liver metabolites of carp at 24 h (A) and 96 h (B) after exposure to cypermethrin. The contribution percent of each component is presented along the x and y axis in relation to pc1 and pc 2 respectively. Ct: control group; L: low concentration; H: high concentration.

Table 1. Corresponding PC1 and PC2 loading for individual metabolites at 24 and 96 hours.

A: 24 hours		
Name	**PC1**	**PC2**
Malate	-0.85797*	0.14649
Lysine	-0.66008*	-0.423
Creatinine	-0.52999	-0.61793*
Alanine	-0.84481*	-0.43612
Aspargine	-0.50666*	-0.29935
Proline	-0.82891*	-0.40608
Ornithine	-0.80498*	0.359076
Valine	-0.97204*	-0.10331
lactate	-0.64619*	-0.21878
Urea	-0.89993*	-0.13616
Histidine	-0.93515*	0.300591
Oxoproline	-0.77729*	0.31973
Glutamate	-0.5358*	-0.07439
Leucine	-0.79748*	-0.50501
Isoleucine	-0.94972*	0.096938
Pyruvate	-0.51516*	-0.53184
Glycine	-0.91475*	0.079476
Methionine	-0.94499*	0.172978
succinate	-0.50878*	0.018358
Tryptophan	-0.82686*	0.450307
Serine	-0.92173*	-0.30686
Glycerol	-0.86238*	-0.26837
Inosine	-0.6052*	0.615485*
Thereonine	-0.98285*	0.016507
Phenylalanine	-0.82766*	0.457088
Tyrosine	-0.93696*	0.034436
Hydroxyproline	-0.71302*	0.550687
aspartic acid	-0.80332*	0.025751
Fumerate	-0.93738*	-0.07549
Glutamate	-0.63912*	-0.44724
Hypoxanthine	-0.70727*	0.080616
Lysine	-0.85577*	0.247021
Xanthine	-0.82646*	0.440056
glycerol3p	-0.69314*	0.471301
Glucose	-0.76211*	0.032005

B: 96 hours		
Metabolite	**PC1**	**PC2**
Malate	-0.9753*	0.095294
Lysine	-0.68679*	0.515889
Creatinine	-0.88979*	0.211371
Alanine	-0.88751*	-0.41011
Aspargine	-0.31837	0.715191*
Proline	-0.97031*	-0.12149
ornithine	-0.39311	0.315493
Valine	-0.98063*	-0.1487
Lactate	-0.85688*	-0.37907
Urea	-0.57937*	-0.56611
Histidine	-0.79667*	0.571266
Oxoproline	-0.96835*	0.186739
Glutamate	-0.72805*	0.629791
Leucine	-0.84316*	-0.4391
Isoleucine	-0.96643*	0.094864
Pyruvate	-0.73761*	-0.49588
Glycine	-0.94399*	-0.28949
Methionine	-0.96643*	0.073764
succinate	-0.56547	-0.35331
Tryptophan	-0.71416*	0.674728*
Serine	-0.9165*	-0.15294
Glycerol	-0.89188*	-0.03128
inosine	-0.31595	0.625355*
Thereonine	-0.97947*	0.059793
Phenylalanine	-0.91975*	0.10664
Tyrosine	-0.9694*	-0.04208
Hydroxyproline	-0.93652*	0.23991
aspartic acid	-0.91243*	0.098791
Fumerat	-0.91265*	0.175854
Glutamate	-0.9408*	-0.15725
hypoxanthine	-0.41796	-0.54054
Lysin	-0.95003*	0.097352
Xanthine	-0.50054	0.726341*
glycerol3p	-0.69828*	0.048967
Glucose	-0.84863*	-0.13604

P<0.05*

Energy Balance and Tricarboxylic Acid Cycle (TCA)

