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NMR-based Metabolomic Responses of Zebrafish (*Danio Rerio*) by Fipronil Exposure

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Abstract Fipronil, the phenylpyrazole insecticide, is effective and used in various fields. Especially, fipronil was reliable because it was known to be specific on invertebrate animals than vertebrate animals including mammals. However, fipronil had potential risks that affect vertebrate animals as it blocks the gamma-aminobutyric acid (GABA) receptors that also exists in vertebrates as well as invertebrates. Therefore, it was necessary that harmful effects of fipronil on vertebrates are clarified. For this purpose, the zebrafish (Danio rerio) were used on behalf of vertebrate animals in present study. The zebrafish were exposed to 5 μg/L, 25 μg/L, and 50 μg/L of fipronil during 12, 24 and 72 hours. To closely observe toxic process, 12 hours and 24 hours of additional time point were set in the exposure test. Nuclear magnetic resonance (NMR)-based metabolomics is an approach to detect metabolic changes in organism resulted from external stimuli. In this study, NMR-based metabolomics showed the metabolic changes in zebrafish caused by fipronil exposure. Metabolic analysis revealed that fipronil interfered with energy metabolism and decreased the antioxidant ability in zebrafish. Antioxidant ability decline was remarkable at high exposure concentration. In addition, metabolic analysis results over time suggested that reactions for alleviating the excessive nerve excitation occurred in zebrafish after fipronil exposure. Through this study, it was elucidated that the adverse effects of fipronil on vertebrate animals are evident. The risk of fipronil on vertebrates can be no longer ignored. Moreover, this study has a meaning of practically necessary research for organism by examining the effects of fipronil at low concentrations existed in real environment.

Keywords High resolution-magic angle spinning, nuclear magnetic resonance spectroscopy, metabolomics, *Danio rerio*, Fipronil

Introduction

The insecticides are chemical substances used in various fields around the world. The insecticides have increased productivity in agriculture and livestock industry and have risen the level of public health through the control of pests[1]. But indiscriminate use of insecticides bring us headwinds. Due to excessive usage of insecticides, residual insecticides flow into environment and harm the non-target organism such as fish and bird[2,3]. Moreover, incorrect use of insecticides increases the risk of exposure to insecticides.

Fipronil is a broad-spectrum insecticide which belongs to the phenylpyrazole chemical family. It is a

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relatively new compound that was first introduced to the U.S. in 1996 for pest extermination[4]. Fipronil gained popularity because it is effective for pest resistant to conventional insecticides such as organophophate and carbamate[5]. It was used in farms to control pests such as red mite, ant, and cockroaches[4,5]. Furthermore, it was an ingredient of pet care product used in house to remove fleas and ticks of pets[6]. But as fipronil has wide range of use, concerns for potential adverse effects of fipronil have been raised.

The major mode of action of fipronil is known to block the gamma-aminobutyric acid (GABA)-gated chloride channel of neurons in the central nervous system[7]. Fipronil acts as noncompetitive inhibitor of GABA, thereby it interferes with the neural inhibitory function of GABA[8]. Hence, fipronil causes excessive excitation of the nervous system and results in convulsion, paralysis and eventually death[4,9]. However, the toxicity of fipronil was known to differ between invertebrates and vertebrates. This is because fipronil has stronger binding affinity for GABA receptors of invertebrates than GABA receptors of vertebrates[10]. Therefore, fipronil shows more mild toxicity to vertebrates than invertebrates[11]. In addition, it was reported that fipronil induces neurotoxicity by blocking glutamate-activated chloride channel present in invertebrates not in vertebrates[12,13]. For these reasons, fipronil has the selectivity for toxic action. Based on this selectivity, the risk of fipronil exposure to vertebrate animals has been ignored. However, it is clear that fipronil binds to GABA receptors in vertebrates even if it has low binding affinity. Consequently, fipronil could affect vertebrates adversely[14].

Studies of fipronil on vertebrates support this fact[15-17]. It was reported that fipronil caused hepatotoxicity, renal toxicity and neurotoxicity on vertebrates both in vivo and in vitro[18-21]. Therefore, vertebrate animals including human are also not free under the influence of fipronil. In addition, small amount of fipronil present in the environment was a factor of neglecting the effects of fipronil on vertebrates[4,9]. However, low-level of fipronil cannot be also ignored as it was revealed that fipronil is toxic to vertebrates. To elucidate these questions for potential effects of fipronil on vertebrates, this study was designed and performed.

The zebrafish (*Danio rerio*) are suitable experimental animals for purpose of this study. The zebrafish are typical vertebrate animals and have similar genetic information and organs to human such as heart, liver, and kidney. Besides the zebrafish are used in many environmental toxicity tests as they are sensitive to toxicity[22]. The adult male zebrafish are known to be more stable physiologically than the adult female zebrafish[23]. Accordingly, the adult male zebrafish was used to exclude other effects except for fipronil exposure in this study. In addition, motile characteristics between adult zebrafish and larvae dramatically differs. Adult fish continuously swim, while zebrafish larvae can show little or no movement over time, thus their dynamic responses can be disproportion[24-29]. So, in this study, we used adult zebrafish than larvae to observe abnormal mode of action by exposure of fipronil. The zebrafish were exposed to 5 μg/L, 25 μg/L and 50 μg/L of fipronil for 72 hours. 72 hours recommended as acute toxicity time for fish species has been considered too long to observe ongoing toxic process[27]. Therefore, additional time points of 12 hours and 24 hours were set to examine toxic process minutely during stress situation in this study.

Metabolomics is a promising study of omics and has been used for a variety of fields such as disease diagnosis, forensic science, and ecotoxicology[28-30]. Metabolite is the product of cellular metabolism pathways and is sensitively modulated by a variety of external stimuli such as drugs, toxicants, stress, and diets. Metabolomics detects changed metabolites by stimuli and analyzes the pathways involved in altered metabolites[31].

Thus, by using metabolomics, assessment of the influence on organisms caused by external stimuli is possible. Nuclear magnetic resonance (NMR) spectroscopy is one of useful tools to study metabolomics. NMR spectroscopy shows metabolic changes in organism in highly reproducible manner. In addition, NMR spectroscopy has advantages of short acquisition time and simple preparation[32,33].

