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RESEARCH ARTICLE

GC-MS BASED METABOLIC PROFILE OF BRAIN TISSUE IN COMMON CARP FOLLOWING ACUTE EXPOSURE TO CYPERMETHRIN.

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Abstract

In the current study, the GC-MS metabolomics analysis was employed to explain the neurotoxic effect of cypermethrin on the metabolic profile in the brain of carp fish. The carps exposed to two concentrations of cypermethrin (0.1 and 1 µg/L) for 24 and 96 hours. The levels of glutamate, tryptophan, tyrosine, glutamine and histidine showed marked increase, while levels of GABA and glycine were decreased compared to control group. Additionally, cypermethrin significantly reduced the level of glucose at 24 h suggesting a disruption in energy production with subsequent usage of lactate as an energy source. Collectively, the present work is the first that proposes a possible mechanism of cypermethrin neurotoxicity in carp fish and reflects the risk of cypermethrin existence in an aquatic environment.

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Introduction:-

The usage of synthetic pyrethroids increases continuously due to their minimal toxicity to mammals and their robust insecticidal activity compared to organophosphorus compounds and other insecticides. Cypermethrin (CYP) is extremely toxic type II synthetic pyrethroid insecticide that is frequently detected at potentially detrimental concentrations in rivers, lakes, sediments and fish body (Marino and Ronco 2005). Previous studies discussed the toxicity of CYP in fish and other aquatic organisms and revealed that it is potentially toxic at environmentally relevant concentrations (Arslan et al. 2017; Kumar et al. 2008).

Although the reports on investigating the toxic effects of CYP in fish are abundant, there are few reports regarding the effect of CYP on the metabolic profile in the brain tissue, the issue that may explain the neurotoxic action of CYP in fish. In fact, exposure to CYP induced aberrant behavioral changes, significant oxidative damage, upregulation of apoptotic genes and histopathological alterations in the brain (Arslan et al. 2017). Cypermethrin increased the transcription levels of brain- derived neurotropic factor (BDNF) gene which is vital for neural cell survival in exposed zebrafish (Özdemir et al. 2018).

Recently, metabolomics technique is used to illustrate comprehensively the response of aquatic organisms to various environmental pollutants such as Malathion (Uno et al. 2012), Butachlor (Xu et al. 2015) and 2,4-dichlorophenol (Kokushi et al. 2017). Metabolomics techniques have been providing new possibilities to figure out the mechanism of action of chemicals, in addition to the discovery of new biomarkers (Kokushi et al. 2017). The metabolomics-

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based toxicity is very effective to detect the effect of different environmental toxicants, even at very low concentrations, on various living organisms. The metabolic stress is the first response to the environmental stressors; therefore, metabolomics analysis is a good tool to determine the perturbations of the metabolome that often proceed other biological changes such as histopathological changes, growth retardation and mortality (Uno et al. 2012).

In the current study, we assessed the toxic effects of CYP on the metabolic profile in the brain of Japanese carp, *Cyprinus carpio* under the laboratory conditions, using metabolomics strategy with the gas chromatography/mass spectrometry.

Material and methods:-

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

Japanese carp with average weight ranging from 25 to 30 grams were obtained from a private fish farm in Kagoshima Prefecture, Japan. The fish were acclimatized for two months under the laboratory conditions before starting exposure to CYP. During acclimation period, fish were fed a commercial fish meal (Nippon Formula Feed Manufacturing, Yokohama, Japan) a few times at a feeding rate of 1% fish weight per day.

In the current study, thirty-six carp fish were kept in three aquariums (twelve fish per each aquarium). The feeding was stopped 24 hours before the exposure. The first group was the control one (CYP free) and the other two groups were exposed to two nominal exposure concentrations of waterborne CYP of 0.1 (L-group) and 1 µg/L (H-group) for 96 hours. The concentrations of the exposed groups based on the concentrations in the aquatic environment and the 96 hours LC₅₀ of CYP in *Cyprinus carpio* as 1.7 µg/L (Marino and Ronco 2005; Saha and Kaviraj 2008). Fish were kept in the glass tanks (40 L) with the mild aeration. The water temperature was maintained at 24±1°C, pH was 6.0±1.0 and dissolved oxygen concentration was 5.0 ±1.0 mg/L. Photoperiod was 14 h light/10 h dark cycles. The water was completely replaced once per day with dechlorinated tap water (left for one day before use) for control and exposed groups. The procedures for the toxicity test were based on the Organization for Economic Co-operation and Development guidelines (OECD) for testing the chemicals with slight modifications (TG203).

