Metabolomics Study with Gas Chromatography-Mass Spectrometry of Hepatotoxicity on Adult Zebrafish (*Brachydanio Rerio*) after Exposure to Diquat

Ye Xiao
Hainan University
Xiang Lin
Hainan University
Zhong-Qun Liu
Hainan University
Mei-Lan Zhou
Hainan University
Tian-Yu Ren
Hainan University
Rui-Li Gao
Hainan University
Wen-Jing Shen
Hainan University
Rong Wang
Hainan University
Xi Xie
Hainan University
Yan-Ting Song
Hainan University
Wen-Ting Hu (Huwt_0@hainanu.deu.cn)
Hainan University https://orcid.org/0000-0003-2561-9769

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Abstract

Although diquat is a widely used water-soluble herbicide in the world, its toxicity to freshwater fish has not been well characterized. In this study, gas chromatography-mass spectrometry (GC-MS) based metabolomics approach combined with histopathological examination and biochemical assays was applied to comprehensively assess the hepatotoxicity in zebrafish (*Brachydanio rerio*) after diquat exposure at two dosages of 0.34 and 1.69 mg·L^{-1} for 35 days. The results indicated that 1.69 mg·L^{-1} diquat exposure cause serious cellular swell and vacuolization with increased nuclear abnormality, and lead to obvious disturbance of antioxidative system and dysfunction in liver; while no obvious pathological injury could be detected, and changes in liver biochemistry were less pronounced at the dose level of 0.34 mg·L^{-1}. Multivariate statistical analysis and pattern recognition showed different GC-MS profiles of zebrafish liver following exposure to diquat, the cluster of the treated groups were both clearly separated from the control samples. The differentially abundant metabolites mainly include carbohydrates, amino acids, lipids, nucleotides, and their derivatives. In the exposure group of 1.69 mg·L^{-1} diquat, severe disturbances of amino acid metabolism played important biological roles associated with inhibition of energy metabolism, reduced immunity, and disorders in neurotransmitters as pathway analysis revealed. Additionally, fluctuation of inositol, creatine, and pantothenic acid, substances associated with stress regulation and signal transduction, participating in metabolic abnormalities in zebrafish with diquat-triggered hepatic damage. Energy metabolism of zebrafish exposed on 0.34 mg·L^{-1} diquat more inclined to rely on anaerobic glycolysis than the normal ones. Amino acid metabolism responses were less affected, but obvious interference effects on lipid metabolism were observed with 0.34 mg·L^{-1} diquat exposure. These results imply increased sensitivity of metabolomics versus histopathology and clinical chemistry in recognizing liver toxicity of diquat. This study will contribute to explore possible mechanism of hepatic damages on nontarget freshwater fish induced by diquat and provide important basis for its environmental risk assessment.

1 Introduction

Diquat is a non-selective contact bipyridyl herbicide widely used in crop protection for control broad-leaved and grassy weeds, to accelerate the ripening of seed and fodder crops, and as aquatic weed control agent for submersed vegetation in aquatic systems (Jones et al. 2000). Despite lower toxicity of diquat were reported compared with paraquat, which use has been prohibited, its environmental risk associated with the large-scale application as a redox cycling compound remains a concern for biological health (Djukic et al. 2007; Fussell et al. 2011; Nisar et al. 2015). The toxicity of diquat is thought to be due to free radical chain reactions and subsequently hydrogen peroxide production, and it can even cause a large amount of reactive oxygen species (ROS) much higher than paraquat (Farrington et al. 1973; Fussell et al. 2011). It was proved that diquat could cause a variety of toxicological outcomes in the liver, kidney, and small intestinein (Smith et al. 1985; Rawlings et al. 1994; Badah et al. 2007; Lu et al. 2010). Moreover, diquat showed a relationship with neurodegenerative diseases such as Parkinson's disease (Bove and Perier 2012).

Diquat as polar and water-soluble compound can enter aquatic body follow rain and surface runoff, and it has been detected as residues in various water environments (Siemering et al. 2008; Bouétard et al. 2013). Massive use of diquat in aquatic and terrestrial environments unavoidably deteriorated water quality, there is a great threaten to non-target fish species in the aquatic ecosystem. Unlike paraquat, mainly causes lung injury and lung fibrosis, the main target organs of diquat poisoning are liver and kidney (Litchfield et al. 1973; Wang et al. 2018). The imbalance of liver metabolism is not only related to disrupting the physiological functions of detoxification and immunity, but also an important cause of hepatic encephalopathy (Haussinger and Schliess 2008). Although only a few research have focused on the aquatic toxic effects of diquat, the results also showed that it had adverse effects on the liver of fish. Diquat administration to three-spined stickleback (*Gasterosteus aculeatus* L.) for 21 days leaded to the alteration of hepatic enzymes for xenobiotic metabolism in the liver (Sanchez et al. 2006). The commercial direct applications of diquat delayed the growth and development of rainbow trout (*Oncorhynchus mykiss*), and affected the proteins involved in metabolism, apoptosis, and energy homeostasis in hepatocytes (McCuaig et al. 2020). As an important metabolic organ of fish, liver is one of the target organs of toxic reactions. However, current studies are insufficient to comprehensively evaluate the effects on the liver of fish resulted from diquat. Metabolomics can assess the profiling of endogenous metabolic compounds with low-molecular weight directly reflect the pathological and physiological state of biological samples. It has been widely used for toxicity assessment of chemicals promoting our understanding of environmental risks on the function and health of organism (Nicholson and Lindon 2008). In this study, chronic effects of sub-lethal diquat exposure with concentrations of 0.34 $mg{\cdot}L^{-1}$ and 1.69 $mg{\cdot}L^{-1}$ were evaluated using adult zebrafish, which had a lot of advantages as a model organism in environmental risk studies of chemical pollutants (Wang et al. 2017). We performed metabolomic analysis with GC-MS of zebrafish liver and investigated the hepatotoxicity to detect the toxicological effects of diquat in different exposure circumstances. The results might provide novel insights into understand the mechanistic complexity of liver injury impact by diquat on non-target fish in environments.

2 Materials And Methods

2.1 Chemicals, reagents and animals

Commercial grade herbicide, 200$g{\cdot}L^{-1}$ diquat dibromate hydrate, was purchased from local pesticide sales center in Haikou, produced by Shengpeng Chemical Co. LTD in China.

Assay kits for methane dicarboxylic aldehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The BCA protein concentration kit was purchased from Beyotime Biotechnology (Shanghai, China).

Adonitol standard (99%) was provided by Sigma-Aldrich Co. LLC (Shanghai, China). Fatty acid methyl esters (FAMEs) and *bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA) reagent (with 1% trimethylchlorosilane,

TMCS, V/V) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and REGIS (Los Angeles, USA), respectively. Methanol, chloroform and pyridine, purchased from Adamas Reagent Co. (Shanghai, China), were of HPLC (High Performance Liquid Chromatography) grade. Methoxyaminatio hydrochloride was of analytical grade and obtained from TCI (Tokyo, Japan).

Adult zebrafish (*Brachydanio rerio*), each weighing 0.2-0.3 g were purchased from a local commercial dealer (Haikou, China). Before the experiment, the fish were acclimated to the experimental conditions for two weeks in a 60 L glass tank in dechlorinated tap water maintained a dissolved oxygen 6 $mg \cdot L^{-1}$. The temperature was controlled at 25±2°C with a light-dark photoperiod cycle of 14 h:10 h. Zebrafish were fed daily with commercial diet at 3% bodyweight once a day. Fish care and experimental protocols were carried out in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and approved by the Animal Ethical Committee of Hainan University.

2.2 Fish exposure and sample collection

After two weeks' acclimation, the zebrafish were randomly divided into three groups: high dose of diquat (HDD) group (1.69 $mg \cdot L^{-1}$), low dose of diquat (LDD) group (0.34 $mg \cdot L^{-1}$) and control group (CON). The selected concentrations for exposure were based on the 96-h LC_{50} value (16.92 $mg \cdot L^{-1}$) from a previous study with a 95% confidence interval of 13.00-22.09 $mg \cdot L^{-1}$. The low concentration,1/50 of the 96-h LC_{50} value, was chosen as it is close to the environmental levels measured after spraying diquat in agricultural fields (Emmett 2002). The high concentration,1/10 of the 96-h LC_{50} value, was chosen as it is comparable to the levels of direct use in aquatic systems (Ritter et al. 2000). Diquat was added according to the experimental concentration. Each group with three replications (twenty fish per tank) and resided in 20 L plastic vessel. The water in each vessel was changed once a day and the experimental conditions were the same as those used during the acclimation period.