Mitochondria are the power house of the cell responsible for energy supply [21]. Glucose is the main source for energy and converted via glycolysis to pyruvate which is then transferred into mitochondria to produce ATP through the TCA cycle and the mitochondrial respiratory chain [22]. TCA cycle is a sequence of chemical reactions take place in all living organisms to get energy and it has a vital importance to many biochemical pathways [23]. Under normal conditions, the glucose oxidation occurs through aerobic respiration; while under hypoxia, the major energy supply shifted to anoxic or anaerobic respiration, which is less efficient than aerobic respiration in energy production [24]. Previous studies found that exposure to CYP caused severe gill alterations; hyperplasia of lamellar cells, telangiectiasis of lamellae and thickening due to cellular infiltration, highly active mucous cells and fusion of secondary lamellae and epithelial lifting [25,26]. Oxygen transport was affected by gill damage predicting a hypoxic condition. Compared to the control group, a reduction in pyruvate level at 96 h (loading values are 0.5 for PC1 and 0.5 for PC2) was observed (Figure 2). Pyruvate supplies energy through both aerobic and anaerobic respiration, and the other TCA intermediates like succinate, fumerate and malate (Figure 2) suggesting that the TCA cycle was negatively disturbed due to the detrimental effect of cypermethrin on the mitochondrial membrane. This observation of high energy consumption is consistent with previous studies in fishes in which neural hyperexcitability and inhibition of the TCA cycle have been proposed to explain the high demand of energy of organisms under various stress conditions[27-30] . In the present study, the level of lactate was decreased at 96 h (loading value is 0.8 for PC1) (Figure 2) revealed its excessive consumption to replenish insufficient energy production [24]. Therefore, its low level may pointed to its use as source of energy in the situation of glucose scarcity. Additionally, cypermethrin decreased the glycerol level at 96 h (loading value is 0.8 for PC1) (Figure 2) in response to such urgent need to glucose which in turn leads to activation of gluconeogenesis to get glucose from non-carbohydrate sources as fats and ketone bodies [31, 32].

Amino Acid Metabolism

Catabolism of proteins and amino acids contribute to a great extent to the total energy production in fishes. Exposure of carp to cypermethrin in the current study resulted in a decline in the hepatic level of most amino acids. These results are in agreement with David et al., 2004 [3] after exposure of carp to 1.2 µg/L cypermethrin for 48 h. Protein depletion in liver is considered as a compensatory mechanism to provide intermediates necessary for kreb's cycle and overcoming the energy crisis occurred as a result of exposure to cypermethrin.

The present outcomes revealed significant reduction in the levels of the BCAAs (leucine, isoleucine and valine) in L- and H- groups at both 24 and 96 h time points compared to control group (Figure 3). Branched chain amino acids (BCAAs) are essential amino acids and are important for protein synthesis. Similar results were observed after exposure of goldfish to lambda-cyhalothrin at concentration 0.012 µg/L for 7 days [24]. The decreased levels of BCAAs suggested their usage in synthesis of proteins in response to their damage. BCAAs could stimulate the ventilatory response, were consumed to survive difficult respiration and were involved in energy metabolism [33]. Leucine can be degraded to form metabolites such as acetoacetate and acetyl-CoA that play a role in the TCA cycle while valine acts as an intermediary metabolite, forming succinyl-CoA [34, 35]. Alanine is a non-essential amino acid that synthetized in the body via conversion of pyruvate. Alanine is a major raw material for gluconeogenesis and is produced by skeletal muscles and erythrocytes [36]. Therefore, changes in the levels of alanine in fish under stress suggest its usage in the pathway of gluconeogenesis. In the present study, a reduction in the level of alanine was observed at 96 h (loading value is 0.8 for PC1) (Figure 4).

Glycine and serine are interconverted primarily in liver and kidneys and they participate in gluconeo genesis, sulfur amino acid metabolism and fat digestion [37]. Both amino acids work to maintain blood sugar level. In the present study, their levels were decreased after cypermethrin exposure may refer to the disturbance in serine glycine pathway and glucose synthesis during CYP stress (Figure 4). Similar effects

