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PROPANIL-INDUCED NEUROTOXICITY IN FRESHWATER COMMON CARP (*Cyprinus carpio*)

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ABSTRACT

Herbicides are known to affect the metabolic parameters in fishes. For this reason a study was designed to assess the effects of herbicide propanil (3',4'-dichloropropionanilide) on common carp (*Cyprinus carpio* L.) through antioxidant indices and other neurotoxicological parameters. Fishes were exposed to two concentration of propanil (2 and 4 mg L^{-1}) for 6 and 48 h and effects on neurtoxicity biomarkers [acetylcholinesterase (AChE), Na^+/K^+-ATPase] and oxidative stress parameters [reduced glutathione levels, superoxide dismutase, catalase, thiobarbituric acid reactive substances and protein oxidation (protein carbonyl) were studied. Study revealed that propanil exposure disturbed antioxidant status, increased lipid peroxidation and protein carbonyl levels in fishes at both doses. Activity of neurotoxicity enzymes AChE and Na^+/K^+-ATPase was inhibited at both the doses of propanil exposure. Propanil was more toxic at higher doses at both the lengths of exposure.

Key words: Antioxidant, *Cyprinus carpio,* herbicide, neurotransmitter, propanil

INTRODUCTION

Aquatic systems are frequently affected by agricultural and industrial activities. Some aquatic organisms often serve as end point indicators for contaminations. The environmental contaminations are intensified by the use of chemicals including herbicides which affect the health and survival of aquatic life (Dezfuli *et al*., 2003; Figueiredo-Fernandes *et al*., 2006). Therefore, it is imperative to assess the toxicity of such herbicides on non-target aquatic organisms. During pesticide use, aerial drift or accidental spills often expose the nearby non-target areas like ponds, rivers, lakes, wetlands, etc. to these chemicals. Herbicides are known to affect fish by the formation of reactive oxygen species (ROS). For instance, propanil (3',4'-dichloropropionanilide) induces oxidative stress in fishes as a result of imbalance between ROS and antioxidant system (Nordberg and Arne´r, 2001). Oxidative stress may also be due to the damaged biological system or failure of antioxidant defense systems. Both peroxidation of lipids (LPO) and carbonylation of proteins have been used as markers to assess the impact of pollutants on aquatic organisms; especially hydroxyl radical (OH), a product of reaction with free radicals, reacts quickly with nearby molecules and leads to oxidative changes in protein, lipid and nucleic acid (Cardoso *et al*., 2006). Pertinently, nervous

system is mainly susceptible to oxidative damage since brain has high oxygen consumption rate and its membrane is enriched with highly oxidizable polyunsaturated fatty acids. Further, the activity of antioxidant enzymes (SOD, CAT and GPX) in brain is comparatively lower than in other tissues; and also these have high Fe content.

The enzymes like Na^+/K^+-ATPase and acetylcholinesterase (AChE) are widely used as biomarkers for pesticide toxicity (Dutta, 2003; Filho, 2006). Since neurotransmitters are sensitive to redox imbalance so these have often been used to assess the exposure of contaminants. AChE is a key enzyme in cholinergic transmission in nervous system. The function of this enzyme is to catalyze hydrolysis of acetylcholine into acetate and choline in synaptic cleft (Yi *et al*., 2006). The sodium pump, Na+/K+-ATPase, present in animal cells regulates intracellular ionic gradients in cellular homeostasis and secondary transport (Skou and Esmann, 1992). Herbicides reportedly inhibit ATPases including cell membrane associated Na^+/K^+-ATPase (Davis and Wedemeyer, 1971). In present study, fresh water common carp *Cyprinus carpio* was chosen because the effect of propanil on this particular fish species has scarcely been studied. Further, in terms of methodology, little is known about the changes in AChE, Na+/K+ ATPase, thiobarbituric acid-reactive substances (TBARS) and oxidative response to exposure of brain tissues of *Cyprinus carpio* to herbicides. This species is commercially grown in Himalayan temperate region and is considered resistant and adaptable to different temperatures. The present study was aimed to investigate the neurotoxic effects of two concentrations of propanil on fresh water fish, *Cyprinus carpio*.