Being exposed to diquat for 35 days, all zebrafish were fasted for 12 h, anesthetized with 100 mM tricaine methane sulfonate (MS-222), sacrificed and liver were collected. For the metabolomics study, three fish were removed and polled as one sample, and five replicates at each concentration level. Three replicates of fish devoted to the histopathology analysis. Six fish one sample that dedicated to the biochemical assays were sampled from each vessel.

2.3 Biochemical analysis and histopathologic examination

For biochemical assays, the livers were dissected out quickly and homogenized in 75 mM phosphate buffer (pH 7.6) to obtain a 10% (w/v) solution. Levels of MDA, and the activities of SOD, CAT, GST, ALT and AST were determined using commercially kits. The BCA protein concentration kit was used to determine the total protein content. The results are expressed per milligram of total protein.

For histopathologic examination, the excised livers were removed as quickly and intactly as possible, rinsed with cold phosphate buffered saline solution, fixed in 4% paraformaldehyde solution. Then the

tissues embedded with paraffin wax and sectioned (4-5 mm). The sliced sections, stained with eosin and hematoxylin (HandE), examined under light microscope (NIKON Eclipse ci, Tokyo, Japan).

2.4 Sample preparation for GC-MS

The livers were dissected out quickly and immersed in liquid nitrogen immediately, stored at −80°C before test. First, 500 μL volume of pre-cold of methanol/chloroform (3:1, by vol.) with 10 μL of adonitol as an internal standard was immediately added into 25mg of liver sample, mixed by vortexing for 30 s. Add the steel ball grinding for 4 min with 45Hz, and ultrasonic treatment (3 times, 5 min) in ice water bath. After centrifugation at 12000 rpm and 4°C for 15 min, the supernatant was transferred into a fresh tube. Four “quality control” (QC) samples were prepared to evaluate the stability and reproducibility of the instrument by combining 80 μL of each sample. After evaporation in a vacuum concentrator, 60 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) was added and incubated at 80°C for 30 min, then derivatized by 80 μL of BSTFA (1% TMCS, v/v) at 70°C for 1.5 h. Gradually cooled to room temperature, then 10 μL of a standard mixture of fatty acid methyl esters (FAMEs, 0.5 mg/mL in chloroform) was added.

2.5 GC-MS analysis

Liver samples were detected on an Agilent 7890 gas chromatograph coupled with a PEGASUS HT time-of-flight mass spectrometer. 1 μL aliquot of sample was injected into a DB-5MS capillary column (30 m×250 μm×0.25 μm)in splitless mode. Helium was used as the carrier gas, and flow through the column kept constant at 1 mL/min. The initial temperature was maintained at 50°C for 1 min, and increased to 310°C at a rate of 10°C min^{-1}, then kept for 8 min. The injection, transfer line, and ion source temperature were set at 280, 280, and 250°C, respectively. The electron impact mode was ionization at 70 eV. Full scan mass spectra were acquired from m/z of 50 to 500 at a rate of 12.5 spectra per second after a solvent delay of 6.27 min.

2.6 GC–MS data acquisition

Chroma TOF (V 4.3x, LECO) was used to adjust baseline, deconvolute spectral, align and integrate peaks. Peaks detected in less than 50% of QC samples or relative standard deviation greater than 30% in QC samples was removed. The deviation was filtered based on the interquartile range to remove noise. Only the peak data with no more than 50% null value in a single group or no more than 50% null value in all groups were retained. The missing values in the original data were simulated by half of the minimum value. Internal standard method was used for data normalization. Chemometric analysis of the data was performed using SIMCA-P (V16.0.2, Sartorius Stedim Data Analytics AB, Umea, Sweden) software. Principal component analysis (PCA) was applied to the spectral data by logarithmic transformation and center scaling to visualize inherent clustering. Partial least squares discriminant analysis (PLS-DA) was used to further evaluate the level of separation among all the groups. Orthogonal partial least squares discriminate analysis (OPLS-DA) was applied to the spectral data by logarithmic transformation and unit variance scaling to clarify the difference and find the different metabolites. The variables were screened on the basis of variable importance in the projection (VIP) values obtained from OPLS-DA model greater

than 1.0 and *p* values less than 0.05 from Student's t-test, and then assessed by the fold change value with FC values <0.80 or >1.25. The metabolites of zebrafish liver were identified by matching the mass spectrum and retention index with the standard mass spectra on the LECO-Fiehn Rtx5 database (Chang et al. 2015), then further supported by the HMDB database, PubChem database and KEGG database (Davey et al. 2011).

2.7 Metabolic pathway construction

Based on the significantly changed metabolites, integrated enrichment analysis and pathway topolopy analysis were performed using MetaboAnalyst 5.0 (http:// www.metaboanalyst.ca) to assess the toxicity of diquat to zebrafish. Then, the major perturbed biological pathways caused by diquat exposure were constructed on potential functional analysis by searching the KEGG pathway database.

2.8 Statistical analysis

One-way ANOVA was performed using SPSS 19.0. Data were expressed as the mean ± standard deviation (SD). A *p* value less than 0.05 was considered as statistically significant.

3 Results

3.1. Liver histopathology

Zebrafish were exposed to 0.34 mg·L^{-1} and 1.69 mg·L^{-1} diquat for 35 days, images of HandE-stained zebrafish liver sections are shown in Fig. 1. In the blank control group (Fig. 1a), zebrafish revealed normal cell structure, hepatocytes were evenly distributed, the liver sinusoids were orderly, and cell morphology was clear boundaries. No obvious liver cell necrosis and vacuolization were noticed. Compared with the normal group, little change was observed in the liver tissue of LDD grouper treated with 0.34 mg·L^{-1} diquat, which displayed no various signs of liver pathological injuries except for slight vacuolization (Fig. 1b). In the liver tissue of HDD grouper treated with 1.69 mg·L^{-1} diquat (Fig. 1c), the hepatocytes were disordered with serious swollen, vacuolization, and cloudy degeneration. Additionally, the nuclei of the liver cells were darkly stained and pyknotic, and nuclear lysis were noted.

3.2 Biochemical analysis

The oxidant responses in the liver exposure to diquat were measured by assessing the levels of MDA, and the activities of SOD, CAT, and GST (Fig. 2a-d). MDA assay was performed to evaluate lipid peroxidation that occurred in the liver tissues as shown in Fig. 2a. MDA level of the HDD group was increased compared with the control ($p < 0.01$), which indicated that lipid peroxidation occurred in zebrafish liver induced by 1.69 mg·L^{-1} diquat. Antioxidant enzymes were determined as shown in Fig. 2b-d, no significant changes in hepatic SOD, a significant increase in CAT activity and a significant decrease in GST activity was observed in the HDD group as compared with the control ($p < 0.05$). LDD group was less responsive to diquat, in which the oxidative stress indexes were tested showed no significant change.

The functional state of zebrafish liver was assessed by the levels of ALT and AST in the groups (Fig. 2e and 2f). Compared with the normal group, the activities of ALT were markedly enhanced ($p < 0.05$) in the HDD group exposed to 1.69 mg·L^{-1} diquat, while the AST levels have no obvious change. The activities of ALT and AST were maintained at normal levels in the liver of LDD grouper exposed to 0.34 mg·L^{-1} diquat.