Metabolites existed in sample can be detected simultaneously by using NMR spectroscopy[34,35]. In particular, High resolution-magic angle spinning (HR-MAS) can measure metabolites without sample pre-preparation such as extraction process, and thus sample is maintained.

Up to date, the effects of low-level exposure of fipronil on vertebrates have not been deeply studied. At real environment, concentration of residual insecticide is low[36,37]. Accordingly, it is necessary to determine the effects of insecticide at low concentrations as well as to examine the mortality, reproductivity perturbation and biomass change in toxicology studies. In this respect, this study has the meaning that figured out the effects of low concentrations of fipronil close to real life. Furthermore, this study is important because it uncovered that fipronil, selective insecticide on invertebrate animals, can affect vertebrate animals including human.

Experimental Methods

Chemicals and Experimental animal- Fipronil (CAS No. 120068-37-3, purity >97.0%) was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Fipronil was dissolved in dechlorinated tap water that is acclimation environment of zebrafish for exposure experiment. The extraction solvents, methanol, and chloroform were obtained from Honeywell Burdick & Jackson (MI, USA). Distilled water used in the experiment was provided by Milli-Q system (Millipore Corp., MA, USA). For NMR measurements, deuterium oxide (D_2O, 99.9 atom % D) and 3-(trimethylsilyl) propionic acid-d_4 sodium salt (TSP-d_4) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All chemicals were of analytic grade. Adult male zebrafish (Danio rerio) were obtained from Green Fish (Seoul, Korea). They were acclimated for a week, maintaining in dechlorinated tap water at 28 ± 1 ℃, and light/dark cycle was kept at 14/10-h. The water in fish tank was aerated and changed three times a week during the acclimation period. The fish were fed a commercial diet (Green Fish, Seoul, Korea) twice a day. During the exposure period and one day before exposure experiment, zebrafish were fasted according to OECD guideline for acute toxicity test[27].

Chemical exposure- After a week acclimation, zebrafish were randomly divided into four groups with 15 fish each and were immersed in a 10 L tank filled with 8 L chemical water. The fish were exposed to 5 μg/L, 25 μg/L and 50 μg/L of fipronil along with control (0 μg/L) for 12 hours, 24 hours and 72 hours. Experimental conditions in exposure period were equal to conditions in acclimation period except for feeding. Exposure experiment was proceeded in the form of static non-renewal test that did not change chemical water during exposure period. After 12 hours of exposure, five zebrafish in each tank were transferred into another tank filled with fresh water. They were rinsed for 1 hour to remove chemical residue on the body surface. After rinsing process, all zebrafish were weighed and flash-frozen immediately in liquid N_2 and then lyophilized overnight. Each dried fish was grinded to powder and it was collected in a conical tube. Metabolite extraction was performed by methanol, chloroform, and distilled water. This method is the modified protocol of bligh and dyer's method that has been used in variety of tissue sampling[38]. After extraction, the aqueous layer of each sample was transferred to a glass vial and flash-frozen immediately in liquid N_2 and then lyophilized overnight. Completely dried samples were reconstituted with 700 μL of D_2O containing 2 mM TSP-d_4 as an internal reference of 0.0 ppm and quantification. Prior to NMR measurements, resulting samples were transferred into 5 mm NMR tubes. This procedure was also performed after 24 hours and 72 hours of exposure, respectively.

NMR measurements- All extracts of zebrafish were measured using 600 MHz Agilent NMR spectrometer (Agilent Technologies, Palo Alto, CA, USA). To obtain clear metabolite peaks, Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence accompanied by PRESAT pulse sequence was used in this study. CPMG pulse sequence can filter out peaks of macromolecules by regulating the T_2 relaxation

delay[39]. This process dramatically decreases the macromolecule peaks that make metabolite peaks uncertain. Accordingly, acquisition of clear metabolite peaks is possible by using CPMG pulse sequence. PRESAT pulse sequence was applied to suppress the signal of water that appears large in sample of zebrafish extracts. All ^{1}H-NMR Spectra were obtained in conditions of 13.188 μs 90° pulse, 3 s relaxation delay and 3 s acquisition time at 298 K. Total measurements take 13 min 9 s. ^{1}H-NMR spectra were measured with 128 transients consist of 4 dummy scans over a spectral width of 9600 Hz. After total acquisition, all ^{1}H-NMR spectra were manually phased, and baseline corrected. ^{1}H-NMR spectra were arranged based on TSP-d_4 peak of reference peak using VnmrJ 4.2 software (Agilent Technologies, Palo Alto, CA, USA).

Statistical Analysis of ^{1}H-NMR Spectra - For multivariate statistical analysis, all ^{1}H-NMR spectra of zebrafish were binned into buckets of 0.001 ppm between 0.5 ppm to 9.5 ppm. Residual areas affected by water suppression (from 4.6 ppm to 5.34 ppm and from 6.05 ppm to 6.20 ppm) were excluded. The segmented spectra were aligned by MATLAB 8.0 (MathWorks Inc., Massachusetts, USA) and were imported into SIMCA-P+ 12.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. As a result of multivariate statistical analysis, orthogonal partial least squares discriminant analysis (OPLS-DA) model, a supervised pattern recognition method, was obtained. OPLS-DA model visualizes the difference among groups in the form of score plot. The property of OPLS-DA model was exhibited by R^2Y and Q^2 parameters.

For quantitative analysis of metabolites, each metabolite in ^{1}H-NMR spectra were identified and quantified using Chenomx NMR suite 7.1 (Chenomx Inc., Edmonton, AB, Canada). By matching peaks with 600 MHz NMR library database in Chenomx and the Human Metabolome Database (HMDB), peaks were assigned. Peaks were quantified by comparing to standard peak of 2 mM TSP-d_4 at 0.0 ppm. Quantified concentrations were extracted from assigned spectra. Based on extracted absolute concentration, relative concentration was calculated to minimize the error resulted from different weight of each animal. Then metabolic difference was compared by mean value (AVG) of calculated metabolites concentration and dispersion of set was measured by standard deviation (STE) which was calculated by the square root of the variance.

Results

Statistical analysis

Pattern recognition method, multivariate statistical analysis, represents NMR spectra in condensed form. One spectrum is marked as one dot in score plot. Spectra with similar pattern are located closely in score plot. The t[1] axis, vertical axis on score plot, is principal axis that represents most correlated variation among groups. The t[2] axis is perpendicular to the t[1] axis and represents the next most correlated variation[40].