MATERIALS AND METHODS

Fish and treatment

Healthy and active common carp (*Cyprinus carpio*) were obtained from Chattabal fish market, Srinagar (Kashmir) in the year 2015. No apparent disease or abnormality was found during routine analyses of these fish. Fish were acclimatized to laboratory conditions for 10 days in glass tanks (30 L) prior to the experimentation. They were kept in continuously aerated water with a static system and under a natural photoperiod (12 h light/12 h dark). The temperature and oxygen content in water was 22-24°C and 7.1-8.3 mg L^{-1}, respectively. During acclimatization, the fish were fed commercial fish food twice a day *ad libitum*. Group I was control fishes while group II and III were treated with 4 and 2 mg propanil kg^{-1} body weight (bw), respectively, for 6 h. Group IV and V were treated with 4 and 2 propanil kg^{-1} bw, respectively, for 48 h.

Chemicals

A commercial formulation of herbicide propanil was used in both the experiments. Acetylthiocholine (AChE) (Merck), 5,5'dithio-bis (2-nitrobenzoic acid) (Merck), 1-chloro-2,4 dinitrobenzene (Merck), hydrogen peroxide (Loba Chemie), trichloro-acetic acid (Titan Biotech), 2-thiobarbituric acid, and sodium chloride (Himedia) and all other chemicals used were of highest purity grade.

Experimental design

Thirty fish were randomly divided into five groups (mean weight of each fish was 130 g in the range of 100-140 g). Fishes were exposed to two concentrations (2 and 4 mg propanil L^{-1}) for 6 and 48 h. A control set was also run with the same number of fish in the same volume of water but without propanil. The fish were sampled at the end of experiment and their brain tissues collected. After preparation of post-mitochondrial supernatants, all the enzyme assays were performed in triplicate in a completely randomized design.

Preparation of post-mitochondrial supernatant

Brain tissues were removed and cleaned with ice-cold saline (0.85% NaCl). A 10% homogenate of brain tissue was obtained in buffer solution [10 mM tris-HCl and 250 mM sucrose; pH 7.4] using a homogenizer and centrifuged at 3,000 rpm for 10 min. in a refrigerated centrifuge (Remi, C-24 BL) to separate nuclear debris. The aliquot so obtained was further centrifuged at 10,000 rpm for 20 min to obtain post-mitochondrial supernatant (PMS), which was used as a source of enzymes.

Assay for catalase

The catalase activity was assessed as per Claiborne (1985). For this, the reaction mixture used consisted of 0·05 mL PMS, 1·0 mL H_2O_2 (0·019 M) and 1·95 mL phosphate buffer (0·1 M, pH 7·4) in a volume of 3 mL. The absorbance was measured with the help of a UV-Vis spectrophotometer (Shimadzu UV-1280) at 240 nm and enzyme expressed as nmol H_2O_2 consumed min^{-1} mg^{-1} protein.

Estimation of lipid peroxidation

The lipid peroxidation was assayed as per the method of Wright *et al.* (1999). The reaction mixture (1.0 mL) contained 0.58 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL homogenate, 0.2 mL ascorbic acid (100 mM), and 0.02 mL ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h and reaction stopped by the addition of 1.0 mL 10% trichloroacetic acid (TCA). Following the addition of 1.0 mL 0.67% TBA, all the tubes were placed in boiling water-bath for 20 min. and shifted to crushed ice-bath before centrifuging at 4500 rpm for 10 min. The amount of malondialdehyde formed in each sample was assessed by measuring optical density of supernatant at 535 nm using spectrophotometer (Shimadzu UV-1280) against a reagent blank. The results were expressed as n mol MDA formed h^{-1} g^{-1} tissue at 37°C.

Measurement of superoxide dismutase (SOD) activity

SOD activity was assessed as per the method of Marklund and Marklund (1974). The reaction mixture used had 2.9 mL tris-HCl buffer (50 mM, pH 8·5), pyrogallol (24 mM in 10 mM-HCl) and 0.1 mL PMS in a total volume of 3 mL. Enzyme activity was measured at 420 nm and expressed as unit mg^{-1} protein. One unit of SOD enzyme is defined as the enzyme activity that inhibits the auto-oxidation of pyrogallol by 50%.