3.3 GC–MS metabolic profile of zebrafish liver

GC-TOF-MS based metabolomics analysis was conducted to evaluate the metabolic changes resulted from diquat exposure. Typical total ion chromatogram (TIC) of liver samples from zebrafish of each group is shown in Supplementary Fig. S1. A total of 533 peaks were detected in this study. After exclusion of noise on data pre-processing, there was 459 valid peaks selected for further analysis. Unsupervised PCA analysis was performed to enable the initial overview of the dataset, visualizing group trends in the liver metabolome (Fig. 3a). The analytical platform showed good reproducibility and stability as evident by tight QC sample clustering, which indicated the reliability of the metabolomic approach. The PCA score plot showed a partial separation and clustering between the three experimental groups. The metabolic phenotypes of the control group and the high dose of diquat group formed two distinct clusters, indicating there were differences in liver metabolic fingerprints. The low dose of diquat treatments shifting towards those in the high dose of diquat group. To better classify the liver metabolic profiles of three experimental groups, we further assessed the differences in metabolite profile by supervised PLS-DA method to guarantee a more reliable result output. Scatter plots of PLS-DA were shown in Fig. 3b, the endogenous metabolites in the liver of the exposure groups demonstrated clearly significant changes compared with the control group. The model ($R^2Y = 0.997$, $Q^2 = 0.856$) has good discrimination and prediction degree. The results suggested that 0.34 mg·L^{-1} and 1.69 mg·L^{-1} diquat exposure both induced disturbance of small molecule metabolites in the liver of the treated zebrafish compared with the control ones.

After removing and integrating some compounds according to similarity and other information, a total of 128 metabolites in zebrafish liver were identified and quantified. The intensities of metabolites were listed in a heat map (Fig. S2), and the separate clades between groups consistent with the PCA analysis.

3.4 Metabolic responses to diquat in liver of zebrafish

Score plot of PCA showed visually displays differences between liver samples of the control and test groups at different exposure concentrations (Fig. S3). The results of PCA analysis suggested that disturbance of small molecule metabolites occurred following exposed to diquat in the liver of zebrafish. To interpret the discrimination of the global liver metabolic profile upon diquat treatment, OPLS-DA model was performed for further analysis. Score plot of OPLS-DA exhibits clear differences between the control and exposure groups at both 0.34 mg·L^{-1} and 1.69 mg·L^{-1} diquat (Fig. 4a and 4c). The evaluation indexes of two models indicating faithful representation of the data and good cumulative predictive power, expressed as follows: $R^2Y = 0.989$ and $Q^2 = 0.390$ at 0.34 mg·L^{-1} diquat; $R^2Y = 0.979$, and $Q^2 = 0.663$ at 1.69 mg·L^{-1} diquat (Fig. 4b and 4d). Based on the results of VIP and p value, 17 and 22 differentially abundant metabolites were identified under diquat stress at 0.34 mg·L^{-1} and 1.69 mg·L^{-1},

respectively (Table 1 and Table 2). Six commonly altered metabolites screened out in two diquat treatment groups were aspartic acid, ribose, uridine, creatine, 6-phosphogluconic acid, and monostearin. Except for creatine, elevated levels were consistently found for all shared metabolites in two groups (Fig. 5). In addition, the change trend in glutamine and glutamic acid, which transform into each other in the organism, decreased consistently after exposure.

Table 1
OPLS-DA VIP values and fold-changes of the differential metabolites in the zebrafish exposed with 0.34 $mg{\cdot}L^{-1}$ diquat compared with normal controls

Metabolites	Similarity	Rt (min)	Mass	Mean LDD	Mean CON	VIP	Fold Change
palmitic acid	926	19.53	117	2.5772	1.9374	2.011	1.330 ↑
creatine	918	14.21	115	0.8499	0.5982	1.8	1.421 ↑
oleic acid	914	21.15	117	0.1624	0.0855	2.605	1.898 ↑
stearic acid	914	21.32	117	1.132	0.8118	2.1259	1.394 ↑
aspartic acid	909	13.78	232	0.1616	0.0459	1.86	3.517 ↑
palmitoleic acid	876	19.26	117	0.0969	0.0468	2.621	2.072 ↑
ribose	876	15.59	103	0.0504	0.0337	1.914	1.496 ↑
glycerol 3-phosphate	872	16.56	357	0.7187	0.3466	2.22	2.073 ↑
myristic Acid	826	17.55	117	0.0278	0.0206	1.892	1.345 ↑
squalene	769	25.82	69	0.0258	0.0544	1.114	0.746 ↓
monopalmitin	729	23.83	129	0.0182	0.0091	2.007	2.004 ↑
6-phosphogluconic acid	693	22.68	318	0.1813	0.0839	1.231	2.162 ↑
monostearin	654	25.52	399	0.0051	0.0038	1.888	1.337 ↑
GMP	561	27.41	169	0.0056	0.0028	1.423	1.974 ↑
uridine	399	23.04	387	0.0022	0.001	1.695	2.160 ↑
glutamine	378	14.02	71	0.016	0.0442	1.986	0.361 ↓
asparagine	316	14.57	324	0.0115	0.0052	1.477	2.215 ↑

Rt, retention time. The LDD represents test group treated with 0.34 $mg{\cdot}L^{-1}$ diquat, and the CON represents control group.

Table 2
OPLS-DA VIP values and fold-changes of the differential metabolites in the zebrafish exposed with 1.69 $mg \cdot L^{-1}$ diquat compared with normal controls

Metabolites	Similarity	Rt (min)	Mass	Mean HDD	Mean CON	VIP	Fold Change
alanine	964	8.15	116	2.9529	5.0656	2.0689	0.583 ↓
isoleucine	932	10.82	158	0.2902	0.9486	1.7945	0.306 ↓
creatine	918	14.21	115	0.2857	0.5982	1.8909	0.478 ↓
myo-inositol	911	19.86	217	0.0315	0.0574	2.1467	0.548 ↓
aspartic acid	909	13.78	232	0.1398	0.0459	1.4505	3.043 ↑
sorbose	893	17.70	103	0.0466	0.0326	1.6461	1.429 ↑
serine	892	11.70	204	0.2720	0.6515	1.9729	0.418 ↓
phenylalanine	881	15.08	218	0.0500	0.1845	1.7194	0.271 ↓
ribose	876	15.59	103	0.0506	0.0337	1.5576	1.502 ↑
pantothenic acid	818	18.94	103	0.0073	0.0111	1.7207	0.656 ↓
tyrosine	800	17.94	179	0.1354	0.2938	1.7843	0.461 ↓
6-phosphogluconic acid	693	22.68	318	0.1531	0.0839	1.0699	1.826 ↑
3-aminoisobutyric acid	660	9.67	131	0.0006	0.0032	1.3388	0.179 ↓
monostearin	654	25.51	399	0.0052	0.0038	1.3978	1.359 ↑
L-glutamic acid	629	13.96	84	0.0349	0.0612	1.7184	0.571 ↓
putrescine	617	16.33	174	0.0038	0.0125	2.0274	0.303 ↓
4-aminobutyric acid	550	13.92	174	0.0060	0.0106	1.0168	1.746 ↑
uridine	399	23.04	387	0.0023	0.0010	1.3932	2.226 ↑
2-ketoadipate	370	10.10	96	0.0081	0.0106	1.8256	0.756 ↓
galactose	286	18.08	254	0.0023	0.0007	1.8670	3.085 ↑
glycocyamine	250	16.22	180	0.0187	0.0101	1.9104	1.845 ↑
dihydrouracil	227	13.38	128	0.0239	0.0175	1.2672	0.266 ↓

The HDD represents test group treated with 1.69 $mg \cdot L^{-1}$ diquat, and the CON represents control group.

Low dose diquat induced an increase in the levels of 7 fatty acids and fatty acids derivatives (palmitic acid, oleic acid, stearic acid, palmitoleic acid, monopalmitin, monostearin, and myristic acid). At the same time, there were 3 kinds of amino acid and amino acid derivatives (aspartic acid, asparagine, and glutamine) changed in this group. In the high dose diquat group, amino acids and derivatives were greatly affected, with 11 kinds (alanine, isoleucine, aspartic acid, serine, tyrosine, phenylalanine, 3-aminoisobutyric acid, putrescine, 2-Oxoadipate, 4-aminobutyric acid and glycocyamine) changes, yet only 1 fatty acids derivative (monostearin) was found to be elevated. In addition, the content of myo-inositol and pantothenic acid had lower concentrations, and changes of 2 carbohydrate metabolite (sorbose and galactose) and one kind (dihydrouracil) of pyrimidines levels only in zebrafish treated with high dose of diquat. The content of 1 energy substances (glycerol 3-phosphate) and 1 nucleotide (guanosine-5'-monophosphate) were increased, together with low level of squalene only caused by exposure to low dose of diquat.

