Haematological and histopathological alteration on ***Cyprinus carpio*** (Hamilton) after exposure to organophosphate pesticide Thimet (10%CG)

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Abstract

The present study contemplated evaluating and assessing the effects of Phorate (Thimet 10%CG) on common carp (*Cyprinus carpio* L.) from the results of acute toxicity tests and haematological, and histological examinations. Carp fishes were exposed to the pesticide preparation for different time intervals for acute toxicity tests. The results of the test and the examinations of exposed carp were compared with the results of carp from the control group. The exposure of common carp to phorate caused significant haematological and biochemical profile changes and histopathological changes in organ tissues. The organophosphate-based pesticide phorate (Thimet 10%CG) is toxic to fish.

Keywords: Cyprinus carpio Thimet 10%CG pesticide haematology histopathology.

Introduction:

Pesticides are extensively used globally in agriculture, forestry, public health, and veterinary practices to control insects, pests, and disease-causing vectors. Indiscriminate use of pesticides leads to their accumulation in the soils leading to environmental pollution and finally, they ultimately find their way into aquatic habitats such as rivers, lakes, and ponds. The majority of pesticides are made up of organophosphates, which are highly toxic to fish and other aquatic species. Hence, it is necessary to study the immediate and chronic effects of pesticides on fish, which form a part of the food web and human diet (Van der Oost, R *et al*., 2003). Many of the organophosphates were developed initially as insecticides. They are commercially available as water-soluble liquids or granules, have low volatility, can be applied before or after planting and are effective against nematodes. A few examples of organophosphate insecticides include phorate (Thimet), disulfoton (Disyston), ethoprop (Mocap), fensulfothion (Dasanit), fenamiphos (Nemacur), isazofos (Triumph 4E), Terbufos (Counter), and a few others (Zhang Z.Y *et al*., 2010).Phorate is a phosphorodithioate insecticide with the chemical name O, O-diethyl Sethyl thiol methyl phosphorodithioate, CAS No. 298-02-2. Phorate is commonly used in agriculture as soil and systemic insecticide and sold commercially under several names, the most common of which is Thimet (Wayne, NJ, 1987).The present study is contemplated to evaluate the phorate toxic effects on *Cyprinus carpio* (Hamilton) which is commonly called the Eurasian or European carp and previously known as the common carp. This fish is a freshwater fish of eutrophic waters and is present in lakes and large rivers in Europe and Asia. They naturally live in temperate climates in fresh or slightly brackish water with a pH range of 6.5-9.0 and salinity up to about 0.5% (Balon Eugene K 1974) and temperatures of 3 to 35°C. The best temperature is 23 to 30°C, with spawning beginning at 17 to 18°C. Carp can tolerate water with very low oxygen levels, by slugging air at the surface. The carp has a robust build, with a dark gold sheen most prominent on its head. Common carp can grow to very large sizes if given adequate space and nutrients. They can grow to a maximum length of 120 centimetres, a maximum weight of over 40 kilograms and an oldest recorded age of 38 years.We have evaluated the haematological and histopathological alteration of *Cyprinus carpio*. Histopathological changes have been widely used as biomarkers in the evaluation of the health of fish exposed to contaminants, both in laboratory and field studies. Changes in tissues of different organs of fish have been widely used as biomarkers in the evaluation of the quality of fish exposed to contaminants both in the laboratory (Thophon SM *et al*., 2003) and in field studies (Teh SJ *et al*., 1997). Tissue histology is considered an indicator of toxicant exposure and is a useful tool to assess the degree of pollution regarding lethal and sublethal effects (Cengiz, EI. and Unlu, E. (2006). These investigations have been proved to be a sensitive tool to find the direct effects of pesticides or herbicides on the target organisms of fish in laboratory experiments (Sark SA *et al.*, 2005). Different concentrations of insecticides are present in water bodies and are found to be toxic to aquatic organisms, especially fish. Using of histopathological biomarkers for environmental monitoring is one of the great advantages of water pollution detection (Thompson, H.M. and Walker, C.H. (1994).

Materials & Methods:

Test Species: The present investigation was conducted on test species a freshwater fish with the following classification:

Phylum	Chordata
Super Class	Pisces
Class	Osteichthyes
Super class	Actinopterygii
Order	Cypriniformes
Family	Cyprinidae
Superfamily	Cyprininae
Genus	Cyprinus
Species	carpio

Table: 1. the scientific classification of *Cyprinus carpio*

Specimen Acclimatization:
The healthy freshwater fish *Cyprinus carpio* (length, 8.2 ± 0.8 cm; weight, 5.9 ± 0.6 g) fingerlings were collected from a private fish farm at Buddam village in Bapatla, Mandal, Guntur District of Andhra Pradesh, India. The fish were maintained in large circular plastic tubs with reconstituted water for 10-15 days under standard laboratory conditions for acclimatization. The water was constantly aerated with oxygen in a static system. The fish were fed with rice bran and commercial fish pellets once a day after cleaning the faecal matter and other waste materials from the tub to avoid the accumulation of ammonia and methane gas.