Estimation of reduced glutathione (GSH)

GSH was determined as per Jollow *et al.* (1974). The samples of 1.0 mL PMS were precipitated with 1.0 mL sulphosalicylic acid (4%), then kept at 4°C for 1 h and centrifuged at 3000 rpm for 15 min. at 4°C. The assay mixture consisted of 0.4 mL supernatant, 2.2 mL phosphate buffer (0.1 M, pH 7.4) and 0.4 mL 5, 50-dithio bis-[2-nitrobenzoic acid] (10 mM) in a total volume of 3.0 mL. The yellow colour developed was spectrophotometerically (Shimadzu UV-1280) read immediately at 412 nm. GSH concentration was expressed in terms of n moles GSH conjugates g^{-1} tissue.

Carbonyl assay

Protein carbonyl was assayed as per Yan *et al.* (1995) with slight modifications. The PMS sample (1.0 mL) was reacted with 10 mM DNPH (dissolved in 2N HCl). After incubation at room temperature for 1 h in dark, 0.5 mL denaturing buffer [150 mM sodium phosphate buffer (pH 6.8) containing SDS 3.0%], 2.0 mL heptane (99.5%) and 2.0 mL ethanol (99.8%) were added sequentially, vortexed for 40 sec and centrifuged at 18000 rpm for 15 min. The protein isolated from interface was washed twice by its re-suspension in ethanol: ethyl acetate (1:1) and suspended in 1 mL denaturing buffer. The blank tubes, incubated with 2 N HCl without DNPH, were included for each sample. The carbonyl content was measured spectrophotometrically at 370 nm.

Acetylcholinesterase (AChE) activity assay

The AChE activity was assayed as per Ellman *et al*. (1961) and modified by Miron *et al.* (2005). The brain sample (30 mg) was homogenized in a Potter–Elvejhem glass/teflon homogenizer in

sodium phosphate buffer 50 mM (pH 7.2) and triton X-100 (1%). The homogenate was centrifuged at 8000 rpm for 10 min at 4°C and the supernatant used as enzyme source. Aliquot (50 mL) was incubated at 30°C for 2 min with a solution containing 0.1M sodium phosphate buffer (pH 7.5) and 1 mM DTNB. After incubation, the reaction was initiated by the addition of AChE (0.5 mM). The absorbance at 412 nm measured by spectrophotometer (Shimadzu UV-1280). The enzyme activity was expressed in terms of m mol AChE hydrolyzed min^{-1} mg^{-1} protein.

Estimation of Na+/K+ ATPase activity

The Na^+/K^+ ATPase activity was assayed as release of inorganic phosphate (Pi) by the method of Saleem *et al*. (2006). The PMS was prepared in 0.2 M tris-HCl buffer (pH 7.4). The reaction mixture consisted of 0.2 mL 0.2 M KCl, 0.1 mL 0.1 M $MgCl_2$, 0.2 mL 1 M NaCl, 0.1 mL tissue and 1.2 mL tris-HCl. The mixture was incubated at room temperature for 5 min. and 0.2 mL 0.025 M ATP added to start reaction. The mixture was re-incubated at 37°C for 15 min and 1 mL 10% TCA added to stop the reaction. The tubes were centrifuged at 3000 rpm for 10 min, pellets discarded and 0.5 mL supernatant taken along with 3.5 mL distilled water, 0.5 mL ammonium molybdate and 0.5 mL ANSA. The mixture was incubated at room temperature for 30 min and optical density measured at 660 nm. The activity was expressed as μg Pi liberated min^{-1} mg^{-1} protein.

Statistical analysis

The results were expressed as mean ± standard error. All data was analyzed using analysis of variance, followed by Tukey's test. All the statistical analyses were performed using Graph Pad Prism 5 software (Graph Pad Software Inc., San Diego, USA).

RESULTS AND DISCUSSION

The herbicide propanil (3',4' dichloropropionanilide) was found toxic to common carp (*Cyprinus carpio*) at both test doses (2 and 4 mg L^{-1}) as revealed by antioxidant indices and neurotoxicological markers. Higher concentration was more toxic than lower one. Fishes are often used as biomarker in eco-toxicological studies as they play vital role in tropical web, accumulate toxic substances and respond to low levels of toxicants (Cavas *et al.,* 2005). The use of fish as indices of pollution are on increase as these permit early detection of aquatic environmental problems (Van *et al.,* 2003). Brain being the integral component of central nervous system is highly susceptible to oxidative stress due to the presence of a large amount of PUFA which are vulnerable to ROS induced damage. Further, brain consumes 20% body's oxygen which contributes to the pathogenesis (Travacio *et al*., 2005).

