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Manifestation of Toxic Effects of Propoxur on Acetylcholinesterase and Histology of Liver of *Tilapia mossambica* (Peters)

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Abstract : The present study was carried out to evaluate the deleterious effects of propoxur (2- isopropoxyphenyl N-methyl carbamate) on the liver of *Tilapia mossambica* (Peters) to assess the toxicity to its behaviour, histology and enzyme acetylcholinesterase (AChE, EC 3.1.1.7). Propoxur can be considered as highly toxic to *Tilapia mossambica* with a median lethal concentration (LC_{50}) of 0.52±0.01 ml/L. Various parameters of liver AChE and histology were studied after exposure to a sublethal concentration (0.065 ml/L) for 15 and 30 days. Propoxur showed significant (P<0.001) AChE inhibition of 22.77% for 15 days and 43.89% for 30 days of exposure. Kinetic studies reveal competitive inhibitory nature of propoxur. Recovery upto 69.3% was noticed after 30 days. The alterations in hepatic tissues like cellular vacuolization, hemorrhage, narrowed and disappeared wall of central vessel were observed. The impaired behavioural responses showed adverse consequences of propoxur on the fishes. The results are indicative of propoxur harmful nature to environment and subsequently to human population.

Key Words: Propoxur, Liver, *Tilapia mossambica,* Acetylcholinesterase inhibition, AChE kinetics, Histology.

Introduction

The variety of toxicants including pesticides are constantly discharged into water bodies from major sources like domestic, agricultural and industrial wastes. The accumulation of such toxicants especially synthetic pesticides yields some physiological, biochemical as well as histological detrimental effects in fresh water fauna by affecting various activities like metabolism and enzymes (Cope, 2004; Wang *et al.,* 2009). Different kind of biological responses can be used as biomarkers to assess the toxic effects of pollutants. Estimation of AChE and histopathology of fish liver is increasingly being employed as biomarkers of xenobiotic exposure (Fernandes *et al.,* 2008, Sheikh and Tembhre, 2018). Carbamates are most preferred pesticides on account of its less persistent life and effectiveness on pests and insects. Carbamates are anticholinergic agents that bind to the esteric site of the AChE. Because of the specificity of this relationship, AChE inhibition is widely used as a specific biomarker for these insecticides (Fukuto, 1990; Hualing Fu, 2018). Propoxur is a carbamate pesticide, widely used for protection of crops from variety of insects and pests. It is also used in pest control as a replacement for DDT against malaria vector. However, the use of propoxur on a large scale has caused serious environmental pollution. The primary effect of propoxur is the inhibition of the acetylcholinesterase (AChE), which causes accumulation of acetylcholine (ACh) at nerve synapses and disrupts nerve function (Fernandes *et al.,* 2015; Srivastava and Singh, 1982). Propoxur has an ecotoxicological potential on fish, a non-target organism. Propoxur have been reported to inhibit blood and brain AChE activity to 50% after oral administration of 10 mg/kg b.wt. propoxur (Yadav *et al.,* 2010). At 0.0538 ppm concentration Propoxur significantly lowered brain AChE activity in the fish *Tor tambroides* (Ahmad *et al.,* 2016). Kinetic studies on fish tissue acetylcholinesterase were performed earlier by many investigators using wide range of pesticides (Satyadevan *et al.,* 1993; Silva Filho *et al,.* 2004). The liver performs many vital functions and detoxifies the xenobiotics, so it is much affected and possesses histopathological alterations under pesticide stress (Ortiz, 2011). Singh and Srivastava (1998) reported vacuolation and heavy necrosis in the hepatic cells. However, swollen hepatocytes and necrosis of cell with granular cytoplasm, and detached hepatic cells appeared in the liver of tilapia, *Oreochromis mossambicus* due to exposure of chlorpyriphos (Kunjamma *et al.*, 2008). Study of recovery of pesticide induced AChE inhibition is important as it reveals status of pesticide-AChE complex (Tembhre *et al.,* 2006). Detailed studies of the toxic effect of propoxur on AChE of aquatic species are lacking. Therefore, the present investigation was aimed to estimate the effect of propoxur on *Tilapia mossambica* AChE inhibition and its recovery, kinetics and histopathology in liver tissues to explore the potency of propoxur exposure.