Figure. 1 is OPLS-DA score plots about zebrafish exposed to fipronil after 12 hours, 24 hours and 72 hours, respectively. These figures show the distinction between control group and treatment groups at each exposure time. After 12 hours of fipronil exposure, samples came together for each group (Fig. 1a). As exposure time became longer, separation between control group and exposure groups became clear based on the t[1] axis (Fig. 1b and c). These results indicate that there is greater metabolic difference between control group and exposure groups depending on time. Figure 2 shows the distinction among exposure times at each concentration unlike Fig. 1. There was no significant separation among control groups, however as exposure concentration became higher, separation among groups of different exposure times became clear based on the t[1] axis (Fig. 2b-d). These results indicated that there was greater metabolic difference among fipronil exposure times as concentration increases. To figure out the significant metabolic difference between control group and fipronil treatment groups, one-way analysis of variance (ANOVA) was performed using Metaboanalyst 4.0.

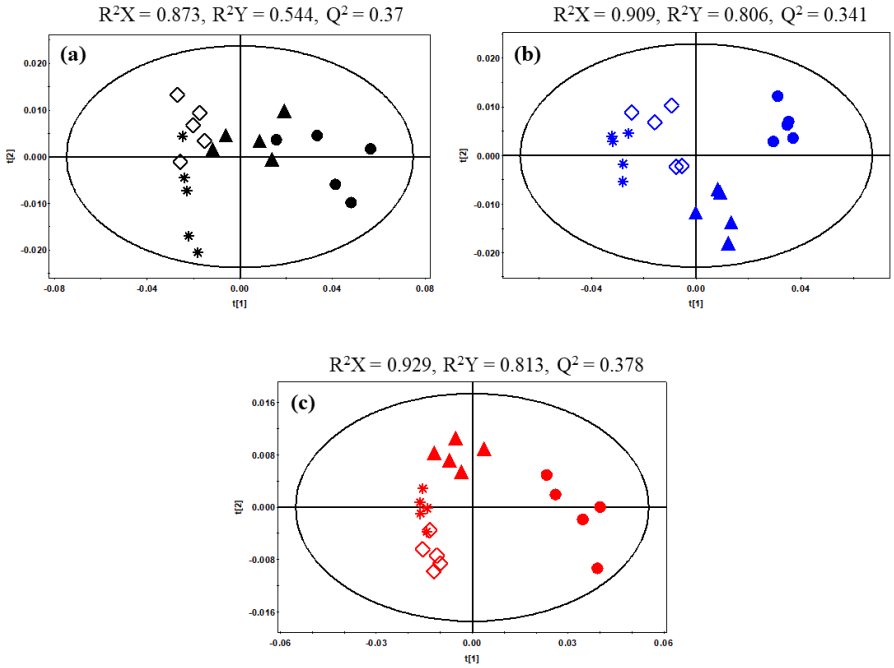

Figure 1. OPLS-DA score plots of ^{1}H-NMR spectra from zebrafish extracts depending on exposure time of fipronil. The symbols are as followed : Control (●), Fipronil-5 μg/L (▲), Fipronil-25 μg/L (◇) and Fipronil-50 μg/L (*). The colors are as followed : 12 hours of exposure (black), 24 hours of exposure (blue) and 72 hours of exposure (red). (a) 12 hours of fipronil exposure, (b) 24 hours of fipronil exposure and (c) 72 hours of fipronil exposure.

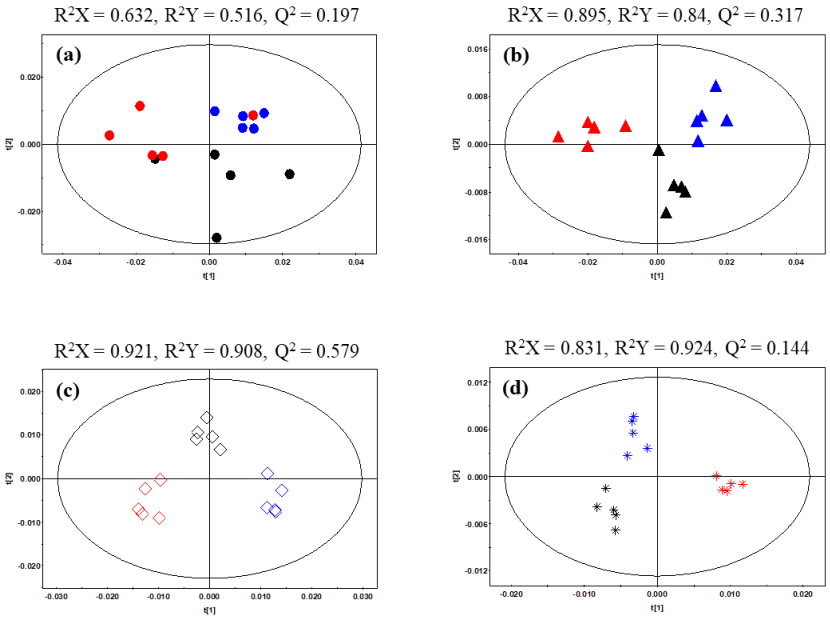

Figure 2. OPLS-DA score plots of ^{1}H-NMR spectra from zebrafish extracts depending on exposure concentration of fipronil. The symbols are as followed : Control (●), Fipronil-5 μg/L (▲), Fipronil-25 μg/L (◇) and Fipronil-50 μg/L (*). The colors are as followed : 12 hours of exposure (black), 24 hours of exposure (blue) and 72 hours of exposure (red). (a) Control (0 μg/L), (b) fipronil concentration of 5 μg/L, (c) fipronil concentration of 25 μg/L and (d) fipronil concentration of 50 μg/L.

Pathway analysis

To investigate pathways associated with significantly changed metabolites among groups, pathway analysis was performed in Metaboanalyst 4.0. Significant metabolites that obtained through one-wayANOVA and VIP score were used for pathway analysis.

The results of pathway analysis are shown in Fig. 3 and Fig. 4, indicating the significantly changes pathway in zebrafish extracts.

Significance of each pathway is decided by impact value calculated from pathway topology analysis[43] and –log (p) value related to p-value.