In present study changes in enzymatic parameters were studied in common carp *Cyprinus carpio* using two propanil concentrations (2 and 4 mg L^{-1}) normally used in plant protection. As compared to the GSH value of 24.5±1.72 n moles GSH conjugate g^{-1} tissue observed in unexposed control fish, 4 mg propanil L^{-1} exposed-fish showed significant decrease in GSH levels when exposed for 6 and 48 h (16.83 ± 0.98 and 15.17 ± 0.94 n moles GSH conjugate g^{-1} tissue, respectively) (Fig. 1). GSH is a main non-protein thiol and is a primary reductant in cells (Yilmaz *et al*., 2006). It possess antioxidant properties and its protective effect against oxidative stress induced toxicity in aquatic animals is well established (Droge, 2002).

Lipid peroxidation is perhaps the most extensively investigated process induced by free radicals and hence considered a valuable indicator of oxidative damage of cellular components (Ferreira *et al*., 2005). In present study a significant increase in LPO values was observed in brain due to 6 and 48 h propanil exposure both at 4 mg L^{-1} (5.14 ± 0.47 and 5.77 ± 0.55 n mole MDA formed h^{-1} g^{-1} tissue) and 2 mg L^{-1} (4.07 ± 0.16 and 6.05 ± 0.13 n moles MDA formed h^{-1} g^{-1} tissue) as compared to 2.98 ± 0.10 n mole MDA formed h^{-1} g^{-1} tissue in non-exposed control fish (Fig. 2). Our results are in agreement with previous reports showing increase in LPO by pesticides like alachlor (Peebua *et al.,* 2007),

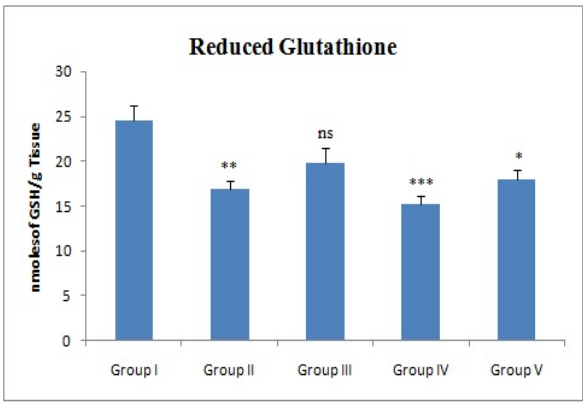

Fig. 1: Effect of propanil on GSH in brains of fish, exposed for two durations. Values are expressed as means ± SE (n = 6). GSH is measured as nmoles GSH g^{-1} tissue. Significant differences are indicated by $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ as compared to control. Group I = control fish, Group II = fish treated with 4 mg propanil kg^{-1} for 6 h, Group III = fish treated with 2 mg propanil kg^{-1} for 6 h; Group IV = fish treated with 4 mg propanil kg^{-1} for 48 h; and Group V = fish treated with 2 propanil mg kg^{-1} for 48 h.

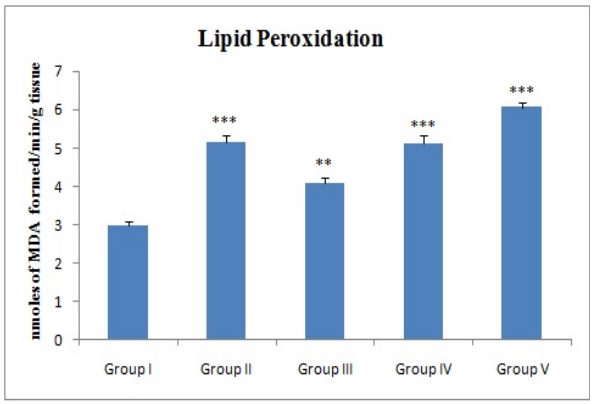

Fig. 2: Effect of propanil on LPO in brains of fishes exposed for two durations. Values are expressed as means ± SE (n = 6). LPO is measured as n moles MDA formed min^{-1} g^{-1} tissue. Significant differences are indicated by $^{***}p<0.001$, $^{**}p<0.01$ as compared to control. Group I = control fish, Group II = fish treated with 4 mg propanil kg^{-1} for 6 h, Group III = fish treated with 2 mg propanil kg^{-1} for 6 h; Group IV = fish treated with 4 mg propanil kg^{-1} for 48 h; and Group V = fish treated with 2 propanil mg kg^{-1} for 48 h.