During sampling of brain tissue, 500 µL/L of 2-phenoxyethanol was used as an anesthetic agent. Brain tissue was collected from each sampled fish. Six fish were sampled at 24 and 96 hours from each group. Immediately after isolation of brain, the tissues were frozen by the liquid nitrogen to stop the metabolic and enzymatic activities and finally stored at - 80°C until metabolite analysis.

For metabolomics analysis, brain samples (about 10 mg for each) were placed in a 2-mL polypropylene microtube together with a zirconia ball (diameter 5 mm) in addition to 1 mL of a mixture of chloroform, methanol, and Milli-Q water (1:2:0.8, v/v/v). The microtube was vigorously shaken for six min by a sample disruption system (Tissue Lyser II, QIAGEN, 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 centrifugal concentrator (TAITEC, VC-15SP, Japan) (Uno et al. 2012).

Derivatization was performed in two main steps: oximation and silylation. The dried residue in the microtube was derivatized using 10 µL of a methoxylamine hydrochloride solution prepared by dissolving methoxylamine hydrochloride in pyridine (40 mg/mL). This is by means of continuous shaking in a water bath at 30°C for 90 min. Silylation was also done by addition of 90 µL of MSTFA + 1% TMCS at a 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. Unfortunately, the derivatization in a few samples could not be conducted well, and added the present evaluations. However, we deemed the missing only slightly affected the whole evaluations of metabolic disturbance resulted from the exposures of CYP.

Metabolites were analyzed on an Agilent Technologies 6890 Series gas chromatograph equipped with a 5973 MSD mass selective detector and a DB 5-ms capillary column (0.25 mm i.d.× 30 m, 0.25- µm film thickness; J&W

Scientific, USA). One microliter of sample was injected into the column. The temperatures of 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.

Quality Assurance of analytical procedures; Calibration standard curves were created and the quantitative estimation of cypermethrin was done by comparing the test solution with the standard solutions injected under the identical gas chromatography conditions. The standard cypermethrin solution was analyzed during the analysis of samples followed by the same procedure of extraction, clean-up, and analysis. The percentage of recoveries ranged from 80% to 105%. The cypermethrin level was corrected for the recovery values.

After getting the peaks on the chromatogram of GC-MS, the baseline was drawn to obtain the peak areas of each metabolite, deconvoluted, and aligned using MetAlign™ (ver. 080311, Wageningen University, Wageningen, Netherlands). All the peak area was divided by the weight of each sample and normalized to the internal standard (Uno et al. 2012). The resulted value was multiplied by 10^5 and analyzed by R programming language (<http://www.rproject.org/>) for one-way analysis of variance (ANOVA) ($p < 0.05$) and principal component analysis (PCA) to evaluate the differences of the toxic effects among 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 CYP.

Results and discussion:-

The current study is the first to investigate the effect of CYP on amino acids, intermediates of energy generation and neurotransmitters in brain of carp using GC/MS metabolomics analysis.

On the PCA score plot at 24 h (Fig.1-A), H-group was clustered on the negative part and separated from control and L-groups along with PC1. Additionally, L-group was clustered on the negative direction from control along with PC2. However, at 96 h (Fig.1-B), slight separations were recognized among the groups. Although H-group showed slight separation from others groups along with PC1, the differences were not remarkable compared to those at 24 h. The PCA analysis revealed the disturbance exerted in the metabolic system in the brain of carp after CYP exposure.

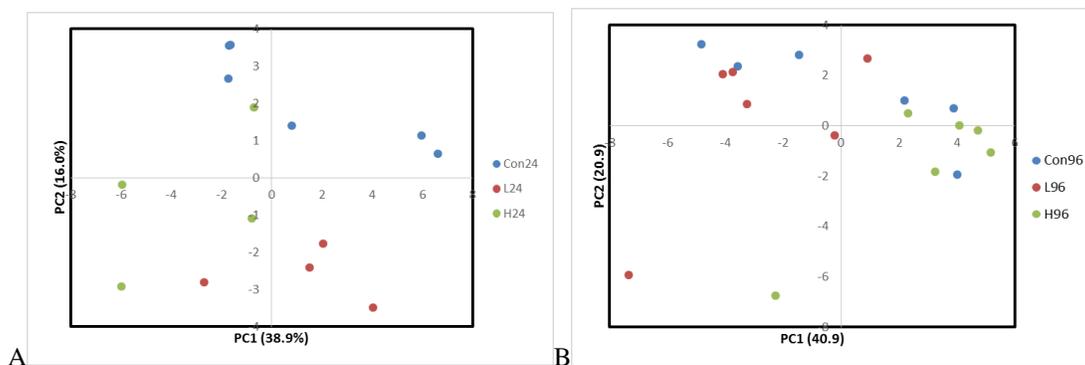


Fig.1- Principle component analysis (PCA) score plots derived from variations of metabolites in brain of carp fish at 24 h (A) and 96 h (B) after exposure to cypermethrin. The contribution percent of each component is presented along the X and Y axis was related to PC1 and PC2, respectively.