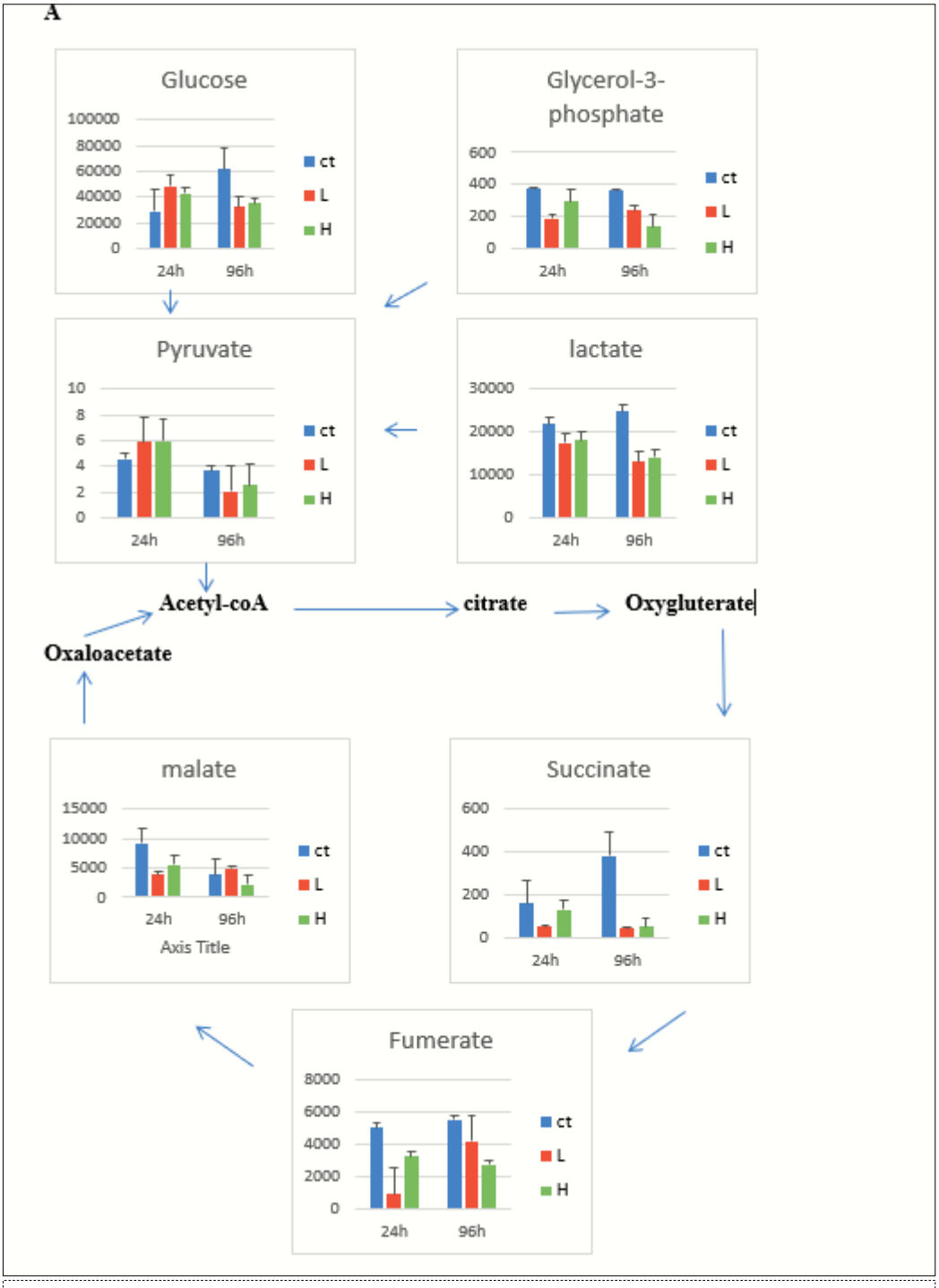

Figure 2. Disorders of TCA cycle, glycolysis and gluconeogenesis at 24, and 96 h of exposure for control group (blue), L group (red), and H group (green) (A) and the variations of creatinine level (B) in liver of carp fish. The values on the y-axis were normalized, divided by fish weight, and multiplied by 10^5 (unit, mg−1). Results are represented as means ± SE.