malathion (Chandra *el al.*, 2008), deltamethrin (Koprucu *et al.*, 2008) and butachlor (Farombi *et al.*, 2008) in fish. It was also noticed that the LPO activity was more at longer duration of exposure even at lesser dose (Fig. 2). Less increase was observed in shorter exposure (6 h). During the same period, the protein carbonyl levels were elevated at 4 mg propanil L^{-1} exposure for both 6 and 48 h (1.31 ± 0.17 and 1.40 ± 0.09 n moles DNPH mg^{-1} protein) as compared to 1.16 ± 0.38 and 1.20 ± 0.17 n moles DNPH mg^{-1} protein at 2 mg propanil L^{-1} exposure for the same periods (Fig. 3). Thus protein carbonylation causing protein oxidation could be linked to increased TBARS levels due to ROS formation which, in turn, could directly attack protein and lead to carbonyl formation. The findings are in line with Parvez and Raisuddin (2005) who demonstrated increased protein carbonyl levels in fish (*Channa punctata*) exposed to pesticides.

SOD plays a crucial antioxidant role in animal and constitutes primary defense against the toxic effects of superoxide radicals in aerobic organisms. SOD is the first enzyme to cope with oxi-radicals (Kohen and Nyska, 2002). A significant decrease in tissue SOD activities at 4 mg propanil L^{-1} exposure for 6 and 48 h (2.77 ± 0.24 and 2.98 ± 0.17 IU L^{-1}) and 2 mg propanil L^{-1} exposure for the same periods (3.31 ± 0.10 and 3.21 ± 0.14 IU L^{-1}) was observed in fish (Fig 4). A similar effect on SOD activity in carp tissue following pesticide exposure has been reported by Yonar *et al.* (2012).

Catalase activity was significantly decreased in brain of fish exposed to both concentrations (4 and 2 mg propanil L^{-1}); however, higher dose 4 mg propanil L^{-1} exposure for 6 and 48 h (3.12 ± 0.11 and 3.04 ± 0.07 n moles H_2O_2 consumed min^{-1} mg^{-1} protein) showed more inhibition. Similar effect has been observed by Hai et al. (1995) in carp (*Cyprinus carpio* L.). Catalase is an enzyme located in peroxisomes and facilitates the removal of hydrogen peroxide, which is metabolized to molecular oxygen and water (van der Oost *et al.*, 2003). In agreement with our findings, Oruç and Usta (2007) reported that diazinon caused a decrease in catalase activity in *Cyprinus carpio*.

AChE activity was significantly decreased in brain of fish exposed to propanil at both the concentrations (4 and 2 mg L^{-1}). Exposure of fish to higher dose of propanil 4 mg L^{-1} for 6 and 48 h duration showed AChE activity of 13.17 ± 1.94 and 9.83 ± 1.47 n moles ATC hydrolyzed min^{-1} mg^{-1} protein, respectively, so depicted more inhibition (Fig. 6). Our results are in agreement with Miron

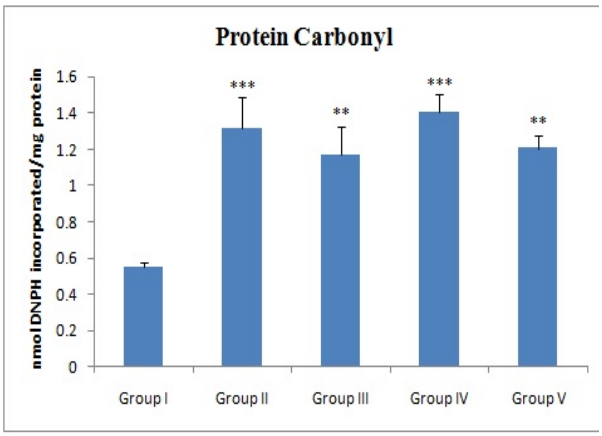

Fig. 3: Effect of propanil on protein carbonyl level in brains of fishes exposed for two durations; Values are expressed as means ± SE (n = 6). Protein carbonyl measured as n moles DNPH mg^{-1} protein. Group I = control fish, Group II = fish treated with 4 mg propanil kg^{-1} for 6 h, Group III = fish treated with 2 mg propanil kg^{-1} for 6 h; Group IV = fish treated with 4 mg propanil kg^{-1} for 48 h; and Group V = fish treated with 2 propanil mg kg^{-1} for 48 h.