Acute Exposure Bioassay:
A common stock solution of Thimet (10%CG) was prepared by dissolving 1 gram (1000 mg) of pesticide in 100 mL of acetone and the required quantity of Thimet (10%CG) was drawn from the stock solution to maintain the standard concentration of 1 mg/L in the container. The acclimatized fish were placed into a separate container containing dechlorinated and aerated water. The Pilot experiments were conducted to derive the LC50 values of 24 h, 48 h, 72 h, and 96 h for Thimet (10%CG). During the entire experiment, a control group was maintained with acetone for comparison. The median lethal concentration and time with their upper and lower confident limits and percentage of mortality were calculated by following Finney's probit analysis method (Finney DJ, 1971). The behavioural alterations in intoxicant exposed fish were observed during the acute toxicity of Thimet (10%CG).

Haematological Studies:
The fish specimens were anaesthetized with methanesulfonate. 1 mL of blood was obtained by caudal vein puncture and placed in glass tubes containing EDTA. Plasma was obtained by centrifugation of blood at 3000rpm for 15 min and non-hemolyzed plasma was stored in a deep freezer for further biochemical analyses. The RBC, WBC, and haemoglobin were determined using a Neubauer hemocytometer. The RBC present inside the five small squares was counted under a 40X lens of the light microscope. The following formula was used to calculate the number of RBC per mm3 (µL) of the blood sample:

$$\text{Number of RBC/mm3} = (N \times \text{dilution}) / \text{area counted} \times \text{depth of fluid.}$$

The WBC present inside the four large squares was counted under a 40X lens of the light microscope. The following formula was used to calculate the number of WBC per mm3/(µL) of the blood sample:
$$\text{WBC} = (N \times \text{dilution}) / \text{area counted} \times \text{depth of fluid.}$$

A thin smear of blood sample was prepared and stained with Giemsa stain. Haemoglobin concentration was determined by the cyanmethemoglobin procedure. To determine PCV/ HCT, fresh blood samples were collected into heparinized microhematocrit tubes sealed with plasticine at one end and centrifuged for 5 min at 3000RPM. The mean values of PCV (%) were measured with a microhematocrit reader. Plasma glucose was determined using assay kits as per the suppliers' instructions. Total protein (TP) content was determined according to the method of Magar, RS and Afsar Shaikh (2012). Total lipid (TL) content was determined calorimetrically according to Joseph A *et al*., (1972). The activity levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined calorimetrically according to Reitman S and Frankel S (1957). The results were represented in table-1.

Histopathological Study:
The healthy freshwater fish *Cyprinus carpio* (length, 8.3 ± 0.6 cm; weight, 5.9 ± 0.6 g) fingerlings were acclimatized to standard laboratory conditions for 10 to 15 days. All the care and precautions were taken according to the standards of APHA (2005) for maintaining the fish. The fish were exposed to Thimet (10%CG) for 1-day lethal, 1-day sub-lethal, 5-day sub-lethal, and 10 days sub-lethal (1/10th of 96 h LC50) concentrations. At the end of the exposure, fish were randomly selected and were subjected to necropsy for histopathological examination.
The gill, liver, kidney, and brain tissues were isolated from control fish (not exposed to the toxicant) and experimental fish. Initially, physiological saline solution (0.75% NaCl) was used to rinse and clean tissue. Then, the tissues were fixed in aqueous Bruin's solution for 48 h, processed through a series of different per cent alcohols, and embedded in paraffin wax. Sections were cut into 4-6 mm thickness, stained with Haematoxylin & Eosin stain, and were mounted in Canada Balsam. Histopathological sections were examined Begum G 2004).

Statistical analysis:

The results were expressed as mean (X) ± standard deviation. The data was analyzed using 'Graph pad instant' (Data set 1, SD) software, and a student t-test was conducted for pair-wise comparisons to determine the significant difference at a 95% level of confidence. For all the tests, values of results with $p < 0.05$ were considered to be of statistical significance.
The graphs were drawn using the software 'Origin 6.0'.