Materials and methods

Fish and Treatment

20% EC propoxur, ($C_{11}H_{15}O_3N$) 2- isopropoxyphenyl N-methyl carbamate, mol. Wt. 209.29, a light yellow liquid, was selected for the present study. Fish (*Tilapia mossambica,* length: 10±2 cm, weight: 10±2 gm) were collected from Patra fish seed farm, located in Bhopal, M.P., India and were acclimatized to the laboratory conditions for 15 days in glass aquaria. They were fed daily with palletized supplementary feed, and water was renewed daily. The physico-chemical characteristics of water were determined (APHA, 1995), and were : temperature- 26 ± 2°C, pH- 7.1 ± 0.2, dissolved oxygen- 6.8 ± 0.3 mg/L, total alkalinity- 170 ± 10 mg/L and total Hardness- 15 ± 1 mg/L . 96 h LC_{50} of propoxur was

determined (Duodoroff *et al.,* 1951), and estimated to be 0.52 ml/L. A sublethal concentration, (0.065 ml/L or 65 ppm), which was 1/8th from 96 h LC_{50} value of propoxur for *Tilapia mossambica,* was selected for 15 and 30 days exposure. Control was also maintained for the same duration. The behaviour of the fish during the experiment was observed.

Enzyme Assay

At the end of the experiment, fish were sacrificed. 5% tissue homogenate was prepared in ice-cold 0.25 M sucrose solution and centrifuged at 12,000 rpm for 7 minutes. AChE activity was measured spectrophotometrically at 540 nm by the method of Metcalf (1951), using Acetylcholine iodide (AChI) as substrate. Protein estimation was done according to Lowry *et al*. (1951) using Bovine serum albumin as standard. Kinetic constants like Km and Vmax were determined by applying Lineweaver Burk plot. The recovery was assessed at similar intervals. Graphs of results were prepared by applying Excel 2016. Data were statistically analyzed by Student's t-test.

For assessment of recovery of inhibited AChE, the test fishes were removed at the end of 15 and 30 days of exposure of 0.065ml/L of propoxur and were transferred to toxicant-free water. Fish were removed and dissected at 15 and 30 days to study recovery of inhibited AChE.

Histopathological Investigation

For assessment of histopathological changes, liver tissues were fixed in aqueous Bouin's fluid for 24 hours, washed, dehydrated in graded series of alcohol, cleared in xylene, infiltrated and embedded in paraffin wax; sections were cut at 5-6 micron thickness and stained with Haematoxylin and Eosin. Slides were photographed at 100 X and 400 X.

Results

Behavioural Changes

The treated fish showed erratic, speedy and jerky movements at lower concentration (0.065 ml/L for 48 h) and at the higher concentration (0.52ml/L for 48 h) fish exhibited hyperactivity, violent behaviour and jumping out of the tanks violently (escape behaviour). Prolonged exposure i.e. 15 and 30 days, the fish became hypoactive, struggled for breathing, restricted swimming movements, finally led to lethargic condition and loss of equilibrium. Normal behaviour was observed during the recovery period of the experiment.

AChE Activity and Inhibition

Acetylcholinesterase activity in the liver tissue was monitored after exposure of 0.065ml/L of propoxur for 15 and 30 days. As shown in Table-1 & Fig.-1 a significant decrease of AChE activity was observed in all tested samples after exposure of 0.065ml/L of propoxur in the liver of *T. mossambica*, and this reduction was time-dependent. The liver AChE activity of *T. mossambica* decreased significantly by 22.7% (15 days) and 43.89% (30 days) as compared to the control. The AChE activity was declined to 1.39±0.15 (15 days) and to 1.01±0.20 (30 days) against the control which was 1.80±0.22 (micro moles of AChI hydrolysed/mg protein/hour) at 0.065ml/L of propoxur (Table-1).

