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The Effect of Fipronil on Lipid Metabolism in the Brain of Fresh Water Fish *Labeo rohita* (Rohu)

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A B S T R A C T

India is one of the major countries in the world that produce fish through aquaculture. In India, carp culture has the major economic importance in freshwater aquaculture practice. Fish are the main source of animal protein and lipids for the growing human population. But the changes in the water quality affected the quality of fish significantly. The indiscriminate use of pesticides in agricultural fields polluted the water and environment and thus the aquatic fauna. In our present study, we analyzed the effect of new generation insecticide fipronil, a phenyl pyrazole chemical on the lipid metabolism in the brain of fresh water fish *Labeo rohita* (Rohu). The result showed significant changes in the lipogenic enzymes like β-hydroxy methyl glutaryl Co-A reductase, glucose-6- phosphate dehydrogenase, and malic enzyme and a significant reduction in lipid profiles like total lipid, cholesterol, triglyceride, phospholipid, and free fatty acids in the fish brain after the exposure of sub-lethal concentration of fipronil. This study revealed the adverse effect of indiscriminate usage of fipronil in the agricultural field.

***Key words*:** *Labeo rohita*, Rohu, Fipronil, Lipids, Enzymes

Today, aquaculture is one of the faster-growing food sectors globally and India is the second-largest fish producer through aquaculture after China [1]. The demand for high-quality fish and fishery products increased every year due to their nutritional value that contain plenty of important nutrients [2]. Most of these demands were sourced from aquaculture and captured fisheries. Aquatic organisms like fish were exposed to various types of insecticides during their life cycle, so the good health of fish was very important for human beings [3]. However, the haphazard use of pesticides in an agricultural field contaminated terrestrial and aquatic ecosystems including surface and ground waters through the leakage, runoff or direct input [4]. Pesticides might have more effect on aquatic environments than terrestrial environments because these chemicals were eventually received by the water bodies easily [5].

Depending upon the species, the adverse effect of chemical pesticides may be varied into lethal (acute) or sub-lethal (chronic) [6]. Many chemical pesticides used in our agricultural field negatively affect non-target organisms like beneficial insects, birds, and aquatic animals such as fish [7]. In this fipronil, a new broad-spectrum insecticide included in the phenyl pyrazole family is considered as most effective to protect the crops such as rice, corn, cotton, potatoes, tulips, etc. from many herbivorous insects [8]. It acted on insect's central nervous system by binding its gamma-aminobutyric acid (GABA) – gated chloride channels and thus blocked the inhibitory action of GABA [9-10]. Biochemical, neurological, hematological, histopathological, and genetic level effects of fipronil on the fish were studied by many researchers [11-15] and found fipronil was severely affected fish after lethal or sub-lethal exposures.

In India, the freshwater fishery has an important role to contribute animal proteins for the growing population, and Indian major carps like rohu Catla and mrigal, common carp, and tilapia are the main source for highly nutritive proteins, vitamins, minerals, and lipids which is beneficial for good human health [16]. Lipids are the most important nutrient which contains a high amount of polyunsaturated fatty acids (PUFAs) especially omega-3 and omega-6 [17]. Lipids and their constituent fatty acids along with proteins have an important role in human nutrition, the fish were considered as the sources of protein and lipids for humans and domestic animals [18-19]. However, the quality like the taste, texture, and characteristics flavour of fish depended on its tissue lipid profiles it varied with its species and habitat [20]. The use of small fish as biological models in toxicology according to the standard protocol has many important advantages over mammalian models [21]. *Labeo rohita* (rohu) belonging to the Cyprinidae family, is one of the major carp cultured extensively in India. Here we used rohu as experimental fish to determine the alteration in lipid metabolism in brain tissue after the sub-lethal exposure to

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fipronil. Our present investigation analyzed the effect of fipronil on lipogenic enzymes and lipid profiles in rohu fish brain after the exposure of sub-lethal concentrations of fipronil (1/5th of LC 50 for 96 hours) for 96 hours and 28 days.

MATERIALS AND METHODS

Labeo rohita **(**7.5 ± 1.5 cm in length and 8.5 ± 0.5 g in body weight, as mean ± S.E) obtained from Patani Fisheries, Marathakkara (Fish Seed dealers in Thrissur, Kerala) were transferred to the laboratory. Fish were acclimatized to laboratory conditions for two weeks in large cement tanks with natural water after providing a dip treatment in 0.1% potassium permanganate solution to prevent infection. They were fed with commercial dry feed pellets (Nutrifry, Premium Larval Feed, Godrej Agrovet Limited, Mumbai), once daily with the ratio of 3% of body weight. The commercial-grade fipronil was procured from agrochemical dealers of Kerala.