Results and Discussion:
In the present study, different period exposure and toxic effects of Phoret (Thimet 10%CG) on haematological and histopathological aspects of *Cyprinus carpio* were evaluated using standard protocols and experiments. The morphological study of RBC reveals the abnormalities are more in lethal exposure of Phorate. Double monopodial projections (DLP) and irregular cells were found in 5 days of sub-lethal exposures to Phorate. Erythrocytes were found to be swollen and spherical (SS) in lethal concentrations exposures of 10 days. The spherical erythrocytes may be referred to as "spherocytes". The number of such spherical erythrocytes increased significantly in lethal exposure. The swollen, oblong, and shrieked erythrocytes were seen at 24 hrs sub-lethal exposure, which increased significantly upon exposure to 24 hrs lethal concentration of phorate. The impact of Phorate was so much more deleterious at the lethal concentration than that of sub-lethal exposure. The results of hematological studies were represented in Table-1 and image-1, and graph-1.Table: 1. Hematological Changes after exposure of Phorate for lethal, 1 day, 5 days, and 10 days.

Parameter	**Control** Mean ± SD	**Lethal** Mean ± SD	**% Change**	**1 Day** Mean ± SD	**% Change**	**5 Days** Mean ± SD	**% Change**	**10 Days** Mean ± SD	**% Change**
RBC Count (10^6/mm^3)	1.82±0.01	1.27±0.01	30.2	1.76±0.008	3.2	1.64±0.008	9.8	1.44±0.01	20.8
WBC Count (10^3/mm^3)	17.51±0.008	13.18±0.01	24.7	16.51±0.008	5.7	18.70±0.005	6.8	5.61±0.09	67.9
HB (gm/100ml)	8.1+±0.010	5.17±0.01	36.9	8.05±0.08	1.7	6.11±0.008	25.3	20.14±01	-11.95
PCV (%)	30.11±0.007	25.1±0.06	16.4	29.5±0.37	2.0	27.73±0.008	7.9	24.18±0.01	19.6
MCV (fL)	168.15±0.009	192.22±0.01	24.7	166.11±0.008	2.04	170.11±0.007	1.96	172.25±0.02	3.9
MCH (pg)	46.60±0.01	40.25±0.014	13.6	44.92±0.013	1.68	36.27±0.008	22.1	38.47±0.01	8.13
MCHC (%)	28.16±0.009	21.11±0.01	25.03	27.47±0.01	2.4	22.18±0.008	21.2	23.7±0.01	15.8
Glucose(mg/l)	51.18±0.01	63.32±0.012	23.7	53.5±0.007	2.32	57.80±0.008	12.9	65.7±0.90	14.5
TP(g/100ml)	2.181±0.01	1.72±0.01	38.7	2.58±0.008	7.18	2.12±0.007	24.5	1.85±0.01	34.1
TL(g/l)	12.49±0.38	17.13±0.015	37.1	13.5±0.008	8.0	15.26±0.318	22.1	16.24±0.02	30.0
AST(IU/l)	80.15±0.009	130.18±0.01	62.4	92.32±0.009	15.1	120.31±0.45	50.1	127.11±0.01	58.5
ALT(IU/l)	30.26±0.31	58.11±0.009	92.0	34.20±0.008	13.0	45.34±0.006	49.8	52.17±0.01	72.4

SD=Standard Deviation, fL = femtoliters, IU= International Unit, values are significant at $P>0.05$.