Because of their lipophilicity, pyrethroids can cross the blood- brain barrier and reach the central nervous system at potentially neurotoxic concentrations. They disrupt the function of the nervous system by their effect on the voltage-sensitive sodium channels (VSSC) as well as the release of neurotransmitters (Rodriguez et al. 2016).

In the present study, CYP exposure caused a significant elevation in the level of glutamine ($P < 0.05$) in L-group at 24 h and in H-group at 24 and 96 h. In addition to, a significant increase in the level of glutamate in H-group at both time points (Fig.2-A, Table.1). Glutamine is the precursor for glutamate synthesis and plays a vital role in removing excess of nitrogen from brain suggesting that CYP allowed excessive accumulation of nitrogen in the brain of carp (Ning et al. 2018). The rise in glutamate ($P < 0.05$), an excitatory neurotransmitter, in brain tissue was previously reported in the brain of goldfish exposed to lambda-cyhalothrin (Li et al. 2014). The increase in glutamine and glutamate in the brain gave rise to an increase in the binding activity to excitatory receptors in sub-synaptic

membranes resulting in hyper-excitation with subsequent excite-toxicity (Zhang and Zhao 2017). The level of aspartate was increased in response to CYP stress in comparison with control group. The increase in aspartate supports the glutamate excitatory role in brain synapses and reveals its co-transmitter role with glutamate in neurotoxicity (Morland et al. 2013).

GABA, the main inhibitory neurotransmitter, is synthesized in the brain from glutamate via the enzyme glutamate decarboxylase (GAD). GABA was extremely decreased ($P < 0.05$) in H-group at 96 h. Such variation suggests that the higher concentration of CYP ($1 \mu\text{g/L}$) kept the brain of carp under an exciting condition (Fig.2.A, Table.1). GABA and glutamate are involved in memory, learning and synaptic plasticity, and are particularly vital for maintaining the neural balance of excitation and inhibition (Watanabe et al. 2002). Thus, the modulation in the levels of GABA and glutamate could affect the activities of carps exposed to CYP.

Exposure to deltamethrin and cyhalothrin (type II pyrethroids) decreased the synthesis of serotonin and damaged serotonergic neurons in rat brain (Martínez-Larrañaga et al. 2003). Tryptophan is a precursor for the synthesis of serotonin and melatonin hormones. In the present work, CYP significantly increased the level of tryptophan in H-group at 96 h ($P < 0.05$) (Fig. 2-B, Table.1) indicating the disturbances of serotonergic neurotransmission in the brain.