Recovery of Inhibited AChE

The recovery in AChE activity in the liver after exposure to 0.065ml/L of propoxur for 15 and 30 days was encouraging in the present study. AChE did not recover significantly after 15 days, which could restore to 7.9 %. However a long duration of 30 days in toxicant free water inhibited AChE recovered to almost 69%.

AChE Kinetics

The Km for AChE in control liver was observed as 1.05± 0.25×10^{-3}M. After propoxur treatment the Km increased to $2.70\pm0.17\times10^{-3}$M for 15 days and $4.30\pm0.98\times10^{-3}$M for 30 days of exposure. The Vmax for propoxur treated and control liver AChE was constant and it was measured to 1.2 Absorbance/mg protein/30 min. (Fig.-1 & Table-1).

Table: 1. AChE specific activity, % inhibition, ACh content, % increase in ACh content, Km and Vmax of the liver of *T. mossambica* treated with 65 ppm of propoxur for 15 and 30 days. Values are mean +/- S.D. of five individual observations. The AChE specific activity is expressed in µ moles of AChI hydrolyzed / mg protein /hour. Vmax expressed in A/mg protein/ 30 min (P values *P<0.05,**P<0.02,***P<0.001).

Parameters	Control	Treatment with 65 ppm of Propoxur		Recovery of inhibited AChE	
		15-day exposure	30-day exposure	15- day recovery	30 –day Recovery
AChE Specific activity	1.80±0.14	1.39**±0.15	1.o1***±0.20	1.50**±0.01	1.71***±0.01
AChE % inhibition	0	-22.77	-43.89	-16.66	-5.0
ACh contents	20.20±0.98	26.40***±0.04	29.80***±0.50	23.2**±0.02	20.40***±0.01
% increase in ACh	0	+30.39	+47.52	+14.85	+1.01
$Km_{X\ 10^{-3}}$ M	1.05±0.25	2.70***±0.17	4.30***±0.98	-	-
Vmax	1.2	1.2	1.2	-	-
% Recovery	-	-	-	7.91	69.30

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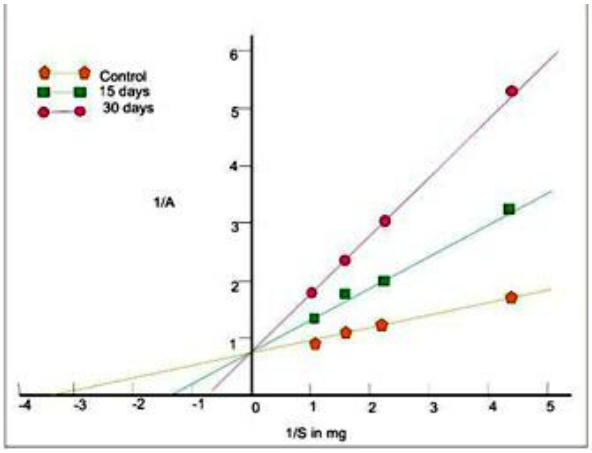
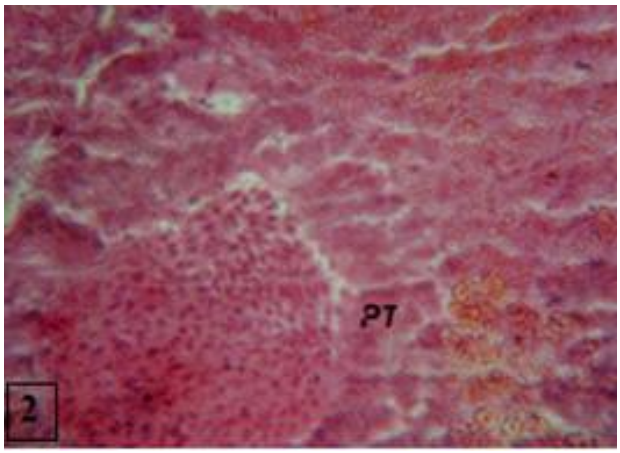
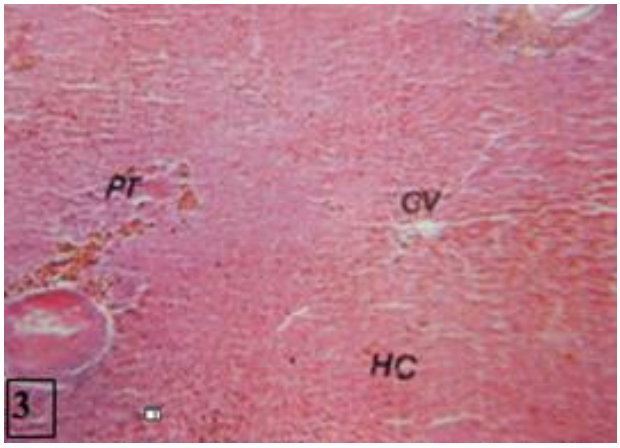