The pathway that has pathway impact value greater than 0.1 is regarded as affected pathway in the organism. Also, if -log (p) value is greater than 1.3, it is considered significant statistically. Therefore, the pathways that have pathway impact value greater than 0.1 and –log (p) value greater than 1.3 are regarded as reflecting the metabolic changes occurred in zebrafish. By using these results, analysis of metabolic changes occurred in zebrafish under conditions such as different concentrations and exposure times was performed.

Discussion

Pathway analysis results about exposure time indicate the changes among exposure concentrations in respective exposure time. In all exposure time results, citrate cycle (TCA cycle) which is the central energy metabolism in organism was common (Fig. 3).

The change of metabolites belong to TCA cycle means that energy metabolism disturbance occurred in zebrafish after fipronil exposure. In result of 72 hours fipronil exposure, intermediates related to TCA cycle such as glucose, pyruvate, 2-oxoglutarate, fumarate, malate and oxaloacetate were changed (Table 3). This energy metabolism disturbance seems to be associated to behavior change of zebrafish. In previous study, it was reported that zebrafish larvae that exposed to fipronil represented anxiety-like behavior including high swimming speed and abnormal photoperiod adaptation[44]. Similarly, the tank of zebrafish exposed to fipronil did swim rapidly than zebrafish of control tank also, suddenly stopped to rest. To retain this pattern, a lot of energy were needed therefore, TCA cycle which is an energy metabolism was influenced. Furthermore, in order to acquire more energy, degradation of proteins and lipids was proceeded[45-47]. Organisms can gain energy not only from carbohydrates but also from proteins and lipids. In result of 72 hours fipronil exposure, amino acids that are final products of protein such as arginine, glycine, histidine, isoleucine, methionine and valine were increased and glycerol, degradation product of lipid was increased in fipronil exposure groups (Table 3). Amino acids and glycerol were converted to intermediates of TCA cycle and participated in energy production. These results suggest that fipronil exposure causes energy metabolism disturbance in vertebrates such as zebrafish.

Unlike common TCA cycle in all exposure times, glutathione metabolism was specific in the result of 72 hours fipronil exposure (Table 3). Glutathione is an abundant antioxidant in the cells, and it treats reactive oxygen species (ROS)[48]. ROS has high reactivity and oxidizing power, so it oxidizes molecules in the body. This cell oxidation process is known as oxidative stress and cells might be damaged by oxidative stress[49]. Glutathione of antioxidant acts to reduce cell damages by controlling oxidative stress[50]. Fipronil is known to induce oxidative stress by generating ROS after influx into the body[51]. Glutathione, an antioxidant, will try to alleviate oxidative stress in this situation. The concentrations of glutathione at 72 hours of fipronil exposure are shown in Fig 5a. The concentration of glutathione increased in concentration-dependent manner up to 25 μg/L. This suggests that the level of glutathione gradually increased in order to reduce oxidative stress caused by fipronil exposure. However, at the highest concentration of 50 μg/L, the level of glutathione was lower than control. The case of exposure in the highest concentration of 50 μg/L for 72 hours is the occasion when toxicity is most serious because of

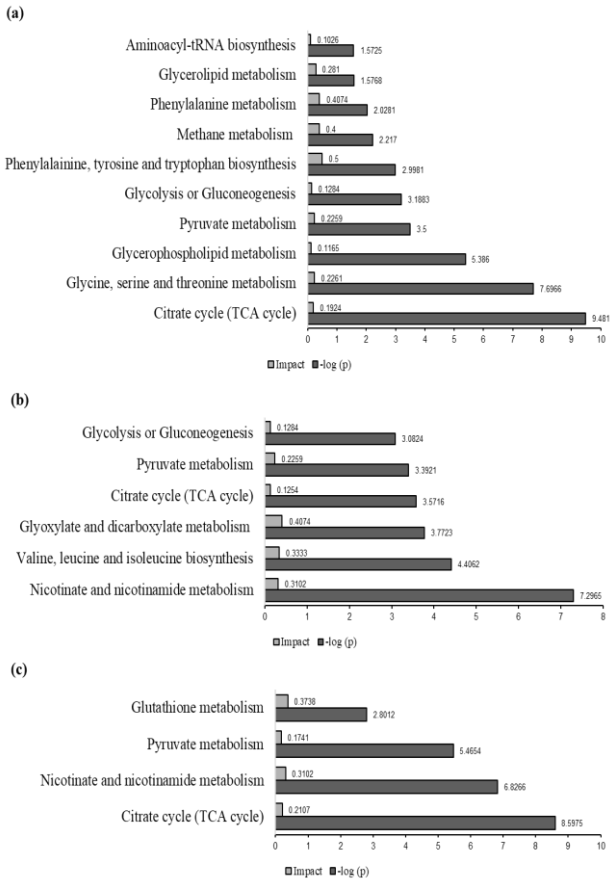

Figure 3. Pathway analysis of zebrafish extracts depending on exposure time of fipronil. (a) 12 h, (b) 24 h and (c) 72 h after

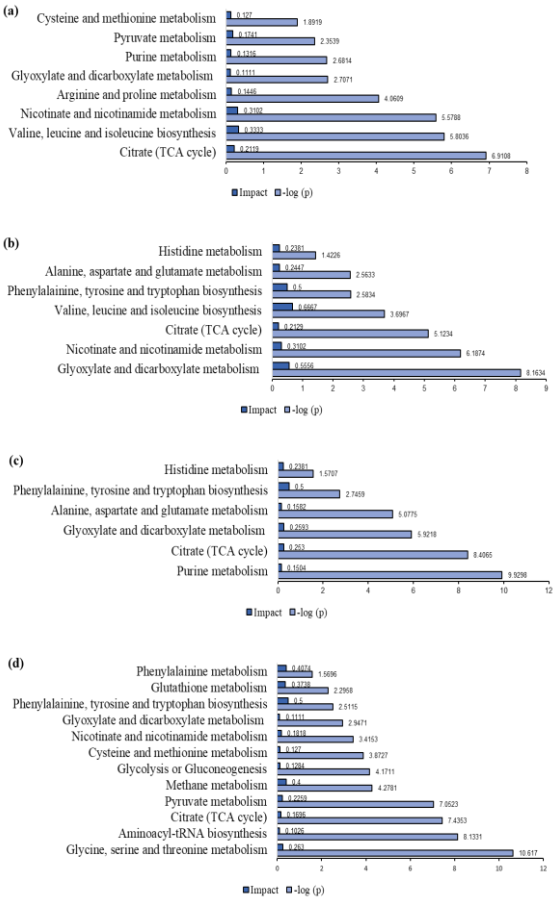

Figure 4. Pathway analysis of zebrafish extracts depending on exposure concentration of fipronil. (a) Control (0 μg/L), (b) 5 μg/L, (c) 25 μg/L and (d) 50 μg/L.

much oxidative stress. In the state that external glutathione influx is absent, antioxidant action has been continued after fipronil exposure, and therefore production of glutathione would have been reached the limit. As a result, the level of glutathione decreased at the highest concentration of 50 μg/L after 72 hours of exposure.