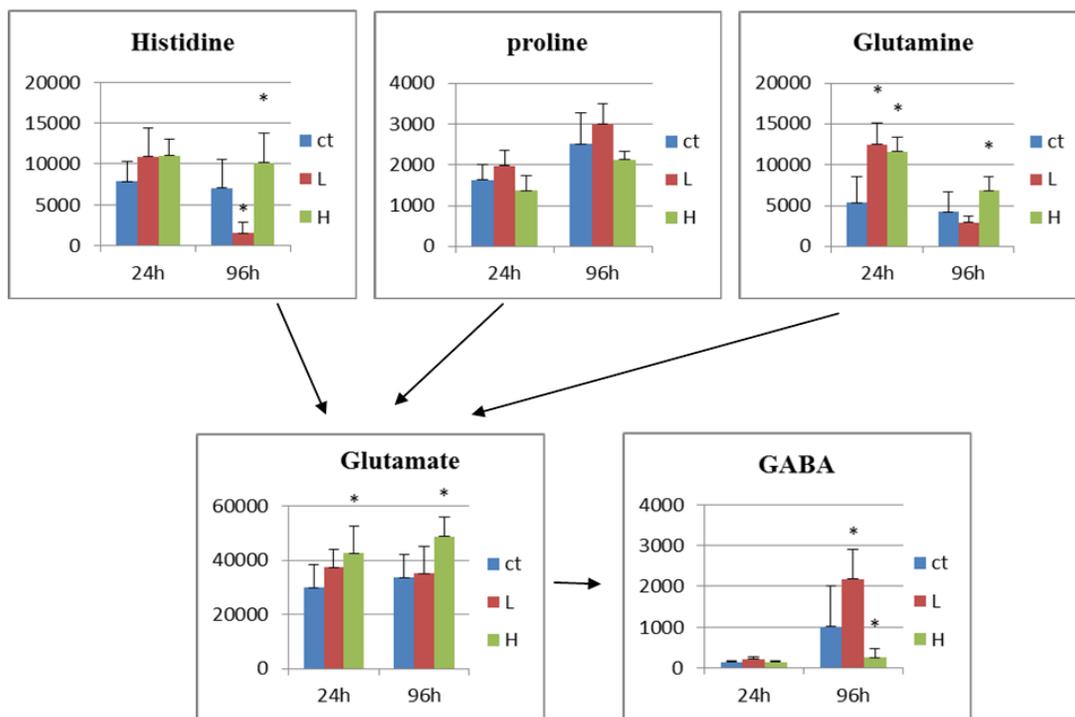


Fig.2- A: The levels of the metabolites (unit, mg^{-1}) involved in GABA synthesis in brain of carp fish induced by CYP; The control group (blue), L-group (red), and H-group (green). The results are represented as means \pm SD.

In the current study, higher concentration of CYP ($1 \mu\text{g/L}$) increased the level of tyrosine ($P < 0.05$) at 96 h (Fig.2-B). In the liver, tyrosine is synthesized from phenylalanine and then converted into L-3, 4-dihydroxyphenylalanine (L-DOPA) in brain tissue (Ning et al. 2018). A previous study in young rat reported that prenatal exposure to fenvalerate resulted in disruption in dopaminergic receptors (Malaviya et al. 1993). The increase in tyrosine level ($P < 0.05$) in the brain of carp could be the evidence of the disturbance in L-DOPA and/or adjacent metabolites (Table.1).

Glycine is an inhibitory neurotransmitter in the nervous system and plays an important role in the swimming performance to overcome muscle disorders. Exposure to high concentration of CYP ($1 \mu\text{g/L}$) significantly decreased the level of glycine ($P < 0.05$) at 24 h and 96 h (Fig.2-B, Table.1) proposing its contribution in muscle excitation

(Stehr et al. 2006). Likewise, the level of glycine was reduced after exposure to fipronil in larvae of zebrafish (Wang et al. 2016). In addition to its role in neurotransmission, glycine participates in gluconeogenesis, sulfur amino acids metabolism and fat digestion (Wang et al. 2016). It is considered one of the most important glycogenic amino acids as it works to maintain blood sugar level. The reduction in the level of glycine after CYP exposure may denote the disturbance in glucose synthesis during CYP stress. A previous study showed similar effects in the head of zebrafish after individual and combined exposure to acetamiprid and halosulfuron-methyl (Zhang and Zhao 2017).

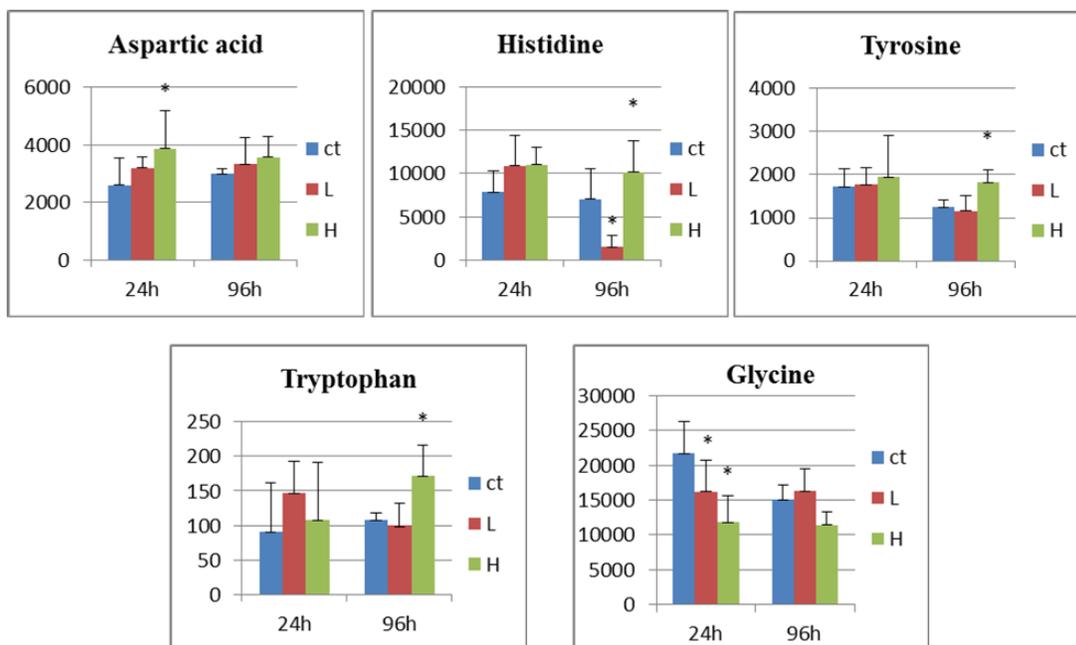


Fig.2- B: The levels of amino acids (unit, mg⁻¹) related to the neurotransmission in brain of carp fish after exposure to CYP; The control group (blue), L group (red), and H group (green). The results are represented as means \pm SD.