Metabolic pathway analysis based on the differentially abundant metabolites was performed. The numbers of pathways impacted by 0.34 $mg{\cdot}L^{-1}$ and 1.69 $mg{\cdot}L^{-1}$ diquat were 9 and 18, respectively (Fig. 6). The pathways disturbed by exposure shared in two treated groups were alanine, aspartate and glutamate metabolism; arginine and proline metabolism; pentose phosphate pathway; and pyrimidine metabolism. The impact values of three shared metabolism pathways, including alanine, aspartate and glutamate metabolism, arginine and proline metabolism, and pyrimidine metabolism, were increased in a dose-dependent fashion following diquat exposure. Fourteen pathways, phenylalanine, tyrosine and tryptophan biosynthesis; D-glutamine and D-glutamate metabolism; phenylalanine metabolism; lysine degradation; tyrosine metabolism; arginine biosynthesis; inositol phosphate metabolism; beta-alanine metabolism; galactose metabolism; phosphatidylinositol signaling system; butanoate metabolism; glutathione metabolism; glycine, serine and threonine metabolism; and pantothenate and CoA biosynthesis, had impact value specific to 1.69 $mg{\cdot}L^{-1}$ treated group. Only five pathways, steroid biosynthesis; glycerophospholipid metabolism; purine metabolism; glycerolipid metabolism; and fatty acid biosynthesis, were specific to 0.34 $mg{\cdot}L^{-1}$ treated group. Although the impact value of D-glutamine and D-glutamate metabolism and glycine, serine and threonine metabolism exposed with low dosage diquat was 0, changes in the intermediate metabolites glutamine and creatine were also detected respectively. The comprehensive metabolic profile changes were illustrated by integrating the altered metabolic processes and associated important metabolites based on KEGG pathway database (Fig. 7). These results suggested diquat perturbed the metabolic status of zebrafish liver linked to amino acid, inositol, pyrimidine, energy and redox at 1.69 $mg{\cdot}L^{-1}$; the effect of diquat at 0.34 $mg{\cdot}L^{-1}$ on the above-mentioned metabolism was slight, while it had an obvious effect on lipid metabolism.

4 Discussion

Although the use of diquat is limited in the European Union, it consumed in high levels in many countries and is often abused (Yuan et al. 2021). The tested 20% diquat aqua is a relatively common and widely used preparation product. Diquat is one of the few herbicides that can be directly used in aquatic

systems, has been found in the surface water of many rivers and reservoirs, but no clear conclusions could be drawn in the risk assessment of aquatic organisms (Hiltibran 1972; Rosic et al. 2020). It is important to understand the sublethal effects of exposure to ecological related concentrations of diquat on fish. As a multifunctional important organ of fish, the liver plays a key role in metabolism and detoxification. The habitat of fish determines that the toxic and harmful substances in the water body are more likely to damage the liver, although the liver possesses an extraordinary repair ability. This work illustrated the liver changes of zebrafish exposed to diquat for a long time.

The high concentration of diquat we used was approximately equivalent to the concentration applied in the water body. The zebrafish liver cells showed obvious enlargement and vacuolization, blurred cell boundaries, cell deformation and disappearance of nucleus, which showed that the liver cells were damaged, the cell membrane system was destroyed, and there was a precursor to cell necrosis or apoptosis. Exposure to herbicide diquat lead to the increase of redox cycling compounds which generate the formation of active oxygen radical, and mediates systemic toxicity (Eklöw-Låstbom et al. 1986). A large number of relevant research results have demonstrated that oxidative stress is the common pathophysiological basis for the pathogenesis of various chronic liver diseases in fish (Stewart et al. 2004; Gonzalez et al. 2005). The changes of biochemical indexes also showed that diquat exposure destroyed the redox state of zebrafish liver and affected liver function. High dosage of diquat treatment significantly increased the MDA content in zebrafish liver cells, indicated that the increased level of lipid peroxidation inducing membrane damage, which is consistent with the cell edema and blurred contour observed in histopathology. It was also observed that there was no significant change in antioxidant enzyme SOD activity and a compensatory increase in CAT activity. Similar to the results in a previous study that diquat exposure caused zebrafish embryos to show higher transcript levels of CAT compared to control fish and without altering SOD gene expression (Wang et al. 2018). Diquat induces oxidative stress and changes the activity of antioxidant enzymes in cell, the degree of damage depends on the exposure time and concentration (Fussell et al. 2011; Nisar et al. 2015). There are evidences that CAT gene expression can be induced by environmental stressors and poisons (Cupertino et al. 2017). Although increased the CAT activity represents a counter-regulatory reaction to toxicity caused by mild stress from diquat, this reaction is insufficient to prevent liver pathological damage. In this case, we also observed decrease of the GST activity at 35 days. Inconsistent with the research of Sanchez et al. (2006) that 222 $\mu g \cdot L^{-1}$ and 444 $\mu g \cdot L^{-1}$ diquat induced increase in activities of GST of *Gasterosteus aculeatus* for 21 days. GST catalyze the conversion of a variety of electrophilic metabolites with glutathione into hydrophilic compounds, and accelerate the detoxification and excretion of harmful compounds. Prolonged stimulation of diquat at high concentration impaired the detoxification ability of the liver and might lead to the accumulation of toxic substances in hepatocyte. Additionally, high dosage of diquat in this experiment induced the increase in liver ALT levels, and AST also increased but no significant difference, indicating that amino acid decomposition increased, liver synthesis function was hindered, metabolism and detoxification ability decreased. Small changes in AST suggested that there was no damage to the liver parenchyma (Eklöw-Låstbom et al. 1986). Significant histopathological injury and changes of biochemical indexes were not detected in zebrafish after 35 d exposure to 0.34 $mg \cdot L^{-1}$diquat.

Environmental concentration of diquat may not elicit the same significant stress response on adult zebrafish as larvae (Wang et al. 2018).

Liver damage caused by any hepatotoxic agent will cause endogenous severely disordered metabolites (Xu et al, 2015; Wang et al, 2017; Liu et al, 2018). We used GC/MS metabolomics to understand the biological principle by identifying important metabolic differences, and discussed the potential mechanism of diquat induced hepatotoxicity based on metabolic networks to provide in-depth insights into the toxic effect. The metabolome approach is very sensitive as an early toxicity identifier based on metabolite changes and can provide useful information (Li et al. 2014; Xu et al. 2015; Wang et al. 2017). In this study, the metabolic profiling of two treatment groups discriminated themselves from the control. Histopathology and clinical chemistry can only observe the significant changes when the concentration of diquat is 1.69 $mg{\cdot}L^{-1}$. The current GC-MS analysis can prove the difference between diquat and normal group at the dose level of 0.34 $mg{\cdot}L^{-1}$. Similarly, it was found that goldfish after long-term exposure to glyphosate had slight changes in the level of metabolites in the brain, but no histopathological damage was observed (Li et al. 2017). Schoonen et al. (2007) used NMR spectroscopy to analyze mouse urine, which increased a 4- to 16-fold sensitivity versus histopathology and clinical chemistry to recognize early events of liver toxicity. Additionally, the present analysis results support that the response of aquatic species under low and high stress may be different, and the generation of compensatory response or serious toxic damage is affected by the dose of toxic substances. The potential response scheme of zebrafish is summarized in Fig. 6.

Noteworthy aspartic acid up-regulation and glutamate/glutamine down-regulation were in the two exposure groups (Table 1 and Table 2). Aspartic acid and glutamic acid are important intermediates in the alanine, aspartate and glutamate metabolism, that was the most influenced common pathway in the two exposure groups, play a role in energy storage (Fig. 5). As a major metabolic fuel source, the decreased glutamate content demonstrated the reduction of energy reserves. Aspartic acid is the precursor of oxaloacetic acid, an important intermediate in the tricarboxylic acid cycle, and its accumulation suggested that aerobic metabolism might be hindered. At low concentration diquat group, the increase of glycerol 3-phosphate, an intermediate in glycolysis, reflected the transition of energy metabolism from aerobic respiration to anaerobic respiration (Li et al. 2014). It has been reported that diquat can damage the gills, the respiratory organs vital to the survival of fish, and definitely cause breathing difficulties and hypoxia (Berry et al. 1984). In addition, diquat was effective at generating ROS in redox cycling assays with recombinant cytochrome P450 reductase, with the reduction of intracellular oxygen available for metabolic processes (Fussell et al. 2011). A significant decrease in oxygen induces a greater reliance on anaerobic glycolysis to obtain ATP supply (Speers-Roesch et al. 2010). It has been proved that the energy metabolism of goldfish exposed on λ-cyhalothrin is more dependent on anaerobic respiration to provide energy (Li et al. 2014). However, the decrease in lactic acid and inositol, as products of anaerobic respiration, were observed in the high concentration diquat group. The result suggested 1.69 $mg{\cdot}L^{-1}$ diquat caused obvious damage to liver cells, not only the aerobic respiration was restricted, but the anaerobic pathway was also affected and cannot played a good role in energy compensation.