After conducting toxicity study, we calculated the median lethal concentration (LC 50 value) of fipronil for 96 hours to the rohu fish was 23 µg/L using probit analysis by SPSS version 22. We considered 1/5th of LC 50 (as 4.6 µg/L) as sub-lethal concentrations for the experiments. After acclimation the fish were divided into three groups such as group-1 as control (fish were not subjected to fipronil), group-2 as an experiment I (fish were subjected to1/5th value of LC 50 for 96 hours), and group-3 as experiment II (fish were subjected to 1/5th of LC 50 for 28 days). Each group consists of 10 juvenile rohu fish in 10 liters of natural well water. Triplicates were used for each experiment. The desired concentration of the toxicant was added directly into the experimental groups. The test media were renewed every 24 hours and maintained all physio-chemical parameters.

After the stipulated periods (96 hours and 28 days) the fish were sacrificed and its brain was dissected out for different toxicity studies. Dissected brains were washed in physiological saline and homogenized using proper buffer solutions for the analysis of different biochemical parameters.

The tissue lipid extraction by the method of Folch *et. al*. [22]

The tissues were homogenized and extracted with chloroform: methanol (2:1). 'n' volume of the tissue homogenate was added drop by drop to '5n' volume of methanol in a stoppered tube. Then '5n' volume of chloroform was added and mixed. This mixture was incubated at 55°C for 15 minutes, after which another '5n' volume of chloroform was added so that the proportion of chloroform to methanol was 2:1 (v/v). After filtration and washing the residue with chloroform: methanol (2:1) at least 3 times, the combined filtrate was washed with 0.7% KCl solution (20% of the total volume of the extract). The aqueous upper phase was removed with a Pasteur pipette and the lower layer was washed with 5 ml of chloroform: methanol: KCl solution (3:48:47 v/v/v) three times. The washed lower layer was evaporated to dryness and the residue re-dissolved in a known volume of chloroform. Aliquots were used for the lipid analysis of total cholesterol, triglycerides, and phospholipids.

Estimation of total lipid by the method of Bradgon [23]

The tissue homogenate was prepared by adding 2 ml of chloroform to 10 mg of the tissue sample. The homogenate was centrifuged at 15,000 rpm for 10 min. the supernatant was transferred to a test tube and evaporated to dryness at room temperature. To this, 3 ml of distilled water and an equal volume of freshly prepared 2% chromic acid reagent were added. The absorbance of the colour developed was recorded at 625 nm. The chromic acid reagent with 1 ml of distilled water was used as a reagent blank. Reagent grade cholesterol was served as standard curve and expressed as mg per ml.

Estimation of Cholesterol by the method Crawford *et al*. [24]

Take a known quantity of aliquot of the lipid extract and in a known quantity of chloroform-ethanol mixture. Add 3ml coloured ferric chloride reagent in sample, standard, and in the blank. Boil for 5 minutes and cool it in ice bath. Stirred slowly. After 30 minutes read absorbance at 540 nm.

Estimation of triglycerides by the method of Van and Zilversmit [25]

2g of chromotropic acid (or 2.24 g Na salt) was dissolved in 200 ml distilled water. 600 ml of concentrated Sulphuric acid was added slowly to 300ml of distilled water which was chilled in ice. This chilled and diluted acid was then added to the chromotropic acid solution (0.25 mg/ml) 2 g of florisil was taken in a glass stoppered tube and 3 ml of chloroform was added. An aliquot of the extract was layered on the top of the florisil and mixed, chloroform was then added to this to a total volume of 10 ml. It was then stoppered, shaken intermittently for about 10 minutes, and then filtered through a filter paper. After filtration 1 ml of the filtrate was pipetted out into each of the three tubes. 1 ml of working standard of glycerol (9 mg/ ml) was pipetted out into each of three tubes. The solvent was evaporated at 60 - 70°C.0.5 ml of ethanolic KOH was then added to 2 out of the three tubes (saponified sample) and 0.5 ml of ethanol was added to the third tube (Unsaponified sample). The tubes were then closed and kept at 60 - 70°C for 15 minutes. 0. 5 ml of 0.2 N H_2S0_4 was added to each tube and the tubes were then placed in a gently boiling water bath for 15 minutes to remove the alcohol. They were then cooled to room temperature. 0. 1 ml of sodium meta periodate was added to each tube and kept for10 minutes. 0.1 ml sodium arsenite was then added and mixed. Yellow colour of iodine appeared and vanished within a few minutes. 5ml of chromotropic acid was added to each tube and mixed. The tubes were closed and then heated in a boiling water bath for 3 0 minutes. They were then cooled and absorbance was read at 570 nm.