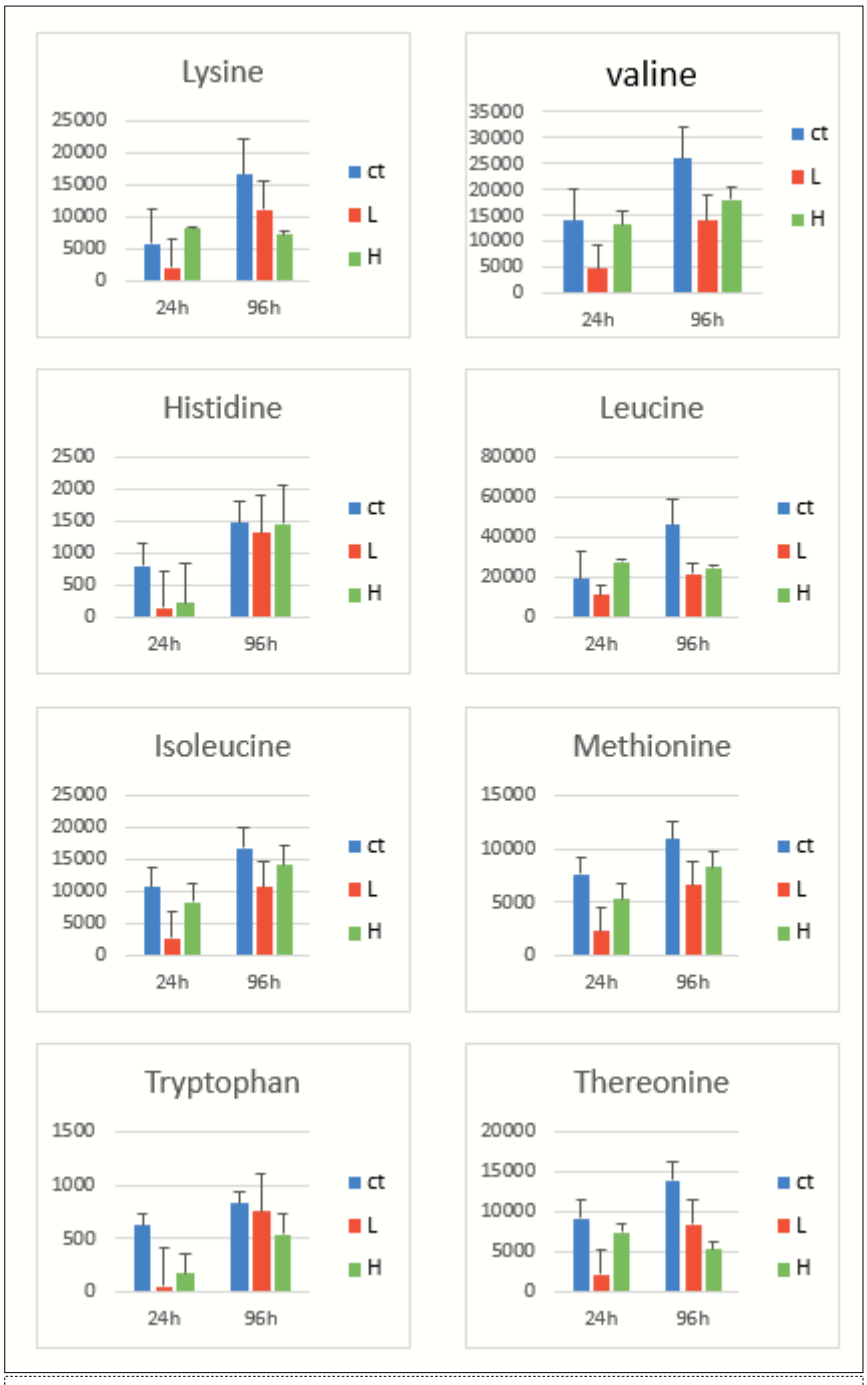

Figure 3. Levels of essential amino acid at 24 and 96 h of exposure for the control group (blue), L group (red), and H group (green) in liver of carp fish. The values on the y-axis were normalized, divided by fish weight, and multiplied by 10^5 (unit, mg−1). Results are represented as means ± SE.