Moreover, this research found that creatine increased in the diquat low-concentration group and decreased in the high-concentration one. Creatine is a well-known energy booster in cellular energy homeostasis (Dworak et al. 2014). A few of studies have reported that the creatine-phosphocreatine system plays a key role in cellular energy metabolism (Oudman et al. 2013). Fluctuations of creatine in zebrafish liver caused by diquat exposure indicated that the disturbance of energy metabolism consistented with the above conclusions. In addition, since creatine also has an indirect antioxidant effect, the activation of creatine at low concentration of diquat could play a certain role in enhancing protection (Junior and Pereira 2008). Energy expenditure is essential for immune function (Eikenaar et al. 2019). The inhibitory effect of diquat exposure on oxygen and energy metabolism severely disrupted the metabolic balance, hindered the synthesis of substances and energy, and posed a challenge to the fish.

D-Ribose and 6-phosphogluconic acid, metabolites in the pentose phosphate pathway, increased in zebrafish liver after exposure to two concentrations of diquat. At the same time, the increase of uridine in the two exposure groups suggested that the enhancement of pentose phosphate pathway, which is the bypass of sugar metabolism provide raw materials for nucleic acid biosynthesis, was reasonable. According to the results of metabolomic analysis, as the raw material for purine and pyrimidine production, the change of pentose affected the downstream nucleotide metabolism. The results of McCuaig et al. (2020) regarding the proteomic of rainbow trout after exposure to diquat showed that the RNA process was enhanced. This imbalance in nucleotide levels is usually associated with immunodeficiency, energy metabolism, and multicellular functions (Aird and Zhang, 2015). In addition, the increased level of metabolism of the pentose phosphate pathway provides more NADPH with reducing power. NADPH provides electrons for the reductive biosynthesis of fatty acids and cholesterol. In the low-concentration diquat group, significant fluctuations in lipid metabolism were observed, and the content of various fatty acids and their derivatives increased. Studies have shown that the chronic exposure of some herbicides and fungicides disturbed the hepatic glycolipid metabolism at sublethal doses and the progression to fatty liver were confirmed (Mesnage et al. 2017; Bao et al. 2020). Diquat and paraquat rely on NADPH for enzymatic single-electron reduction during the redox cycle to form free radical cations (Fussell et al. 2011). The high concentration of diquat occupied a large amount of NADPH for oxidative cycle. Accordingly, the fluctuation of fatty acids observed in metabolomics was weaker than that in the low concentration group. Only one substance monoste arin was observed to increase, which was also included in the differences in the low concentration group. NADPH promotes the regeneration of reduced glutathione, and several studies have confirmed that maintaining glutathione can alleviate the toxicity of diquat to cells and animals (Awad et al. 1994; Rogers et al. 2006; Djurdjevic et al. 2013). Exposure to 1.69 $mg{\cdot}L^{-1}$ diquat reduced pantothenic acid in glutathione metabolism of zebrafish, and the content of precursor glutamate also decreased, indicating that glutathione synthesis was weakened. The decrease of GST activity observed in the detection of biochemical indexes were consistent with these results. Disturbance of the pentose phosphate pathway caused by diquat disrupts the physiological state of the body may be one of the reasons for the serious symptoms of fish.

According to the metabolic results, changes in a large number of amino acids and derivatives were observed in the high-concentration diquat exposure group. Among the 16 impacted pathways, 10 were related to amino acid metabolism, and two pathways were also present at low concentrations diquat group. Significant changes in amino acids are widely observed in the metabolic profiles of aquatic species exposed to various environmental pollutants (Xu et al. 2015; Wang et al. 2017; Li et al. 2018). In the 1.69 $mg{\cdot}L^{-1}$ diquat treatment group, the increase of liver transaminase level indicated the enhancement of amino acid catabolism, which was consistent with the downward trend of most amino acids. Free amino acids could function in energy storage and as molecular modulators involved in important physiological processes (Nagato et al. 2016; Zhang and Zhao 2017). Alanine, as a sugar-generating amino acid, together with glutamic acid generates succinic acid, an important intermediate in the tricarboxylic acid cycle. The reduction of alanine may be used to compensate for the lack of carbohydrates to reduce the dependence on oxygen in hypoxia. Glutamate is not only related to energy metabolism, but also a key modulator in the initiation and development of immune cells (Pacheco et al. 2007; Xue 2011). The decrease of its content will affect the regulation of immune function. Phenylalanine is the precursor of tyrosine, both of which are significantly reduced in the high-dose diquat group. They could be used as an energy substrate to cope with stress, and converted into the neurotransmitter dopamine (Salamanca et al. 2020). Some studies have shown that long-term exposure to diquat causes neurodegenerative diseases such as Parkinson's disease symptoms (Sechi et al. 1992; Baltazar et al. 2014). The decrease of dopamine uptake, one of the important factors of neurodegenerative diseases, could be explained by the lack of these precursor substances. When exposed to 1.69 $mg{\cdot}L^{-1}$ diquat, putrescine, the downstream substance of arginine and proline metabolism, decreased and guanidinoacetate, the upstream substance, accumulated, which indicated that the pathway might be inhibited in zebrafish liver. It has been reported that the obstruction of arginine and proline metabolism might be one of the reasons for liver injury mediated by reactive oxygen (Tzirogiannis et al. 2004; Tkachenko et al. 2012). Additionally, glycine aminotransferase catalyzed the amino transfer of arginine to glycine to produce glycocyamine, the direct precursor of creatine, decreased in this study and creatine showed an increase (Item et al. 2001). The research of Tachikawa et al. (2015) showed that significant change in creatine was also associated with abnormal behavior caused by neurotransmitter metabolism disorder in fish.

The accumulation of galactose at 1.69 $mg{\cdot}L^{-1}$ diquat exposure supported the hypothesis that anaerobic respiration was inhibited, and correspondingly, inositol, as a by-product of galactose metabolism, was observed to decrease. Inositol is not only related to energy metabolism, but also a precursor molecule for many secondary messengers, including inositol phosphates. Inositol phosphates mediates important intracellular signal transduction and play key roles in a variety of cell physiological processes, such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. The metabolic analysis of liver showed that inositol decreased after exposure to diquat, which may lead to the weakening of signal pathway. Park and Koh (2019) proposed that the apoptosis of PC12 cells induced by diquat was related to the activation of caspase-3 and the inhibition of mTOR, which both are downstream molecules of the PI3K/Akt pathway. PI3K/Akt signaling pathway disorders are closely related to the onset and

subsequent development of neurodegenerative diseases (Nakano et al. 2017). In addition, some studies have shown that supplementation of free inositol as an immune enhancer help increase feed conversion and reduce liver fat level for fish to cope with adverse conditions (Shiau and Su 2005). Therefore, low levels of inositol in 1.69 $mg \cdot L^{-1}$ diquat exposure group may be harmful to energy utilization, cause signal conduction to be blocked, and impair a variety of physiological functions.

In conclusion, the hepatotoxic effects of chronic exposure to sublethal doses of diquat were investigated. The results showed that diquat induced hepatic damage and dysregulation of various metabolic pathways involving energy metabolism, lipid metabolism, nucleotide metabolism and amino acid metabolism. The metabolome method is very sensitive as a toxicity identifier to the environmental concentration of diquat according to the change of metabolites. The difference in the effects of two exposure doses on metabolic characteristics reflected different mechanisms. This work provides new insights into the toxic effects of diquat, enabled a comprehensive evaluation of the toxicology beyond the physiological and biochemical changes on zebrafish upon diquat exposure and to propose a potential explanation regarding a series of metabolic pathways disruptions. The specific toxic effects observed in this study, such as neurotransmitter disorders and fat accumulation in the liver, should be further investigated to evaluate toxicity.