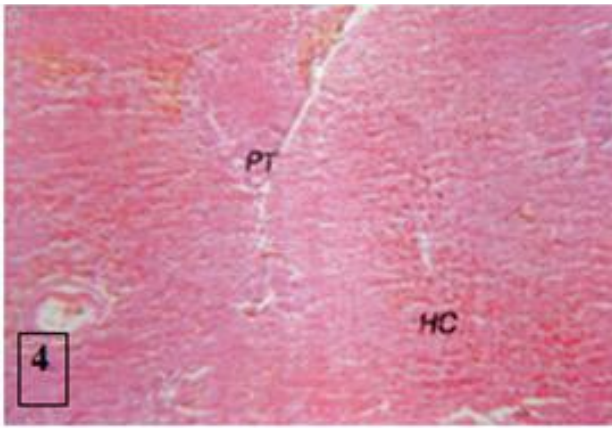
Fig. : 1 Lineweaver-Burk plot of inhibitory effect of 65 ppm propoxur on AChE of liver of *T. mossambica* treated for 15 and 30 days. S is the concentration of AChI. Each point is the mean (N=5).

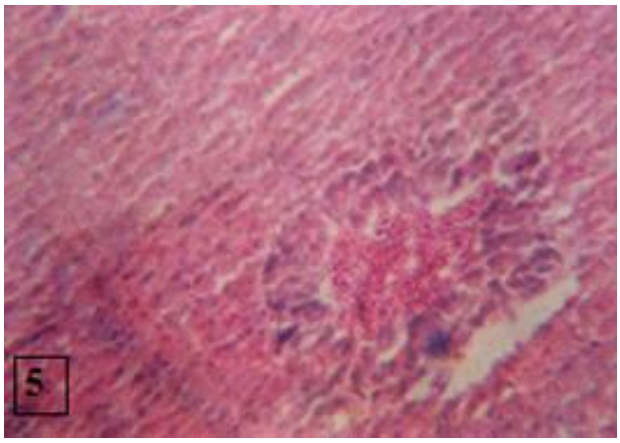
Histological Alterations in Liver

A histopathological examination was carried out to determine the extent of hepatotoxicity as a consequence of propoxur treatment in liver tissue. As illustrated in **Fig.** 2, 3 & 4, the liver in the control group presented normal features and showed hexagonal hepatic cells within the network of bile canaliculi. Portal vein covered with pancreatic acini known as hepatopancreas was noticed. Hepatocytes surrounding the central vessels like rosette pattern and in lumen there were erythrocytes.