On the other hand, the result of 12 hours fipronil exposure showed opposite tendency to the result of 72 hours fipronil exposure in terms of glutathione.

The level of glutathione decreased in concentration-dependent manner and increased at the highest concentration of 50 μg/L (Fig. 5a). In the body, there are two ways that biomolecules are released to target organs or cells. One of ways is that consumes biomolecules from existing pool located in the cell and the other way is that produces new biomolecules from precursors. Since exposure time was short and concentration was low, at concentrations of 5 μg/L and 25 μg/L after 12 hours of exposure, it seems that zebrafish attempted to use glutathione present in the body first in order to alleviate oxidative stress. However, at 50 μg/L of the highest concentration, it was presumed that oxidative stress was not controlled by existing glutathione in the pool and so glutathione generation was begun newly in zebrafish.

Because glutathione production was started from the beginning of exposure in condition of no external influx, at 72 hours where time passed from 12 hours, glutathione depletion would have been caused. The level of glutathione in 50 μg/L of fipronil exposure

concentration clearly showed depletion of glutathione over time (Fig. 5b).

In addition, to investigate the metabolic changes over time, differences among groups of 12, 24 and 72 hours were analyzed in each concentration. Phenylalanine, tyrosine and tryptophan biosynthesis was only detected in fipronil exposure groups (5, 25 and 50 μg/L) (Fig. 4). This result suggests that the effects of fipronil on zebrafish depending on exposure time are related to phenylalanine, tyrosine and tryptophan biosynthesis.

Phenylalanine and tryptophan are essential amino acids. Thus, phenylalanine and tryptophan can be present in the body when they are ingested by diet. Tyrosine is generated from phenylalanine entered into the body. In concentration of 50 μg/L, phenylalanine and tryptophan were decreased over time (Table 4). As phenylalanine and tryptophan are essential amino acids, their concentrations would have decreased due to fasting during exposure period. However, reduction of phenylalanine and tryptophan caused by fasting is same in control group as well as in fipronil exposure groups. Therefore, alteration of phenylalanine, tyrosine and tryptophan biosynthesis is thought to result from other factors besides the effects of fasting.

As mentioned earlier, zebrafish needed more energy than usual because of behavior change caused by fipronil exposure. Phenylalanine is converted to fumarate, an intermediate in TCA cycle, and could participate in energy production process. In this regard, phenylalanine appears to be used for energy supply. In other words, phenylalanine took part in TCA cycle to obtain more energy, in consequence, decreased phenylalanine and increased fumarate were detected (Table 4). Tryptophan is a precursor of serotonin, a neurotransmitter that protect neurons from damage of excitatory neurotoxicity[52]. Fipronil is known to cause neurotoxicity by blocking the GABA receptors. To deal with this neurotoxicity, more serotonin would be needed than usual. As a result, the concentration of tryptophan, a precursor of serotonin, was decreased in order to increase production of serotonin (Table 4). Through the differences among exposure times in each concentration, it is manifested that fipronil disturbs energy metabolism and causes neurotoxicity over time. These results are similar to results inferred from differences between control group and fipronil exposure groups in each exposure time.

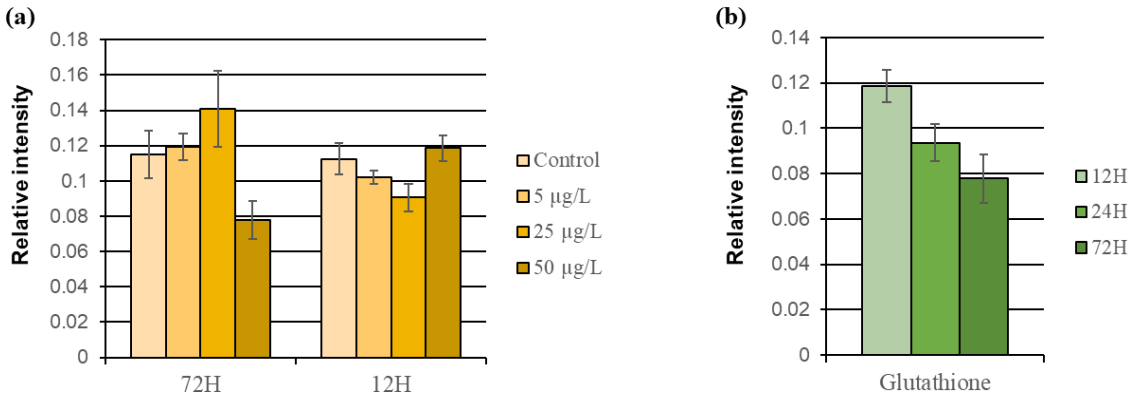

Figure 5. (a) The mean concentrations of glutathione in zebrafish exposed to fipronil during 72 and 12 hours. (b) The mean concentrations of glutathione in zebrafish exposed to 50 μg/L of fipronil. X axis indicates group and Y axis indicates mean relative concentration (%) of metabolites. Error bars represent the standard error.