Declarations

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Conflicts of interest

The authors declare no conflicts of interest.

Contributions

Wen-Ting Hu contributed to the conception of the study; Ye Xiao, Xiang Lin performed the experiment and wrote the manuscript; Zhong-Qun Liu contributed to manuscript preparation; Mei-Lan Zhou, Tian-Yu Ren, Rui-Li Gao participated in the performance of the experiments; Wen-Jing Shen did the initial analysis of the data; Rong Wang, Xi Xie, Yan-Ting Song helped perform the analysis with constructive discussions.

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Figures

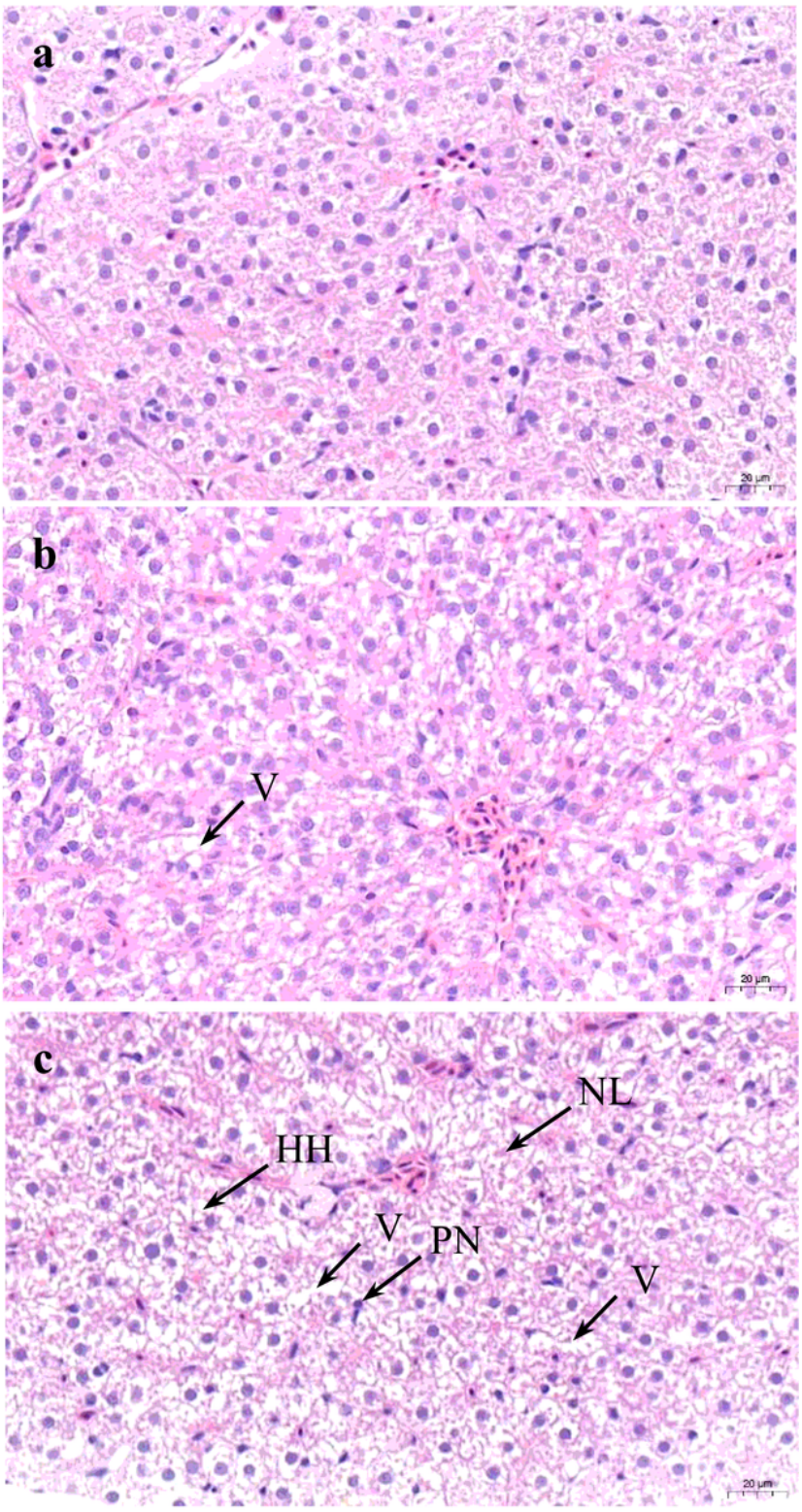

Figure 1

Typical liver histopathology of zebrafish in different groups. **a** Zebrafish liver in the control group. **b** The liver in 0.34 mg·L^{-1} diquat exposure group. **c** The liver in 1.69 mg·L^{-1} diquat exposure group. Sections where the magnification was ×400. *V* vacuolization or non-nuclear cell regions, *HH* hypertrophy of hepatocytes, *PN* pyknotic nucleus, *NL* nuclear lysis and karyorrhexis.

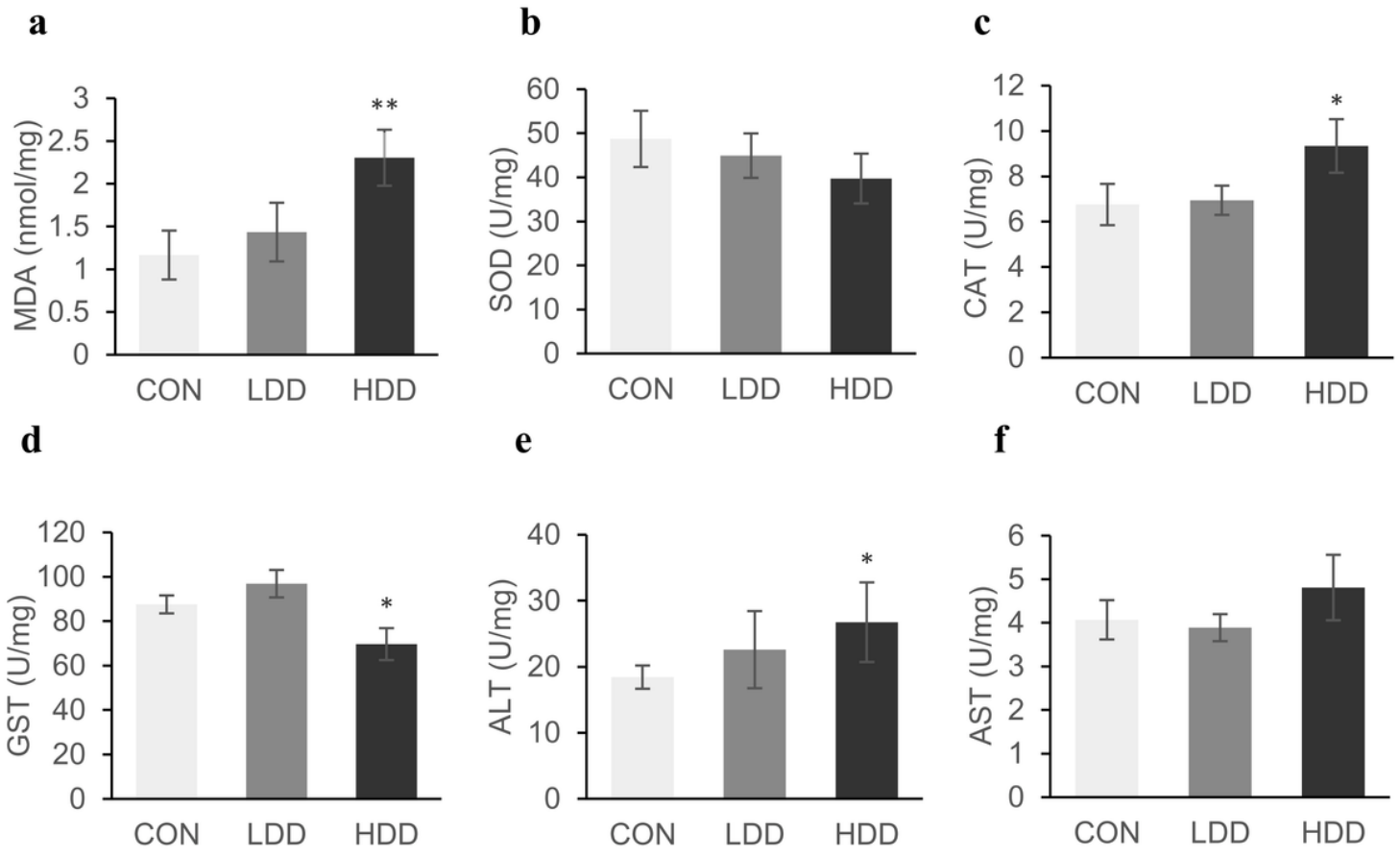

Figure 2

MDA level (**a**), as well as SOD (**b**), CAT (**c**), GST (**d**), ALT(**e**), and AST(**f**) activities of zebrafish in high dose of diquat group (HDD), low dose of diquat group (LDD) and control group (CON). Results of quantitative analysis values are presented as means ± SD (%); n = 3 and 6 fishes per replication; asterisks indicate significantly different from the control group.