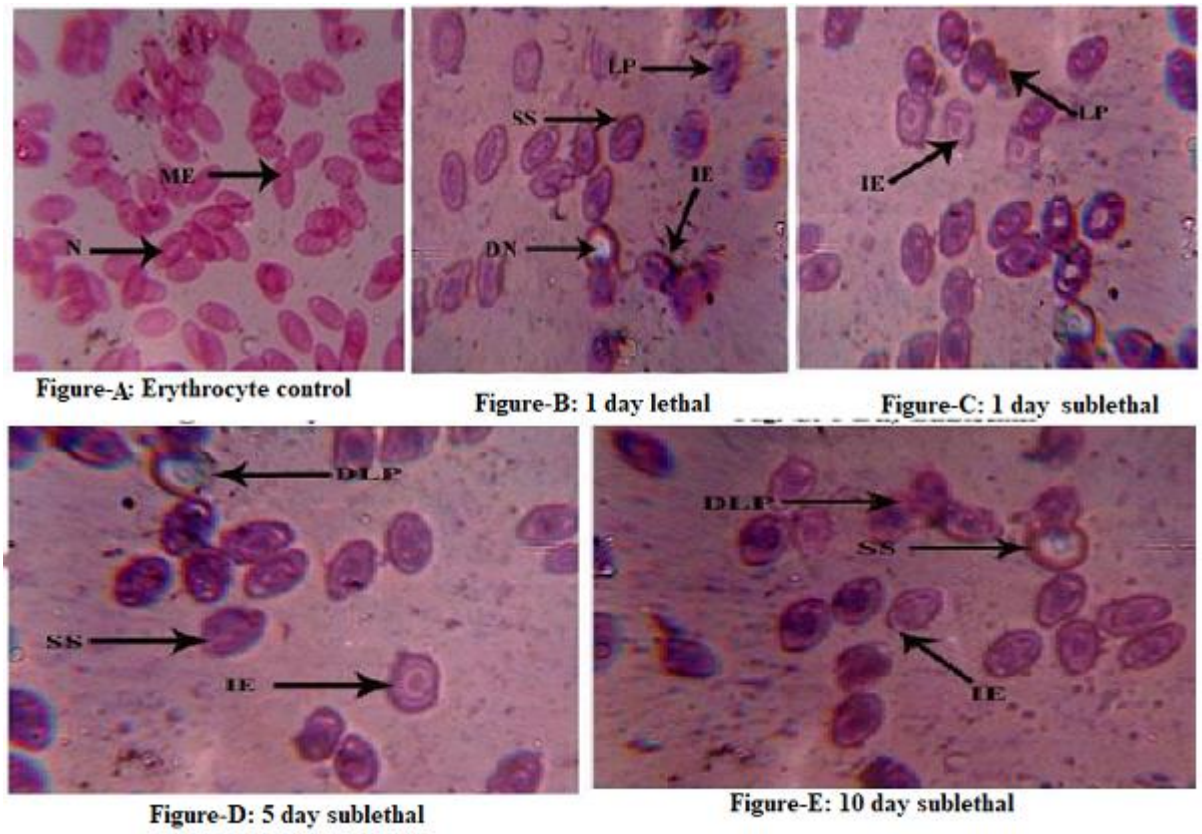


Figure-A: Erythrocyte control

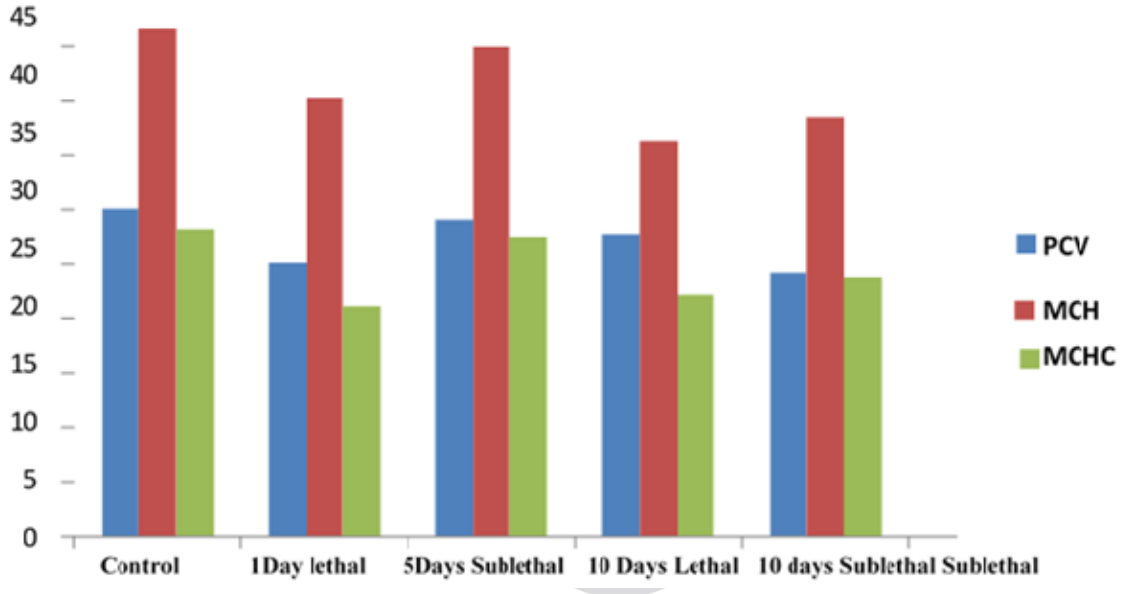
Figure-B: 1 day lethal

Figure-C: 1 day sublethal

Figure-D: 5 day sublethal

Figure-E: 10 day sublethal

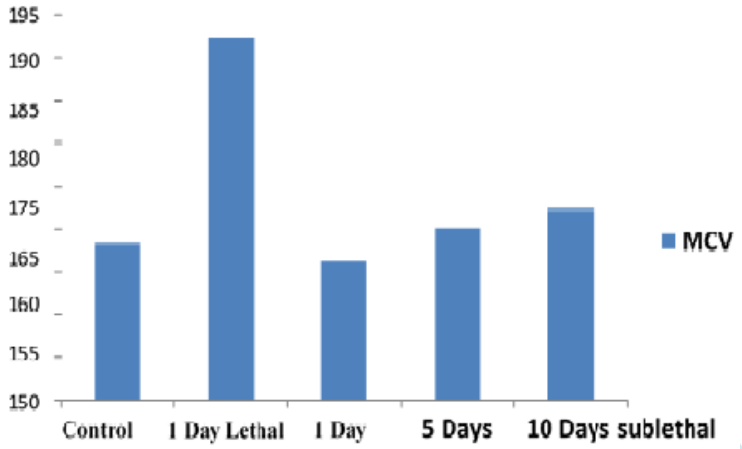
Image-1. Changes in Packed cell volume (%), Mean corpuscular haemoglobin (gm %) and Mean cell haemoglobin (gm %) in the fish exposed to lethal, 1-day Sublethal, 5-day Sublethal and 10 days Sublethal Phorate.



Graph-1: Changes in Mean corpuscular (μm^3) in the fish exposed to Phorate at 1 day lethal, 5 days Sublethal, 10 days lethal, and Sublethal concentrations.

Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) are major indicators of toxicant effects in haematological studies. In the present study, the PCV levels significantly decreased in lethal, 1 day, 5 days, 10 days lethal, and sublethal after exposure to the pesticide. The maximum level of decrease meant observed in 10 days of sublethal and the minimum level of change was observed in 1-day sublethal exposures. The MCV levels increased in lethal, 5 days and 10 days after pesticide exposures but decreased at 1stday. The maximum increment was noted in a lethal and minimum level of increase observed in 5 days sublethal. MCH levels were decreased in lethal, 1 day, 5 days, and 10 days after espouser periods. The maximum level of decrement observed in 5 days sublethal minimum observed in 1 day sublethal. MCHC levels were decreased in lethal, 1 day, 5 days, and 10 days after exposure periods.