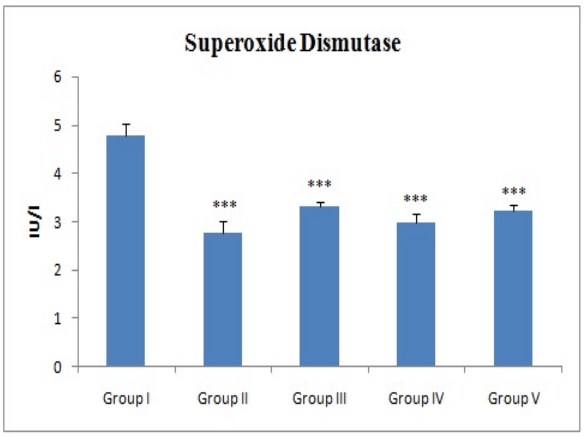

Fig. 4: Effect of propanil on SOD activity in brains of fishes, exposed for two durations; Values are expressed as means ± SE (n = 6). SOD is measured as IU L^{-1}. Significant differences are indicated by $^{***}p < 0.01$ as compared to control. Group I = control fish, Group II = fish treated with 4 mg propanil kg^{-1} for 6 h, Group III = fish treated with 2 mg propanil kg^{-1} for 6 h; Group IV = fish treated with 4 mg propanil kg^{-1} for 48 h; and Group V = fish treated with 2 propanil mg kg^{-1} for 48 h.

et al. (2005) who identified propanil as a potent inhibitor of AChE in brain and muscle of silver catfish. AChE is a vital enzyme involved in cholinergic transmission of nervous system. The enzyme catalyzes hydrolysis of acetylcholine into acetate and choline in synaptic cleft (Yi *et al.*, 2006). Miron

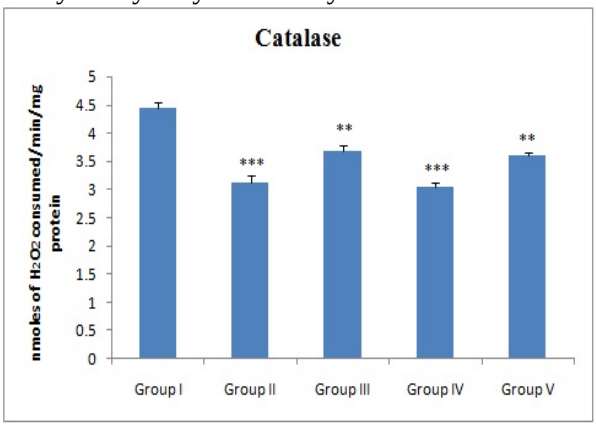

Fig. 5: Effect of propanil on catalase activity in brains of fishes, exposed for two durations. Values are expressed as means ± SE (n = 6). Catalase is measured as n moles H_2O_2 consumed min^{-1} mg^{-1} protein. Significant differences are indicated by $^{***}p < 0.001$ $^{**}p < 0.01$ as compared to control. Group I = control fish, Group II = fish treated with 4 mg propanil kg^{-1} for 6 h, Group III = fish treated with 2 mg propanil kg^{-1} for 6 h; Group IV = fish treated with 4 mg propanil kg^{-1} for 48 h; and Group V = fish treated with 2 propanil mg kg^{-1} for 48 h.