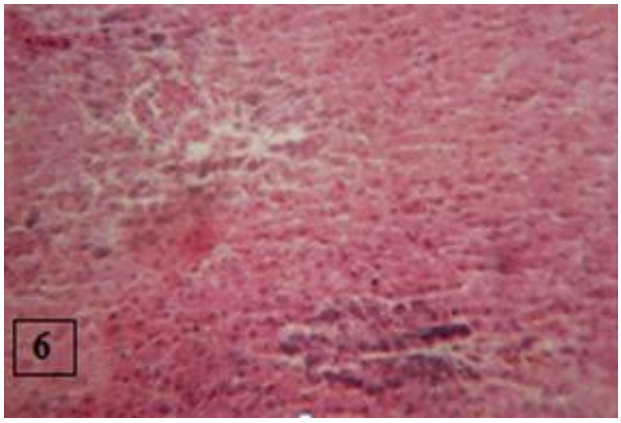


Fig.2 : Photomicrograph of transverse section of the liver of *T. mossambica* Fig. 2: control showing diffused pancreatic tissue; Fig. 3: control hepatic cords and diffused pancreatic tissue; Fig. 4: control showing arrangement of hepatic cells. Fig. 5: Exposure to 65 ppm propoxur for 15 days showing, vacuolization, necrotic patches, extrusion of blood cells from the blood vessels, reduced pancreatic tissue and large space around pancreatic tissue. Fig. 6: Exposure to 65 ppm propoxur for 30 days showing large space around the pancreatic tissue and shrinkage in pancreatic tissue.

Histopathological changes in the liver of fish intoxicated with 65ppm of propoxur for 15 days were conspicuous and included considerable degradation in cellular structure viz. vacuolization, ruptured blood capillaries and hemorrhage, narrowed and disappeared wall of central vessel were observed. Severe necrotic patches, lesions, nucleus enlargement, hepatic cords disarray, complete destruction of hepatic cell membrane, cell proliferation, congestion and sinusoidal spaces were also observed in liver of fish (Fig. 5).

However, exposure to 65ppm of propoxur for 30 days, liver showed karyolysis, necrosis, severe hepatic cords disarray, parenchymal vacuolization, severe irregular shaped cells with irregular nucleus, necrotic patches, ruptured sinusoids and increasing sinusoidal spaces (Fig.6). These results of HE staining in liver tissues suggested that the exposure of propoxur induced hepatic injury.

Discussion

Propoxur is a carbamate and is known to cause neurotoxicity; the exact mechanism by which this effect is mediated is still unknown. The major biological role of this enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine.

The alteration in behaviour has been considered to be related to toxic effects of surroundings. When AChE activity decreases, ACh is unable to break and build up within synapses and cannot function in a normal way (Dutta & Arends, 2003). In the current study we observed some alterations in behaviour of test fishes which include hyperactivity, violent behaviour at short exposure to propoxur, while at long exposure fish showed slow breathing and swimming movements and lethargic condition with loss of equilibrium. The altered locomotor behaviour of fish could be attributed to the accumulation of acetylcholine which interrupted coordination between the nervous and muscular junctions (Rao *et al.,* 2005; Rao, 2006).

The inhibition of AChE leads to excessive ACh accumulation at the synapses and neuromuscular junctions, resulting in overstimulation of ACh receptors (Gupta, 1994). In the present study the liver AChE activity of *T. mossambica* decreased significantly by 22.7% (15 days) and 43.89% (30 days) at 0.065mL/L of propoxur (Table1) as compared to the control. Similarly, AChE inhibition in various tissues of the fish has been reported by various workers (Kumar & Chapman, 2001; Rao *et al*., 2003; Joseph & Raj, 2011). The higher the exposure time, the greater is the negative impact. The inhibition observed in the activity of AChE, is in agreement with the findings of other workers (Das & Mukherjee, 2003; Rao, 2006; Crestani *et al.*, 2007; Joseph & Raj, 2011). The average hepatic activity in *Tilapia mossambica* reared in treated sewage water was significantly lowered (26.6% $P < 0.01$) than that found in control (Saif and Ghais, 2013).