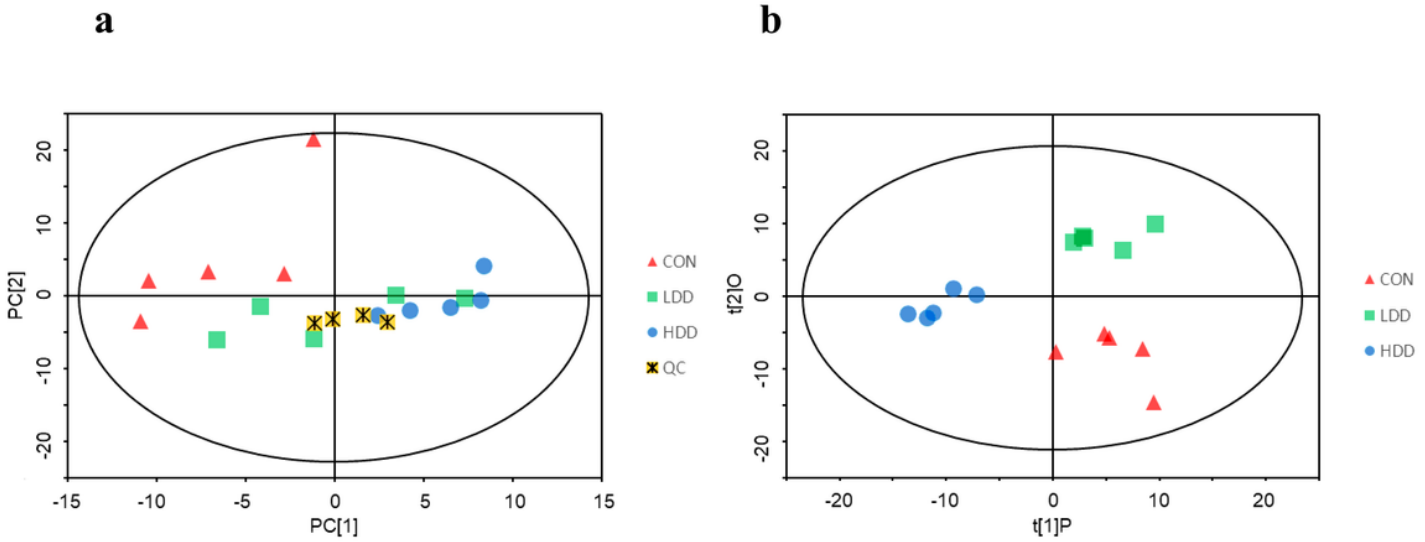

Figure 3

PCA (**a**) and PLS-DA (**b**) score plots based on the GC-MS of the extracts from zebrafish liver (CON: control normal group, LDD: 0.34 mg·L^{-1} diquat exposure group, HDD: 1.69 mg·L^{-1} diquat exposure group, QC: quality control)

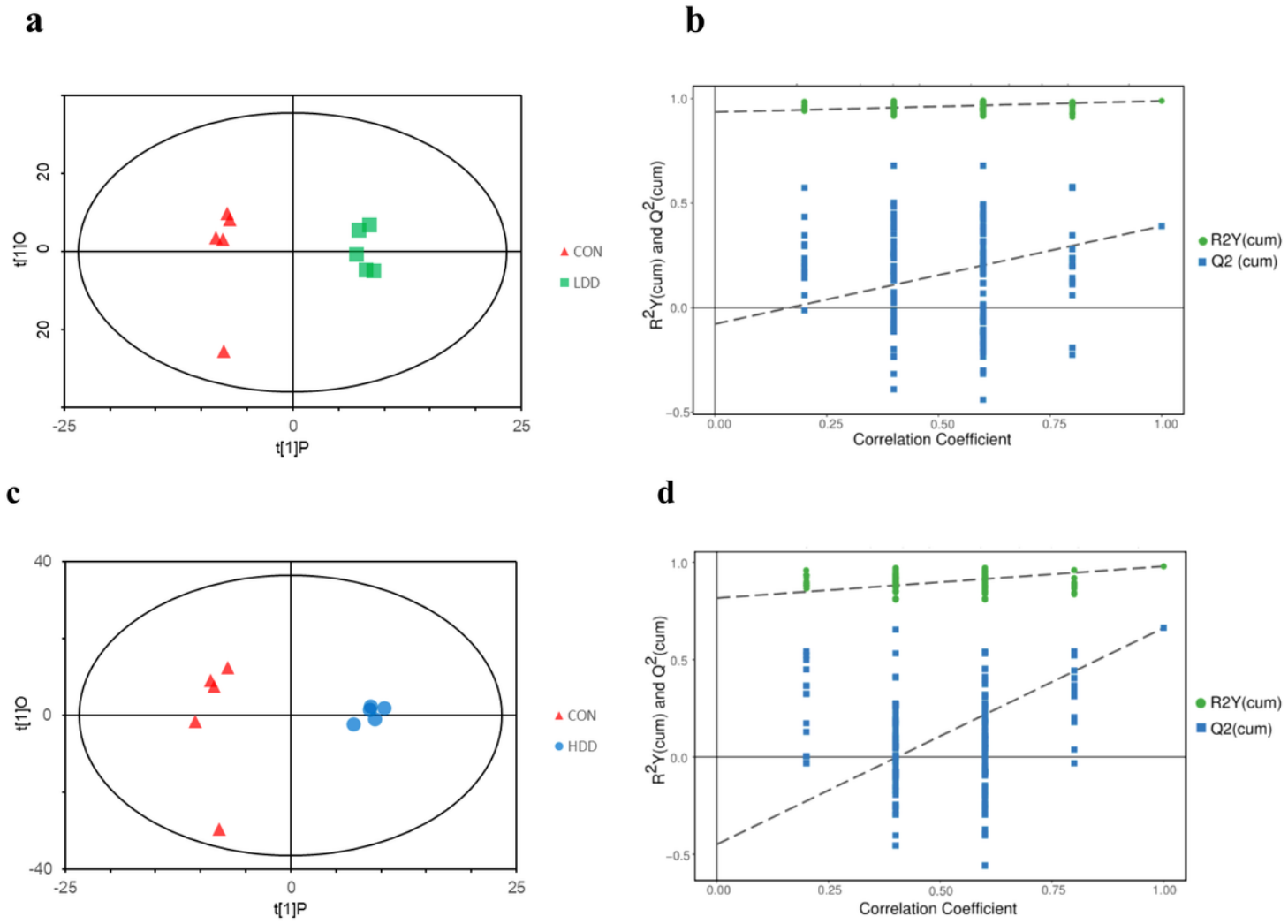

Figure 4

OPLS-DA modeling of GC-MS spectra of zebrafish liver exposed to diquat at doses of 0.34 mg·L^{-1} and 1.69 mg·L^{-1}. S-plots **a** show analysis of the samples exposed to 0.34 mg·L^{-1} diquat and control subjects; S-plots **c** show analysis of the 1.69 mg·L^{-1} diquat tested samples and control subjects; **b**, **d** permutation testing confirmed the good quality of each model.