(a)

12 hours after exposure		24 hours after exposure		72 hours after exposure	
Metabolites	**p-value**	**Metabolites**	**p-value**	**Metabolites**	**p-value**
Acetate	0.0017	Asparagine	0.0124	2-Oxoglutarate	0.0446
Choline	0.0002	Betaine	0.0293	Anserine	0.0102
Dimethylamine	0.0069	Citrate	0.0297	Choline	0.0319
Fumarate	0.0423	Mannose	0.0352	Creatine phosphate	0.0081
O-Phosphocholine	0.0360	NAD	0.0084	Lactate	0.0015
Pyruvate	0.0407	NADP	0.0084	Lysine	0.0151
Sarcosine	0.0176	Niacinamide	0.0043	N,N-Dimethylglycine	0.0350
Serine	0.0198	Pyruvate	0.0076	NADP	0.0454
Succinate	0.0269	Trimethylamine N-oxide	4.83E-08	O-Phosphoethanolamine	0.0095
Trimethylamine N-oxide	6.97E-07	Valine	0.0131	Oxypurinol	0.0168
Uridine	0.0296	Xanthine	0.0012	Succinate	0.0031
		sn-Glycero-3-phosphocholine	0.0039	Trimethylamine N-oxide	2.86E-06
				Tryptophan	0.0100

(b)

Control (0 µg/L)		Concentration of 5 µg/L		Concentration of 25 µg/L		Concentration of 50 µg/L	
Metabolites	**p-value**	**Metabolites**	**p-value**	**Metabolites**	**p-value**	**Metabolites**	**p-value**
Anserine	0.0488	3-Hydroxybutyrate	0.0014	2-Methylglutarate	0.0235	3-Hydroxybutyrate	0.0050
Arginine	0.0287	Acetate	0.0067	Acetone	0.0238	Acetate	0.0199
Asparagine	0.0209	ADP	0.0105	ADP	0.0053	ADP	0.0005
Choline	0.0186	Choline	0.0197	AMP	0.0276	Anserine	0.0306
Formate	0.0200	Citrate	0.0115	Formate	0.0105	Choline	0.0092
Fumarate	0.0008	Creatinine	0.0132	GTP	0.0062	Creatine	0.0247
Leucine	0.0070	Dimethylamine	0.0030	Guanosine	0.0343	Fumarate	0.0343
Lysine	0.0415	Formate	0.0246	Inosine	0.0311	Lactate	0.0119
Malate	0.0176	Glucose	0.0367	Lactate	0.0028	Malate	0.0351
Methionine	0.0358	Histidine	0.0268	Malate	0.0303	Methionine	0.0105
N,N-Dimethylglycine	0.0086	Malate	0.0046	NADP	0.0021	Phenylalanine	0.0239
NAD	0.0385	Mannose	0.0090	Oxalacetate	0.0167	Proline	0.0173
NADP	0.0123	NADP	0.0001	Oxipurinol	0.0169	Sarcosine	0.0022
O-Phosphocholine	0.0302	Trimethylamine N-oxide	0.0009	Succinate	0.0078	Serine	0.0099
Proline	0.0025	Tryptophan	0.0022	Trimethylamine N-oxide	0.0005	Succinate	0.0045
Pyruvate	0.0107	Tyrosine	0.0111	Uracil	0.0481	Trimethylamine N-oxide	0.0429
Sarcosine	0.0382	Valine	0.0489	Xanthine	0.0289	Tryptophan	0.0007
Trimethylamine N-oxide	0.0441	sn-Glycero-3-phosphocholine	0.0493				

Table 1. Significant metabolites on each exposure time group (a) and exposure concentration group (b) of fipronil analyzed by one-way ANOVA results. Threshold of significance was setted by below 0.05 of *p*-value

(a)

12 hours after exposure		24 hours after exposure		72 hours after exposure	
Metabolites	**VIP score**	**Metabolites**	**VIP score**	**Metabolites**	**VIP score**
2-Oxoglutarate	1.0076	ATP	1.2852	Anserine	1.6664
Acetate	1.5665	Acetate	1.0777	Fucose	1.1232
Glucose	1.2638	Anserine	1.0994	Glutathione	1.1179
Glycerol	1.0790	Choline	1.2331	Malate	1.162
O-Phosphocholine	1.1526	Formate	1.4849	NAD	1.0233
Phenylalanine	1.0497	N,N-Dimethylglycine	1.3094	NADP	1.0712
Pyruvate	1.7875	NAD	2.0836	Niacinamide	1.1142
Serine	1.3900	NADP	1.7975	Oxypurinol	1.1585
Succinate	1.1328	Niacinamide	1.8736	Proline	1.1738
Trimethylamine N-oxide	6.2179	Pyruvate	1.6873	Pyruvate	1.1835
Xanthine	1.3463	Trimethylamine N-oxide	5.6859	Succinate	1.3502
sn-Glycero-3-phosphocholine	1.2557			Trimethylamine N-oxide	6.2894
				Xanthine	1.1032

(b)

Control (0 μg/L)		Concentration of 5 μg/L		Concentration of 25 μg/L		Concentration of 50 μg/L	
Metabolites	**VIP score**	**Metabolites**	**VIP score**	**Metabolites**	**VIP score**	**Metabolites**	**VIP score**
2-Oxoglutarate	1.2585	3-Hydroxybutyrate	2.2341	2-Methylglutarate	1.3257	3-Hydroxybutyrate	1.9102
ADP	1.7838	ADP	1.7517	2-Oxoglutarate	1.3171	ADP	1.7767
AMP	1.2821	AMP	1.0840	ADP	2.0911	Choline	1.1427
Anserine	1.2834	Creatine phosphate	1.1418	AMP	1.4468	Creatine	1.0267
Betaine	1.5068	Dimethylamine	2.4018	ATP	1.4951	Formate	1.2562
Choline	1.6434	Formate	1.1336	Creatine phosphate	1.5512	Fumarate	1.0821
Formate	1.1330	Glucose	1.4674	GTP	1.5213	Glutathione	1.1417
Glucose	1.6385	Glutamine	1.2068	Guanosine	1.2403	Lactate	1.6483
Glycerol	1.0445	Histidine	1.0987	Histidine	1.0305	Lysine	1.5441
Histidine	1.5911	Leucine	1.0367	Inosine	2.1237	Malate	1.5114
Hypoxanthine	1.0789	Malate	1.8909	Malate	1.4661	Mannose	1.3027
Lysine	1.8640	Mannose	1.9266	NADP	1.8439	Methionine	1.1070
Malate	1.2732	NAD	1.7820	O-Phosphoethanolamine	1.0913	NAD	1.0857
Mannose	1.7889	NADP	2.0967	Oxalacetate	1.2853	NADP	1.0563
Methionine	1.1991	Niacinamide	1.2971	Oxypurinol	2.0668	Oxypurinol	1.6179
N,N-Dimethylglycine	1.1079	O-Acetylcarnitine	1.0425	Succinate	1.4616	Proline	2.4398
N-Acetylglutamate	1.0081	Oxalacetate	1.0179	Trimethylamine N-oxide	2.7660	Pyruvate	1.3337
NAD	2.0721	Trimethylamine N-oxide	2.7681	Tyrosine	1.5812	Sarcosine	1.5894
NADP	2.0105	Tryptophan	1.4760	Uracil	1.1301	Serine	1.5016
Niacinamide	1.2053	Tyrosine	1.8722	Xanthine	2.0173	Succinate	1.9467
O-Phosphocholine	1.3837					Threonine	1.3325
Proline	1.7655					Trimethylamine N-oxide	2.6670
Pyruvate	1.3203					Xanthine	2.1244
Sarcosine	1.3501					sn-Glycero-3-phosphocholine	1.1256
Threonine	1.0382						
Trimethylamine N-oxide	1.7271						
Xanthine	1.3699						
myo-Inositol	1.0278						