Estimation of phospholipids by the method of Zilversmit and Davis [26]

An aliquot of the extract was pipetted out into a Kjeldahl flask and evaporated to dryness. 1 ml of 5N H_2SO_4 was added and digested in a digestion rack till it became light brown. It was then cooled to room temperature. 1 or 2 drops of 2N $HN0_3$ were added and it was digested again till it became colourless. The Kjeldahl flask was cooled, 1ml of water was added and heated in a boiling water bath for about 5 minutes. 1 ml of 2.5% ammonium molybdate and 0.1 ml of ANSA reagent were added to this. The volume was then made up to 10 ml with distilled water and the absorbance was measured at 660 nm within 10 minutes.

Estimation of free fatty acids by the method of Falholt *et. al*. [27]

0.1 ml sample was evaporated to dryness at 60-65°C in a water bath. Then 1 ml phosphate buffer, 6 ml extraction solvent, and 2.5 ml copper reagent were added. Blank contained 1 ml phosphate buffer, 6 ml extraction solvent, and 2. 5 ml copper reagent. 0. 2 ml of the working standard was pipetted out to a test tube and 1 ml phosphate buffer, 5.8 ml extraction solvent, and 2. 5 ml copper reagent were added. Then the solution was transferred to a stoppered tube and shaken vigorously for 90 sec. It was allowed to stand for 15 minutes, centrifuged at 4000 rpm for 5 minutes and the supernatant (1 ml) was transferred to a tube containing 0. 5 ml di phenyl carbazide solution. The contents were mixed carefully, diluted with 5 ml of the extraction solvent. The OD was read after 15 minutes at 5 50 nm.

Assay of malic enzyme by the method of Ochoa [28]

The chilled tissue was homogenized with three-volume of 0.25 M glycylglycine buffer at 0°C and supernatant obtained by centrifuging at 2000 rpm at 0°C for 10 minutes was used as the enzyme. The reaction mixture in a quartz cell consists of 0.3 ml buffer, 0.06 ml of manganese chloride, 0.2 ml NADP, 0.05 ml of L-malate or enzyme, and water to a final volume of 3.0 ml. the assay was carried out at room temperature (23 – 25°C). The reaction was started by the addition of either malate or enzyme and the optical density (OD) at 340 nm was read against a blank containing all the components except NADP, at intervals of 15 seconds for 1-2 minutes. One unit of the enzyme was defined as that amount that causes an initial change of OD of 0.01/min under the above condition of the assay.

Assay of β- Hydroxy methyl glutaryl Co-A reductase by the method of Rao and Ramakrishnan [29]

Equal volumes of fresh tissue homogenate and dilute perchloric acid were mixed. Kept for 5 minutes and centrifuged at 2000 g for 10 minutes. Take 1 ml of the supernatant into each tubesone for HMG Co-A and one for mevalonate. Add 0.5 ml of freshly prepared hydroxylamine hydrochloride reagents for both into respective tubes and mix. After 5 minutes, 1.5 ml of ferric chloride was added into both tubes and mixed well. The optical density was read after 10 minutes at 540 nm against a similarly treated saline arsenate blank. The ratio between HMG C0-A and mevalonate is taken as an index of the activity of the enzyme which catalyses the conversion of β-hydroxy methyl glutaryl Co-A to mevalonate. Lower the ratio higher the enzyme activity.

Assay of glucose-6- phosphate dehydrogenase by the method of Balinsky and Bernstein [30]

The reaction mixture containing 0.4 ml Tris-HCl buffer, 0.2 ml of NADP, 0.2 ml of magnesium chloride, and 1.0 ml of water were to 0.2 ml of enzyme in a cuvette. The reaction was started by the addition of adding 0.2 ml of glucose-6 phosphate and the increase in the absorbance was measured at 3440 nm. The activity of the enzyme is expressed in terms of units/g in which one unit is equal to the amount of enzyme that brought about a change in optical density of 0.01/minute.

Statistical analysis

All statistical versions were performed with SPSS statistical program (Version -20). All experimental data were expressed as mean ± standard error (SE). Significant differences between control experimental groups were compared by one-way analysis of variance (ANOVA). The P - values that were less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The present study showed the adverse effect of fipronil, on lipogenic enzymes and lipid profile in the brain tissue of rohu. After the exposure of fipronil, there was a significant difference in the activity of lipogenic enzymes and in the number of lipid profiles experienced in the brain of rohu. After each exposure period the activities of malic enzyme and HMG CoA reductase were reduced and the activity of glucose-6- phosphate reductase is increased in the rohu brain. But all lipid profiles such as total lipid, cholesterol, triglycerides, phospholipids, and free fatty acid levels were reduced after each exposure period.