The maximum level of decrease observed in 5 days sublethal minimum observed in 1 day sublethal. The results of MCV were represented in graph-2.



Graph-2: MCV results for different days for lethal and sublethal concentration exposure

Histopathological studies:

(a) Gill: Phorate 10% CG exposures have induced marked physiological changes in fish gill architecture of *Cyprinus carpio*. Exposure of a fish for 1 day, 5 days, and 10 days to sublethal and 1 day to lethal concentrations of Phorate showed significant physiological changes in the gill of a fish. The changes include epithelial cell rupture (ECR) with epithelial lifting (EL). The histopathological stained slides were represented in image-2.

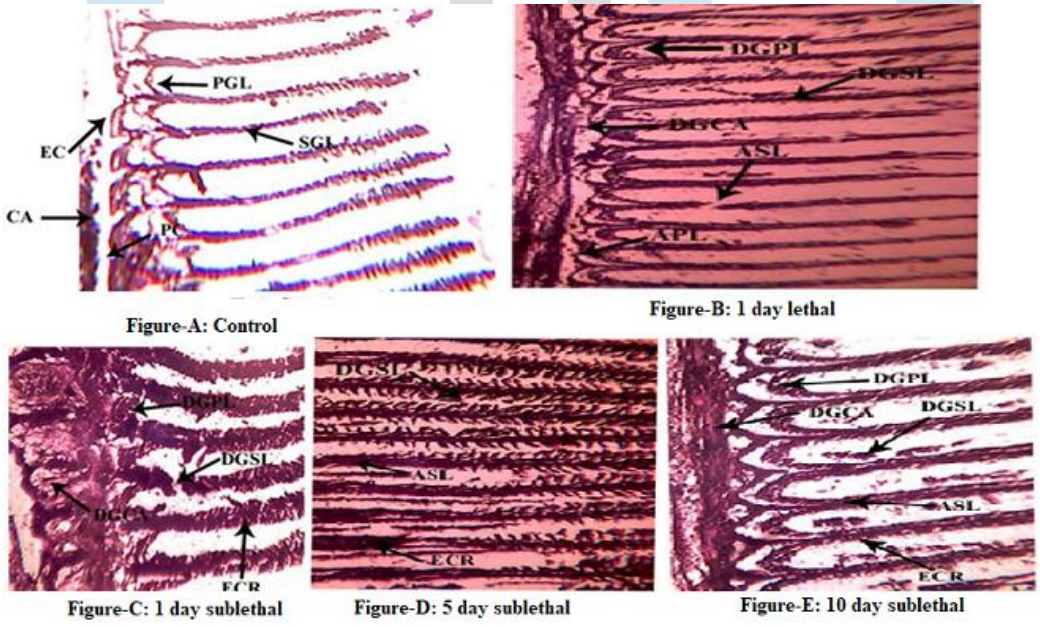


Figure-A: Control

Figure-B: 1 day lethal

Figure-C: 1 day sublethal

Figure-D: 5 day sublethal

Figure-E: 10 day sublethal

Image-2: Gill histopathological staining images. Figure A corresponds to control, B corresponds to 1 day lethal, C corresponds to 1 day sublethal, D corresponds to 5 days sublethal and E corresponds to 10 days sublethal concentration exposure of fish *Cyprinus carpio* to phorate (Thimet 10%CG).