Table 2. The list of metabolites with above 1.0 of VIP value in zebrafish extracts depending on exposure time (a) and on exposure concentration of fipronil.

Metabolites	Control (0 μg/L)	5 μg/L fipronil	25 μg/L fipronil	50 μg/L fipronil
	Mean ± STE	Mean ± STE	Mean ± STE	Mean ± STE
2-Oxoglutarate	0.0733 ± 0.0074	0.0807 ± 0.0068	0.1194 ± 0.0129	0.0745 ± 0.0052
Arginine	0.3535 ± 0.0134	0.3784 ± 0.0238	0.3675 ± 0.0231	0.3709 ± 0.0428
Creatinine	0.1102 ± 0.0081	0.1329 ± 0.0100	0.1569 ± 0.0202	0.1230 ± 0.0194
Fumarate	0.0272 ± 0.0008	0.0306 ± 0.0020	0.0303 ± 0.0027	0.0326 ± 0.0022
Glucose	0.8156 ± 0.1175	0.8769 ± 0.0585	0.9715 ± 0.1857	0.9477 ± 0.0991
Glycerol	0.7628 ± 0.1010	0.8410 ± 0.1075	0.9682 ± 0.0689	0.8164 ± 0.0458
Glycine	3.2276 ± 0.4976	4.7019 ± 0.3159	4.0924 ± 0.5044	4.0373 ± 0.4234
Histidine	4.4765 ± 1.0496	5.4457 ± 0.2967	5.5041 ± 0.7433	5.2713 ± 0.3633
Isoleucine	0.0988 ± 0.0056	0.1127 ± 0.0062	0.1245 ± 0.0200	0.1071 ± 0.0068
Malate	0.2680 ± 0.0155	0.3415 ± 0.0164	0.4222 ± 0.0517	0.3503 ± 0.0326
Methionine	0.0731 ± 0.0026	0.0840 ± 0.0033	0.0859 ± 0.0110	0.0736 ± 0.0044
Oxalacetate	0.4340 ± 0.0715	0.3527 ± 0.0187	0.4000 ± 0.0722	0.3932 ± 0.0317
Pyruvate	0.2042 ± 0.0372	0.1323 ± 0.0174	0.1367 ± 0.0334	0.1328 ± 0.0156
Valine	0.1886 ± 0.0149	0.2286 ± 0.0101	0.2479 ± 0.0532	0.2107 ± 0.0103

Table 3. The concentrations of significantly changed metabolites in zebrafish extracts after 72 hours of fipronil exposure. The data are shown as mean ± STE. STE is standard deviation which means the amount of variation or dispersion of a set of value, and it was calculated by the square root of the variance

Metabolites	12 hours after exposure	24 hours after exposure	72 hours after exposure
	Mean ± STE	Mean ± STE	Mean ± STE
Phenylalanine	0.1152 ± 0.0085	0.0912 ± 0.0109	0.0839 ± 0.0040
Tryptophan	0.0637 ± 0.0018	0.0449 ± 0.0018	0.0487 ± 0.0021
Fumarate	0.0284 ± 0.0015	0.0304 ± 0.0013	0.0326 ± 0.0022

Table 4. The concentrations of phenylalanine, tryptophan and fumarate in zebrafish extracts of 50 μg/L fipronil exposure group. The data are shown as mean ± STE. STE is standard deviation which means the amount of variation or dispersion of a set of value, and it was calculated by the square root of the variance

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References

1. Y. Wang, S. Wu, L. Chen, C. Wu, R. Yu, Q. Wang and X. Zhao, *Chemosphere* **88**, 484 (2012)
2. Ş. Gül, E. Belge-Kurutaş, E. Yıldız, A. Şahan and F. Doran, *Environ. Int.* **30**, 605 (2004)
3. P. Mineau, *Environ. Toxicol. Chem*. **21**, 1497 (2002)
4. A. S. Gunasekara, T. Truong, K. S. Goh, F. Spurlock and R. S. Tjeerdema, *J. Pestic. Sci*. **32,** 189 (2007)
5. A.,Aajoud, P. Ravanel and M. Tissut, *J. Agric. Food Chem*. **51,** 1347 (2003)
6. M. W. Dryden, T. M.Denenberg and S. Bunch, *Vet. Parasitol*. **93,** 69 (2000)
7. A. M. Hosie, H. A. Baylis, S. D.Buckingham and D. B. Sattelle, *Br. J. Pharmacol.* **115,** 909 (1995)