The malic enzyme catalyzes the reversible oxidative decarboxylation of L- malate to pyruvate and CO_2 with accompanying reduction of NADP+ to NADPH in which NADPH is used for the biosynthesis of fatty acids [31-32]. Overproduction of malic enzyme increased the total lipid content [33]. So, in the present study, the reduced activity of the malic enzyme in fipronil treated fish indicated the reduced synthesis of fatty acid which leads to low lipid metabolism.

HMG-CoA is the precursor for cholesterol synthesis, converted into mevalonate by the action of a rate-controlling enzyme 3-hydroxy-3-methyl glutaryl co-enzyme A (HMG-CoA) reductase which is responsible for the biosynthesis of cholesterol and isoprenoid [34-35]. So, the inhibition of HMG-CoA reductase reduced the synthesis of cholesterol [36]. Our present study revealed that decreased activity of HMG-CoA reductase also leads to the reduced synthesis of cholesterol in the brain tissue.

Table 1 Effect of fipronil on lipogenic enzymes in the rohu brain

Group	Control	Experiment I	Experiment II
Malic enzyme	1.29 ± 0.006	1.11 ± 0.002	0.81 ± 0.005
Hmg co-A reductase	0.41 ± 0.009	0.33 ± 0.006	0.07 ± 0.005
G6PD	0.18 ± 0.013	0.21 ± 0.004	6.96 ± 0.074

All values are mean ± SE of five replicates. Since the probability values is less than 0.05, the treatments are found to be significant at 5% level of significance

Glucose-6 phosphate dehydrogenase is a cytosolic enzyme that produces cellular NADPH which is indispensable for the synthesis of cholesterol and fatty acids [37-38]. NADPH is an essential electron donour in all eukaryotes which is essential for the generation of ROS and has a vital role in anti-oxidative defense mechanism [39]. Thus, the highest-level activity of G6PH accompanied with the high production of NADPH which may use in anti-

oxidant defense mechanism. The reduction of lipid level may be either due to the inhibition of biosynthesis of cholesterol in the liver or due to the reduction of dietary cholesterol absorption [40]. During toxicant stress conditions fish may be produced the energy from the lipids for other metabolic function and their products have a key role to mitigate the toxicant stress condition [41]. Reduction of total lipids level in the rohu brain after the exposure of sub-lethal concentration of fipronil in the present study verified by different researchers [42-44].

Table 2 Effect of fipronil in the lipid profiles of rohu brain

Group	Control	Experiment I	Experiment II
Total lipid	3.29 ± 0.023	2.70 ± 0.014	1.34 ± 0.014
Cholesterol	1.16 ± 0.011	0.96 ± 0.009	0.65 ± 0.006
Triglycerides	1.52 ± 0.008	1.27 ± 0.014	0.53 ± 0.006
Phospholipid	1.44 ± 0.114	0.99 ± 0.009	0.46 ± 0.010
Free fatty acid	1.85 ± 0.018	1.44 ± 0.011	0.87 ± 0.004

All values are mean ± SE of five replicates. Since the probability values is less than 0.05, the treatments are found to be significant at 5% level of significance

Cholesterol is also reduced due to the utilization of fatty acids. The use of fatty deposits alternative to glucose as an energy source might reduce the cholesterol level [45]. Reduction of cholesterol in various organs of fish due to the exposure of pesticides proved by [46-49]. Fatty acids, triglycerides, phospholipids are different lipid forms that are included in the major components of biological membranes and tissues of living organisms [50]. In teleost fish tissues, there are two major groups of lipids presented namely phospholipids and triglycerides. In these phospholipids make up the integral structure of the unit membrane in the cells and triglycerides are stored as fat depots for energy sources [51]. Reduction of triglycerides and phospholipids in the brain after the exposure of fipronil indicated that these lipids are used an energy source in stress conditions [52-54].

Free fatty acids (FFA) or non-esterified fatty acids (NEFA) are derived from the hydrolysis of the stored triglycerides [55-56]. These free fatty acids are transported from the bloodstream to the tissues and there it is utilized for energy release and other purposes [57]. In our study the reduction in the level of free fatty acids may be due to the oxidation of FFA for the production of energy [20] to attenuate the stress created by the exposure of fipronil [58-59].

CONCLUSION

Our present study analysed the effect of sub-lethal concentration of fipronil in the lipid metabolism of the rohu brain. The result revealed that the low concentration of fipronil significantly affected the lipid metabolism in the brain tissue of rohu. The use of fipronil in the agricultural field increased every day especially in the rice fields to fight mainly against stem borers. This also affected the natural water bodies near the agricultural field and adversely affected the aquatic fauna in this area. So, our study revealed that control of fipronil in the agricultural field was beneficial for our environment.

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