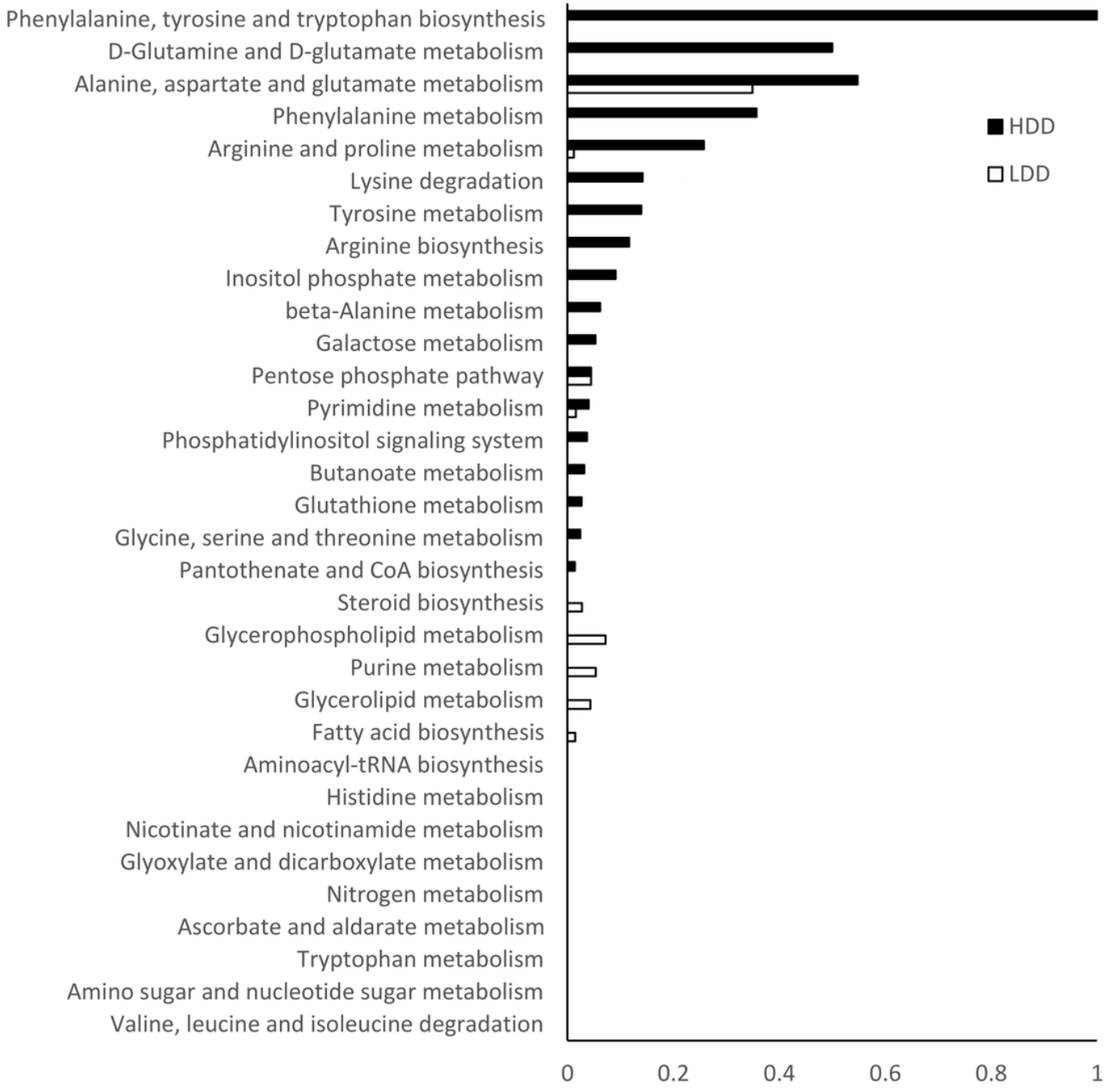

Figure 5

Enriched pathways of differential metabolites in the liver of zebrafish exposed to diquat at doses of 0.34 $mg{\cdot}L^{-1}$ (LDD) and 1.69 $mg{\cdot}L^{-1}$ (HDD)

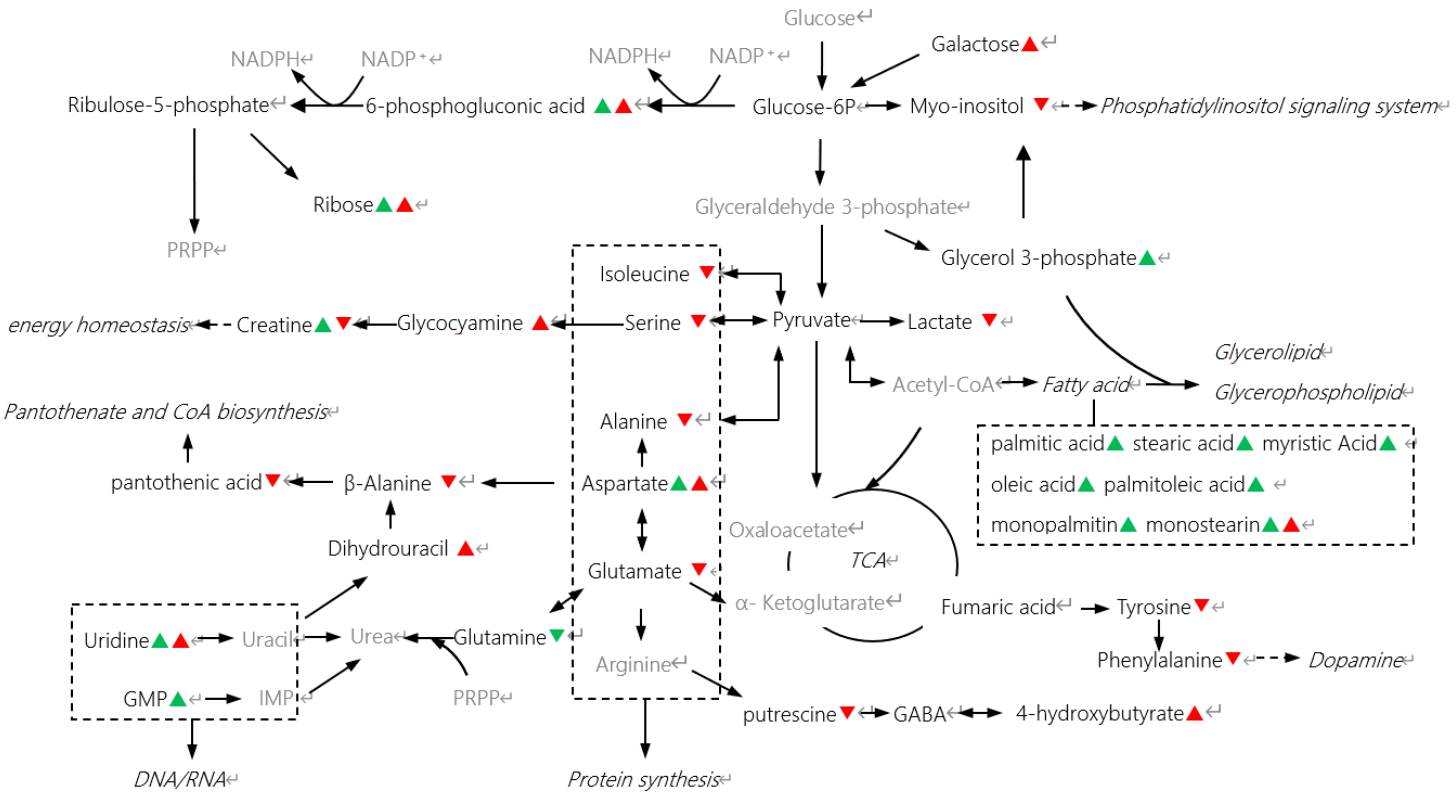

Fig. 6 MetaMap visualization of metabolic regulation in the liver of zebrafish exposed to diquat at doses of 0.34 mg·L^{-1} and 1.69 mg·L^{-1}

Metabolites in black were detected in GC-MS, and in gray were not detected. Markers "▲" and "▼" depict the increased and decreased metabolites in the liver of zebrafish exposed to diquat at doses of 0.34 mg·L^{-1} compared to the normal control, respectively; markers "▲" and "▼" depict the increased and decreased metabolites in the liver of zebrafish exposed to diquat at doses of 1.69 mg·L^{-1} compared to the normal control, respectively.

Figure 6

"See image above for figure legend"

Supplementary Files

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- Supplementarymaterials.docx