Findings of present study reveal that inhibited AChE in liver of *Tilapia mossambica* recover slowly and takes about a month time to restore gradually in toxicant free water (Table-1). This is in concurrence with the findings of Rath & Mishra (1981) and Oruc (2012). The present results as well as previous findings (Sancho *et al.,* 1997; Oruc & Usta, 2007; Patil & David, 2009) reflected the affinity of propoxur for fish AChE. AChE recovery was reported also in fishes exposed to pesticides (Dembele *et al*., 1999; Dutta and Arends, 2003; Rao, 2004; Rao, 2008; Oruc, 2012). Bashamoiddin and Shailbala (1989) observed significant recovery (90-97%) recovery in AChE after 20 days exposure to malathion in the liver of *Cyprinus carpio*. However, Chebbi and David (2009) recorded recovery of inhibited AChE due to quinalphos in a short period of seven days in the liver of *Cyprinus carpio.*

In the current investigation the Km in the liver increased from control fishes to $2.70\pm0.17\times10^{-3}$M at 15 days and $4.30\pm0.98\times10^{-3}$M at 30 days of exposure to propoxur. The Vmax was unaffected with the propoxur and calculated 1.2 A/mg protein/30 min (Table 1). The kinetic constant Km indicates affinity of enzyme and substrate. Large Km indicates less enzyme activity while small Large value denotes higher enzyme activity. The kinetic inhibition activity of propoxur on AChE in the liver of fish has not been reported, therefore this work is first to demonstrate its competitive inhibitory nature to AChE.

Liver plays a key role in storing, metabolism and biochemical transformation of pollutants (Veeraiah, 2001). Liver is mostly associated with detoxification due to its function, position and blood supply and it is most affected organ by toxicants (Camargo and Martinez, 2007). In present investigation at the end of 15 days exposure the cellular degeneration was started with sequence of events like vacuolization, lesions, necrosis, congestion and pyknosis in liver. The sublethal toxicological potential of propoxur causes passive hyperemia, albumin, and hydropic degeneration in the liver of *Cyprinus carpio* L (Gul *et al.,* 2012). The glyphosate exposure develops vacuolation in cytoplasm, large vacuoles and pyknotic nuclei in many areas in liver of Nile tilapia *O. niloticus* (Jiraugkoorskul *et al.,* 2002). These alterations might indicate imbalance between synthesis of biochemical substances and their release (Jiraugkoorskul *et al.,* 2002). Histopathological changes in the liver of *Cyprinus carpio* exposed to dimethoate were conspicuous and included disruption of regular arrangement of hepatocytes, congestion and rupture of vein, extensive hemorrhage, cytoplasmic vacuolization, indistinct cellular boundaries, pyknotic nuclei, nuclear degeneration and necrosis (Singh, 2013). Most of the alterations like lipid infiltration, ruptured blood vessels, blood cells infiltration and disappearance of wall of central vessel as well as severe necrotic patches, karyolysis, hepatic cords disarray and hypertrophy were observed in present study, which are similar to those reported in earlier studies on different fishes exposed to different toxicants. The severe necrosis might be due to inability of fish to regenerate new liver cells (Abubakar *et al.,* 2014). From the results obtained in the present study and that reported by earlier investigators, it is suggested that AChE activity in the liver affected adversely due to exposure to sublethal concentration of propoxur for a prolonged period of 30 days to *T. mossambica.*

Conclusion

Present work showed that propoxur is strongly hepatotoxic and severely affects AChE activity, kinetics and histology of fish liver. Thus it is concluded that liver histology and biochemical parameters can be used as rapid and sensitive biomarkers of pesticide stress in fish.

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