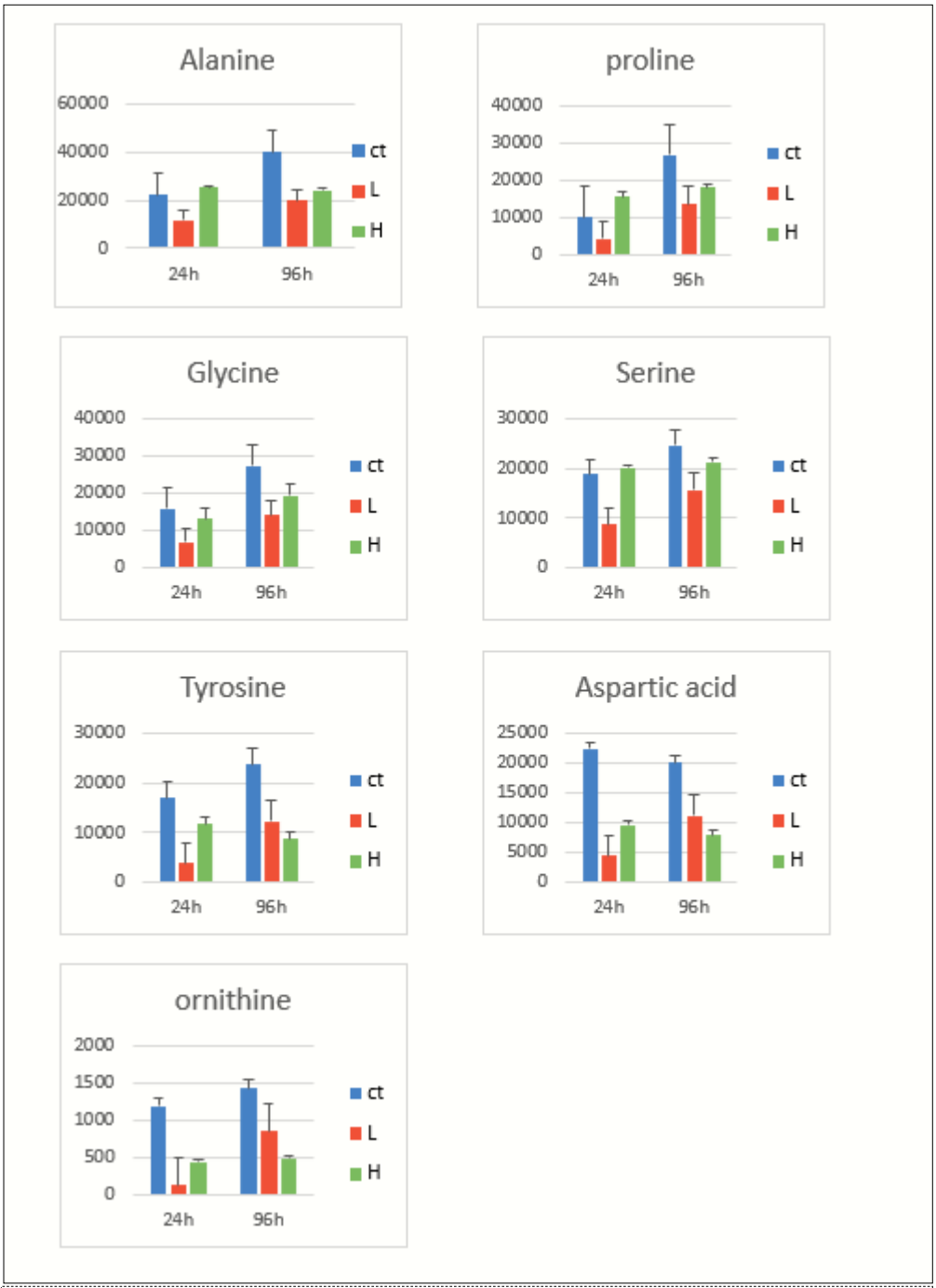

Figure 4. Levels of non-essential amino acid amounts at 24 and 96 h of exposure for the control group (blue), L group (red), and H group (green) in liver of carp fish. The values on the y-axis were normalized, divided by fish weight, and multiplied by 10^5 (unit, mg−1). Results are represented as means ± SE.

were reported in zebrafish exposed to acetamiprid and halosulfuron-methyl either individually or combined [23].

Effect on Purine Metabolism

The purine nucleotides serve as structural components of DNA and RNA molecules, cofactors of many metabolic processes and a source of energy provider adenosine triphosphate (ATP) [38]. In the present work, the reduction of xanthine and elevation of hypoxanthine in liver in H-group at both 24 and 96 h time points (Figure 5) suggesting that cypermethrin considerably disturbed purine metabolism in carp fish proposing neurotoxic effects. Exposure of zebrafish larvae to short-chain chlorinated paraffins (SCCPs), acetamiprid and halosulfuron-methyl resulted in similar results [13]. Particular neuro-studies proved that the alteration of purine metabolism in human body is highly related to numerous nervous system diseases, such as Alzheimer's disease [39] and Parkinson's disease [40].

Conclusion

Using GC–MS metabolomics approach, cypermethrin at environmentally relevant concentrations induced alterations in numerous metabolites in hepatic tissue of zebrafish represented by energy imbalance, disturbance in Kreb's cycle, modification in amino acid metabolism, detrimental effects on nucleotide metabolism and carcinogenic tendency.