Histidine plays a vital role in protein synthesis as well as the synthesis of histamine neurotransmitter. Moreover, histamine is involved in numerous brain regulatory functions such as swimming activity, alertness and memory in vertebrates (Rico et al. 2011). A significant increase in the level of histidine was observed in H-group at 96 h ($P < 0.05$) (Fig.2.B and table.1). Such an increase could be a relevant issue in the excitotoxicity caused by CYP. Alteration in the histamine level was observed after the exposure of zebrafish larvae to triphenyl phosphate (Shi et al. 2018)

Put together, the variations in the levels of histidine, glutamine, glutamate, and other parameters related to neurotransmission were generally close at 24 h in L- and H-groups. However, in H-group, these variations were continuously disturbed the nerve functioning, while in L-group, the recovery was assumed.

Table 1: Corresponding PC1 and PC2 loadings values for individual metabolites at 24 and 96 hours.

Time	24 h		96 h	
Metabolites	PC1	PC2	PC1	PC2
Glucose	0.713**	0.142	-0.061	-0.614**
Glutamine	-0.364	-0.746**	0.912**	-0.233
Glycine	0.780**	0.419	-0.808**	-0.495
Glutamate	-0.620**	-0.515	0.732**	-0.229
Histidine	0.046	-0.695**	0.923**	-0.230
Proline	0.901**	-0.101	-0.883**	-0.314
Tryptophan	0.558*	-0.427	0.619**	-0.501
Tyrosine	0.850**	-0.306	0.386	-0.793**
GABA	0.413	-0.523	-0.887**	0.195

Lactate	0.362	-0.904**	-0.817**	0.151
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*: $p < 0.05$, **: $p < 0.01$.

A dose-dependent decrease was observed in the level of glucose at 24 h (Fig. 3, Table 1). Such decline was possibly due to the excessive consumption of glucose to overcome the stress condition. Glucose is converted via glycolysis to pyruvate which in turn transferred into mitochondria to produce ATP via sharing Krebs cycle and the mitochondrial respiratory chain. The decrease in the level of glucose at 24 h suggests that the brain of carp is maintained using glucose even under exposure to CYP. The level of lactate showed a slight increase in CYP-exposed groups at 24 h without significant effect, while at 96 h, lactate was increased significantly in L-group compared to control one ($P < 0.05$). Meanwhile, the level of lactate showed reduction in H-group at 96 h compared to control group (Fig. 3), proposing its excessive consumption to compensate insufficient energy production (Li et al. 2014). During glucose starvation, lactate was consumed as an energy source in brain tissue of rainbow trout at similar rate to those of glucose in fish (Otero-Rodino et al. 2015).

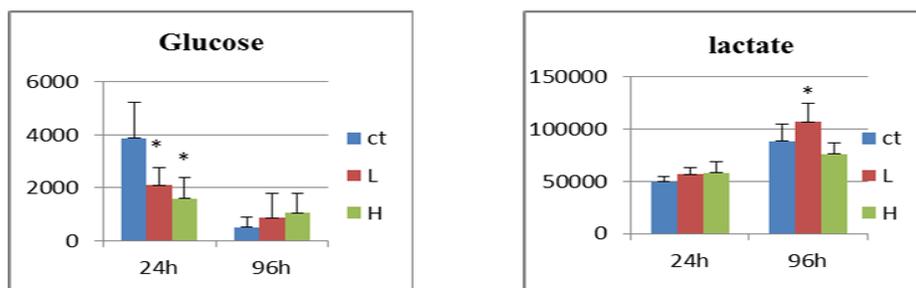


Fig.3- The levels of glucose and lactate (unit, mg^{-1}) at 24 and 96 h in brain of carp fish after exposure to CYP; the control group (blue), L-group (red), and H-group (green). The results are represented as the means \pm SD.

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Disclosure of potential conflicts of interest:

The authors declare that they have no conflict of interest.

Research involving Human Participants and/or Animals:

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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