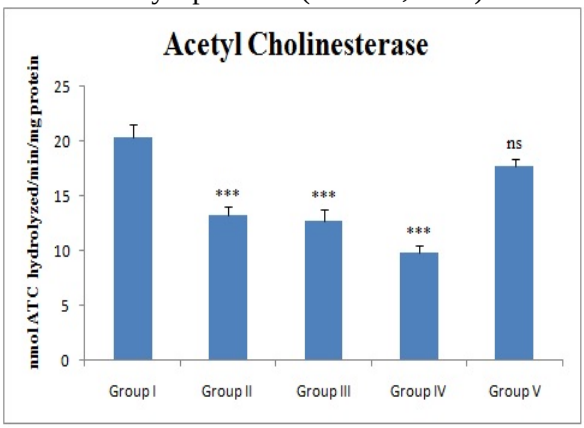

Fig. 6: Effect of propanil on AChE activity in brains of fishes exposed for two durations. Values are expressed as means ± SE (n = 6). AChE activity is measured as n moles ATC hydrolyzed min^{-1} mg^{-1} protein. Significant differences are indicated by $^{***}p < 0.001$ as compared to control. Group I = control fish, Group II = fish treated with 4 mg propanil kg^{-1} for 6 h, Group III = fish treated with 2 mg propanil kg^{-1} for 6 h; Group IV = fish treated with 4 mg propanil kg^{-1} for 48 h; and Group V = fish treated with 2 propanil mg kg^{-1} for 48 h.

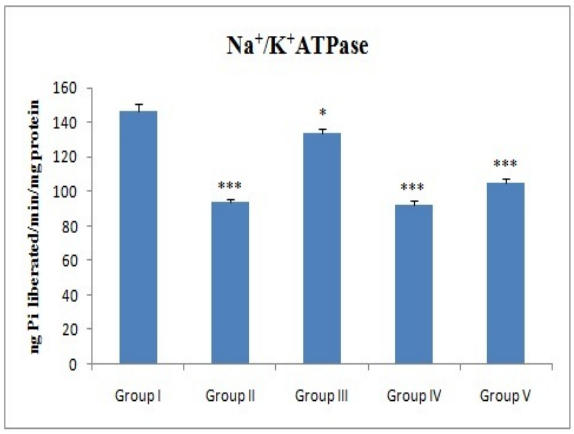

Fig. 7: Effect of propanil on Na^+-K^+ATPase activity in brains of fishes. Values are expressed as means ± SE (n = 6). Na^+/K^+-ATPase activity is measured as µg P_i liberated min^{-1} mg^{-1} protein. Significant differences are indicated by $^{***}p<0.001$ $^{*}p<0.05$ when compared with control. Group I are control fishes. Group I = control fish, Group II = fish treated with 4 mg propanil kg^{-1} for 6 h, Group III = fish treated with 2 mg propanil kg^{-1} for 6 h; Group IV = fish treated with 4 mg propanil kg^{-1} for 48 h; and Group V = fish treated with 2 propanil mg kg^{-1} for 48 h.

et al. (2005) reported increase in brain AChE activity in fish species *Rhamdia quelen* exposed to quinclorac herbicide. AChE activity is also inhibited by some herbicides like glyphosate (Glusczak *et al.*, 2006) while propanil caused AChE inhibition in *Leporinus obtusidens* (Moraes *et al.*, 2009). Using commercial formulation containing propanil, AChE was inhibited in the brain of *L. obtusidens* exposed under field conditions (Glusczak *et al.*, 2007).

Na+/K+-ATPase is a membrane transport protein in mammalian cell membrane and plays a crucial role in sustaining ionic homeostasis. Any deregulation in the activity of Na+/K+-ATPase might be a common feature in neurological pathologies (Naseem and Parvez, 2015). Our results are in agreement with previous reports which showed that ATPase including cell membrane-associated Na+/K+-ATPase are inhibited by herbicides (Davis and Wedemeyer, 1971; Naseem and Parvez, 2015). In our study, Na+/K+-ATPase was significantly decreased in brain of propanil exposed fish that at both the concentrations; however higher doses 4 mg L^{-1} (94 ± 1.98 and 92 ± 2.39 µg P_i liberated min^{-1} mg^{-1} protein) showed more inhibition in enzyme activity (Fig. 7).

Oxidative damage products seemed to be better biomarkers of oxidative stress in fish exposed to pollutants. The present study revealed the hazards of using herbicides propanil in agriculture fields. Propanil caused changes in neuro-toxicological and oxidative stress parameters of carp fish at both the concentrations used. Higher dose was found to be slightly more toxic compared to lower dose however lipid perocidation was higher at lower dose for longer.

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