The labelling on plates is as follows: PGL-Primary gill lamella, SGL-Secondary gill lamella, PC-Pillar cell, EC-Erythrocyte, DGSL-Degenerated secondary lamella, DGCA-Degenerated central axis, DGPL-Degenerated primary lamella ASL-Atrophy of the secondary lamella, APL-Atrophy of the primary lamella, ECR-Epithelial cell rupture.

(b) Liver:

Phorate (Thimet 10% CG) exposures have induced marked physiological changes in liver tissue of fish *Cyprinus carpio*. Exposure of fish for 1 day, 5 days sublethal, 10 days sublethal and 1 day to lethal concentrations of Phorate showed significant physiological changes in the structure of the liver. These changes include degeneration of hepatopancreatic tissue (DGHP), presence of blood cells among hepatocytes (BCH), the appearance of blood streaks among hepatocytes, formation of vacuoles, atrophy, necrosis and disappearance of hepatocytic cell wall and disposition of hepatic cords (Image-3, figure B, C, D and E). More degenerative changes were observed in lethal exposures.

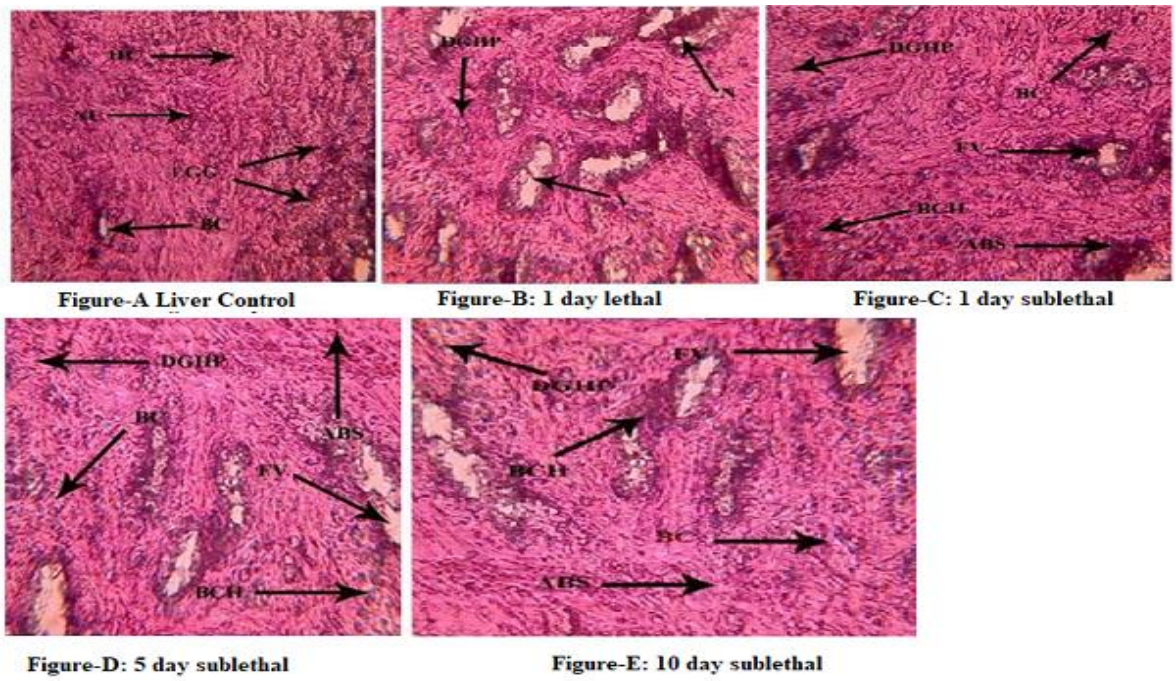


Figure-A Liver Control | Figure-B: 1 day lethal | Figure-C: 1 day sublethal

Figure-D: 5 day sublethal | Figure-E: 10 day sublethal

Image-3: Liver histopathology slides. Figure A corresponds to the normal control slide, Figure B corresponds to 1-day lethal concentration exposure, C represents the1 day sublethal concentration exposure, D illustrates the 5 days sublethal and E represents the 10 10-day lethal concentration exposure of fish *Cyprinus carpio* to Phorate

(c) Kidney:

Phorate 10% CG exposures have induced some common pathological changes in kidney tissue of fish *Cyprinus carpio*. The pathological changes include degeneration of hemopoietic tissue (DGHTC) which caused shrinkage of the glomerulus (SG), formation of the vacuole (FV), hypertrophied cells (HTC) and hypertrophy of distal convoluted segment (HDCS). At higher and lethal concentrations severe necrosis and cloudy swelling in renal tubules and glomerular cytoplasm were observed (Image-4, Fig. B, C, D and E). In fish, the waste products are eliminated from the body through the kidney. The non-detoxified pesticide molecules must be eliminated through the kidney of fish and hence it is susceptible to toxicants during sublethal and lethal exposures. Phorate accumulation in the kidney might have caused degenerative changes in the renal tubule and glomerulus.

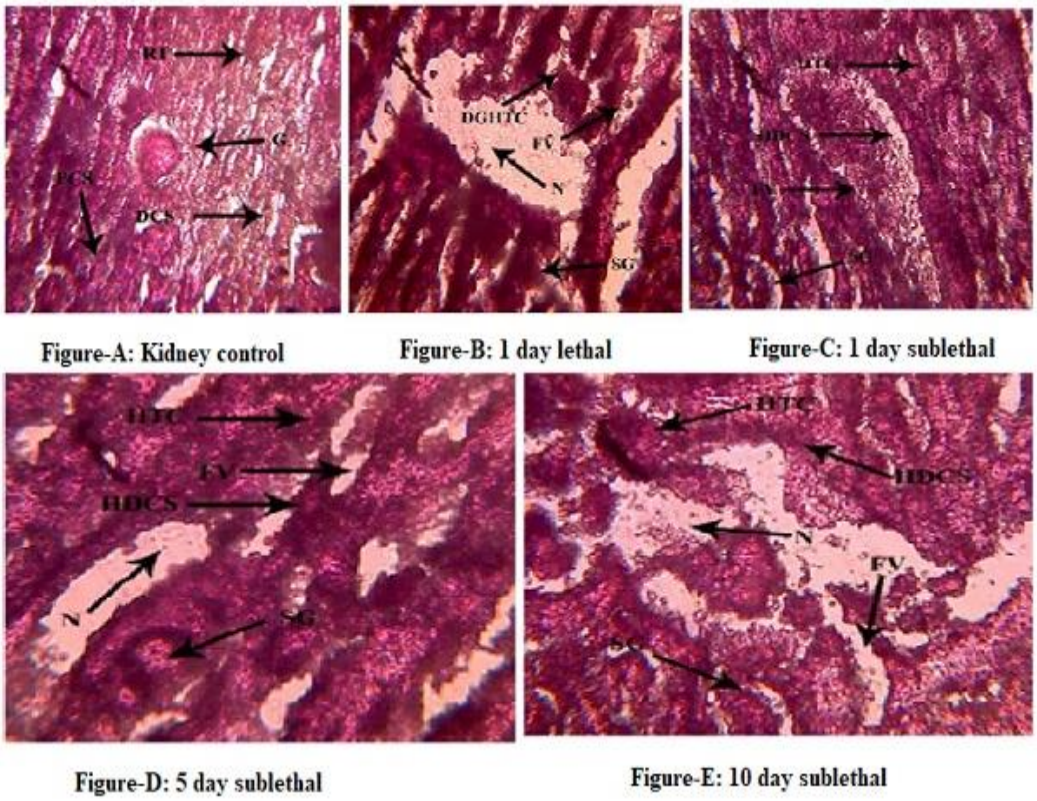


Figure-A: Kidney control | Figure-B: 1 day lethal | Figure-C: 1 day sublethal

Figure-D: 5 day sublethal | Figure-E: 10 day sublethal

Image-4: Kidney histopathology slides. Figure A corresponds to the normal control slide, Figure B corresponds to 1-day lethal concentration exposure, C represents the1 day sublethal concentration exposure, D illustrates the 5 days sublethal and E represents

the 10 days sublethal concentration exposure of fish *Cyprinus carpio* to Phorate.

(d) Brain: Phorate 10% CG exposures have induced some common pathological changes in brain tissue of fish *Cyprinus carpio*. These changes include degenerated granular layer (DGL), rupture of Purkinje cells (RPC), the appearance of blood streaks (BS), formation of the vacuole (FV) and necrosis (N) of brain tissue (Image-5, Fig. B, C, D and E). Since Phorate is neuro poison the toxicant exposure caused atrophy of brain tissue along with rupture of Purkinje cells. Congestion in the hindbrain or medulla oblongata causes abnormalities in the blood circulation. The extent of damage to the brain in the present study was more in 1day lethal and 10-day sublethal concentrations than in 1 day sublethal concentrations of Phorate. The damage to the brain tissue may alter the physiological and behavioural functions of fish.