Overall, the present outcomes showed the potential of GC-MS based metabolomics approach to

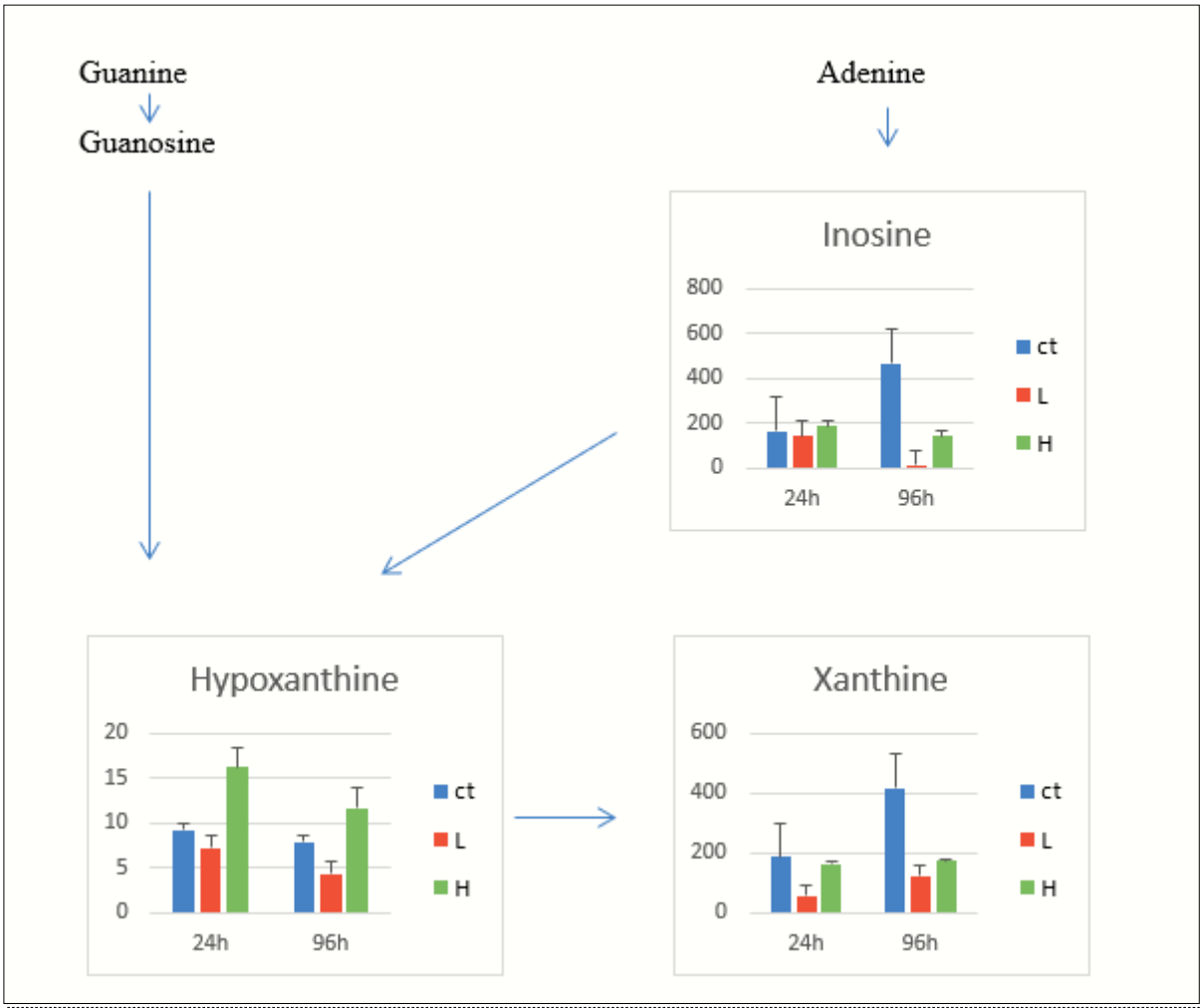

Figure 5. Disorder of nucleoside metabolism at 24 and 96 h of exposure for the control group (blue), L group (red), and H group (green) in liver of carp fish. The values on the y-axis were normalized, divided by fish weight, and multiplied by 10^5 (unit, mg−1). Results are represented as means ± SE.

unravel the toxicological effects of cypermethrin and its underlying mechanisms.

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