8. Y. Ozoe and M. Akamatsu, *Pest Manag. Sci*. **57,** 923 (2001)
9. A. Bobé, J. F. Cooper, C. M. Coste and M. A. Muller, *Pestic. Sci.* **52,** 275 (1998)
10. D. Hainzl, L. M. Cole and J. E. Casida, *Chem. Res. Toxicol.* **11,** 1529 (1998)
11. L. M. Cole, R. A. Nicholson and J. E. Casida, *Pestic. Biochem. Phys.* **46,** 47 (1993)
12. T. Narahashi, X. Zhao, T. Ikeda, V. L. Salgado and J. Z. Yeh, *Pestic. Biochem. Phys.* **97**, 149 (2010)
13. X. Zhao, J. Z. Yeh, V. L. Salgado and T. Narahashi, *J. Pharmacol. Exp. Ther.* **310**, 192 (2004)
14. K. Haya, *Environ. Toxicol. Chem.* **8**, 381 (1989)
15. S. Beggel, I. Werner, R. E. Connon and J. P. Geist, *Sci. Total Environ.* **426**, 160 (2012)
16. M. Kitulagodage, W. A. Buttemer and L. B. Astheimer, *Ecotoxicology* **20**, 653 (2011)
17. P. R. de Oliveira, G. H. Bechara, S. E. Denardi, R. J. Oliveira and M. I. C. Mathias, *Exp. Toxicol. Patho.* **64**,569 (2012)
18. C. M. Stehr, T. L. Linbo, J. P. Incardona and N. L. Scholz, *Toxicol. Sci.* **92**, 270 (2006)
19. P. C. Das, Y. Cao, N. Cherrington, E. Hodgson and R. L. Rose, *Chem.-Biol. Interact.* **164**, 200 (2006)
20. C. Vidau, J. L. Brunet, A. Badiou and L. P. Belzunces, *Toxicol. In Vitro* **23**, 589 (2009)
21. R. A. Ardeshir, H. Zolgharnein, A. Movahedinia, N. Salamat and E. Zabihi, *Toxicol. Rep*. **4**, 348 (2017)
22. G. Kari, U. Rodeck and A. P. Dicker, *Clin. Pharmacol. Ther.* **82**, 70 (2007)
23. Y. Wang, W. Zhu, D. Wang, M. Teng, J. Yan, J. Miao and Z. Zhou, *Chemosphere* **168,** 1571 (2017)
24. X. Wang, E. Cheng, I. S. Burnett, Y. Huang and D. Wlodkowic, *Sci. Rep.* **7**, 1 (2017)
25. Y. Liu, P. Ma, P. A. Cassidy, R. Carmer, G. Zhang, P. Venkatraman and Y. F. Leung, *Sci. Rep.* **7**, 1 (2017)
26. C. Wyatt, E. M. Bartoszek and E. Yaksi, *Eur. J. Neurosci.* **42**, 1746 (2015)
27. *OECD guideline for the testing of chemicals: Fish, Acute Toxicity Test, Guideline 203*
28. S. Tiziani, V. Lopes and U. L. Günther, *Neoplasia* **11**, 269 (2009)
29. L. S. Castillo-Peinado and M. L. de Castro, *Anal. Chim. Acta* **925**, 1 (2016)
30. J. G. Bundy, M. P. Davey and M. R. Viant, *Metabolomics* **5**, 3 (2009)
31. O. Fiehn, *Brief Funct Genomics* (pp. 155-171). Springer, Dordrecht (2002)
32. O. Beckonert, H. C. Keun, T. M. Ebbels, J. Bundy, E. Holmes, J. C. Lindon and J. K. Nicholson, *Nat. Protoc.* **2**, 2692 (2007)
33. O. Beckonert, M. Coen, H. C. Keun, Y. Wang, T. M. Ebbels, E. Holmes and J. K. Nicholson, *Nat. Protoc.* **5**, 1019 (2010)
34. A. A. De Graaf, W. M. M. J. Bovee, N. E. P. Deutz and R. A. F. M. Chamuleau, *Magn. Reson. Imagin.* **6**, 255 (1988)
35. T. Rudi, G. Guthausen, W. Burk, C. T. Reh and H. D. Isengard, *Food Chem.* **106**, 1375 (2008)
36. D. Schlenk, D. B. Huggett, J. Allgood, E. Bennett, J. Rimoldi, A. B. Beeler and P. Bedient, *Arch. Environ. Contam. Toxicol.* **41**, 325 (2001)
37. J. Gan, S. Bondarenko, L. Oki, D. Haver and J. X. Li, *Environ. Sci. Technol.* **46**, 1489 (2012)
38. H. Wu, A. D. Southam, A. Hines and M. R. Viant, *Anal. Biochem.* **372**, 204 (2008)
39. Y. Wang, M. E. Bollard, H. Keun, H. Antti, O. Beckonert, T. M. Ebbels and J. K. Nicholson, *Anal. Biochem.* **323**, 26 (2003)
40. T. P. Dunkley, R. Watson, J. L. Griffin, P. Dupree and K. S. Lilley, *Mol. Cell Proteomics **3***, 1128 (2004)
41. N. K. Khoo, A. Iravani, M. Arjmand, F. Vahabi, M. Lajevardi, S. M. Akrami and Z. Zamani, *Lasers Med. Sci.* **28**, 1527 (2013)
42. N. A. Karp, J. L. Griffin and K. S. Lilley, *Proteomics* **5**, 81(2005)
43. X. Chen, C. Hu, J. Dai and L. Chen, *Evid. Based Complementary Altern. Med*. (2015)
44. C. Wang, Y. Qian, X. Zhang, F. Chen, Q. Zhang, Z. Li and M. Zhao, *Environ. Pollut.* **211**, 252 (2016)
45. M. R. Viant, E. S. Rosenblum and R. S. Tjeerdema, *Environ. Sci. Technol*. **37**, 4982 (2003)

46. L. Zhang, X. Liu, L. You, D. Zhou, H. Wu, L. Li and J. Yu, *Mar. Environ. Res*. **72**, 33 (2011)
47. H. D. Xu, J. S. Wang, M. H. Li, Y. Liu, T. Chen and A. Q. Jia, *Aquat. Toxicol.* **159**, 69 (2015)
48. K. E. Hillyer, S. Tumanov, S. Villas-Bôas and S. K. Davy, *J. Exp. Biol.* **219**, 516 (2016)
49. M. Ott, V. Gogvadze, S. Orrenius and B. Zhivotovsky, *Apoptosis* **12**, 913 (2007)
50. T. A. Slotkin and F. J. Seidler, *Neurotoxicol. Teratol*. **32**, 124 (2010)
51. X. Wang, M. A. Martínez, Q. Wu, I. Ares, M. R. Martinez-Larranaga, A. Anadón and Z. Yuan, *Crit. Rev. Toxicol.* **46**, 876 (2016)
52. H. Chu, H. Sun, G. L. Yan, A. H. Zhang, C. Liu, H. Dong and X. J. Wang, *World J. Tradit. Chin. Med.* **1**, 9 (2015)