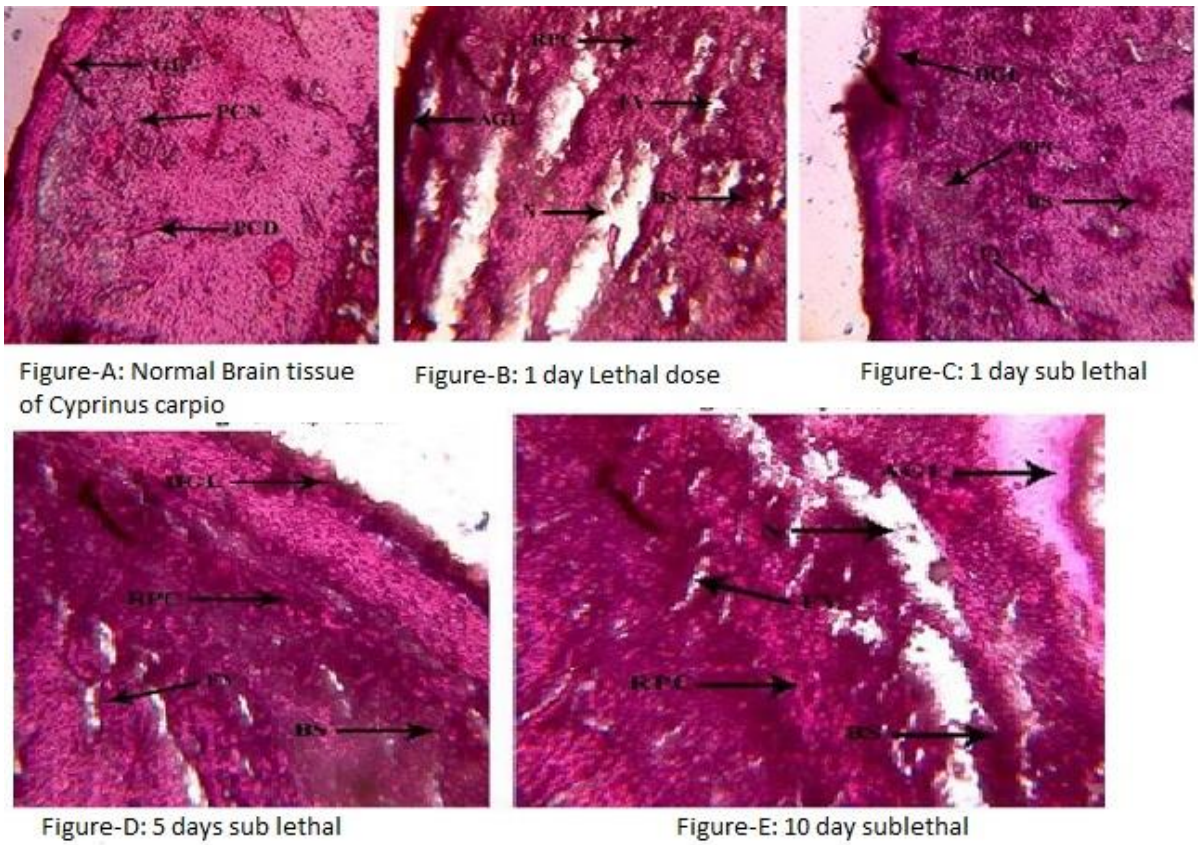


Figure-A: Normal Brain tissue of Cyprinus carpio

Figure-B: 1 day Lethal dose

Figure-C: 1 day sub lethal

Figure-D: 5 days sub lethal

Figure-E: 10 day sublethal

Image-5: Histopathological slide images of *Cyprinus carpio* fish exposed to Phorate (Thimet 10%CG). Figure A corresponds to Control (Norma brain tissue), B corresponds to exposure to a 1-day lethal dose, C corresponds to a 1-day sub-lethal dose, D corresponds to a 5-day sublethal dose and E represents the 10-day sublethal dose exposure.

DISCUSSIONS:

The toxicity of any pollutant is either acute or chronic. The chronic studies include both histochemistry and pathology. Although toxicant impairs the metabolic and physiological activities of the organisms, such studies alone do not satisfy the complete understanding of pathological conditions of tissue under toxic stress. The extent of severity of tissue damage is a consequence of the concentration of the toxicant and is time-dependent. Hence, it is useful to have an insight into the histological analysis. Also, the severity of the damage depends on the toxic potentiality of a particular compound or pesticide accumulated in the tissue.

Conflict of Interest: The authors declare a conflict of interest as none.

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