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Endosulfan induced ultrastructural and biochemical alterations in liver of freshwater catfish, *Clarias batrachus*

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Abstract: *The present study was undertaken to investigate the toxic effect of endosulfan (brand 'Endocel' EC 35%) on few liver function test profile and ultrastructure of liver cell in freshwater catfish, Clarias batrachus. Fish were exposed to the two concentrations of pesticide (0.004 and 0.008 ppm) for 30 days. Biochemical analysis of serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) along with histopathological examination of liver tissues under transmission electron microscope (TEM) were done on 7, 15 and 30 days of exposure. The results showed significant (P<0.05) elevation in SGPT and SGOT during the different days of exposure. The major ultrastructural anomalies incurred in liver tissues due to endosulfan exposure were dilation of nuclear membrane, congregation of heterochromatin at the peripheral zone of inner nuclear membrane, dilated and broken array of cisternae of rough endoplasmic reticulum, increased vacuolation in hepatocytes etc. The extent of biochemical and histopathological anomalies were found to be duration -and dose-dependent. The result of present study demonstrates the toxic effect of endosulfan in fish at environmentally relevant concentration.*

Key Words : Endosulfan, SGPT, SGOT, Liver, TEM, Clarias batrachus.

Introduction

The intensive use of pesticides has adverse effect on the delicate ecosystem-including biodiversity. Fish are sensitive bioindicator species playing significant role in monitoring water pollution. In aquatic organism, the xenobiotics percolate up to the cellular level through cell membrane and interact with cellular macromolecules to inhibit the essential cellular metabolism (Siroka and Drastichova, 2004). Xenobiotics bind to specific receptors on the cell surface or inside the cell either in cytoplasm, nucleus and other cell organelles. The binding of xenobiotics with various cellular receptors may include abnormal cellular processes that have toxic or adverse effects on the cell and gene expression (Kavlock, 1996 ; Danzo,1997; Zachareswski, 1998). It is now established that contamination of water bodies by pesticide adversely affects the life of fish by altering their physiology and reproduction, growth and nutritional value, cellular morphology and physiology (De Vlaming *et al.*, 2000; Parma *et al.*, 2007; Srivastava *et al.*, 2008).

Endosulfan is a chlorinated cyclodine insecticide used worldwide in large variety of crops (Naqvi and Vaishnavi, 1993; Abraham, 2004). Agricultural run off, irrigation water and wetland applications are major sources of this contaminant to the aquatic environment (Scott *et al.*, 1999). It is a persistent organic pollutants (POPs), being highly toxic to fish. Due to lipophilic nature, hydrophobicity and low chemical and biological degradation rates it is accumulated in biological tissues and undergoes biomagnification. The aquatic organisms like fish are able to accumulate several folds higher concentration of pesticides residues than the surrounding water (Siddiqui *et al.*, 2005). The technical grade of endosulfan

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contains two diasteriomers a and b-endosulfan in the ratio of 7:3 respectively that have different physico-chemical properties (EFSA, 2005). In a living body the endosulfan isomers are transformed by chemical or biological system and excreted as oxidation and hydrolysis products like endosulfan sulphate, alcohol, ether, lactone and endosulfan-hydroxy ether. In fish these metabolites have been reported in liver and kidney (Rao and Murty, 1982). The toxicity of endosulfan to the fish is primarily mediated by inhibition of important ion-transport protein in a variety of tissues (Naqvi and Vaishnavi, 1993).Though pollutant related histopathological alterations in the liver of fish have been studied by various workers (Murray and Buttes, 1994; Altinok and Capkin, 2007; Coimbra *et al*., 2007; Ballesteros *et al*., 2009), a correlative approach of histopathological and biochemical alterations in fish due to endosulfan exposure have not been elucidated.

Clarias batrachus, being a state Fish of Bihar with high nutritional and medicinal value, is now worst affected by the consistent endosulfan expsoure. Tissue histology appears to be most sensitive indicator of adverse effects of xenobiotics along with biochemical and haematological indicators of toxicity. Pesticide induced alteration in the enzyme activities have diagnostic significance in evaluation of adverse effects of toxic substances on health. Hence the present investigation was designed to know the deleterious impact of sublethal concentration of endosulfan on fish health using biochemical tests to assess metabolism and histopathology of liver.

Materials and Methods

The technical grade Endocel, (EC35% Excel industries Ltd., Bhawnagar, Gujarat) was used in the present study for evaluation of its toxic effects on freshwater catfish, *Clarias batrachus*.

Healthy adult catfish *Clarias batrachus* of 100 $\pm$10g and 20$\pm$2 cm were collected from Nalanda Medical College and Hospital fish farm Patna, Bihar, situated at 25^{o}23' N and 85^{o}21' E on the southern bank of holy river Ganga during spawning season. The fish were brought to laboratory and maintained in rectangular plastic tub (65 cm x 45 cm x 29.5 cm) filled with 20 litres of dechlorinated tap water. They were disinfected with 0.01% $KMNO_4$ and washed thoroughly prior to their introduction. Fish were acclimatized for 15 days in laboratory condition and fed with mixture of rice bran, suji and eggs with a pinch of starch at rate of 3-4% of their body weight daily. They were alternately fed with goat liver. Feeding was stopped 24 hr before the commencement of the toxicity test.

The 96 hr LC_{50} value of endocel for the fish was determined by Probit analysis method (Finney, 1971) as mentioned in APHA (2005) as 0.02 ppm and one fifth of the said concentration *i.e.*0.004 ppm were considered as sublethal concentration which is nearly equal to environmentally relevant level of endosulfan in the ambient water. Fish were grouped into two -control group and endosulfan treated group. Each group of fish was maintained in separate plexiglass aquaria of 20 litre capacity. The fish were exposed to two concentrations of endosulfan- sublethal concentration of 0.004 ppm and a higher concentration of 0.008 ppm for 30 days.

For dose preparation the stock solution were prepared by analytical method. Firstly, 999 ml water was mixed with 1 ml endocel (EC 35%) and further diluted to thousand times to make one litre of stock solution of 1 ppm concentration. The equivalent amount of stock solution of endocel were added to the respective plexi glass aquaria daily in the morning hours for 30 days at rate of 4 ml of stock solution/litre for 0.004 ppm and at rate of 8 ml of stock solution /litre for 0.008 ppm concentration.

On 7, 15 and 30 days of exposure, six fish of each experimental group were sacrificed by cervical decapitation. Approximately 2 ml of

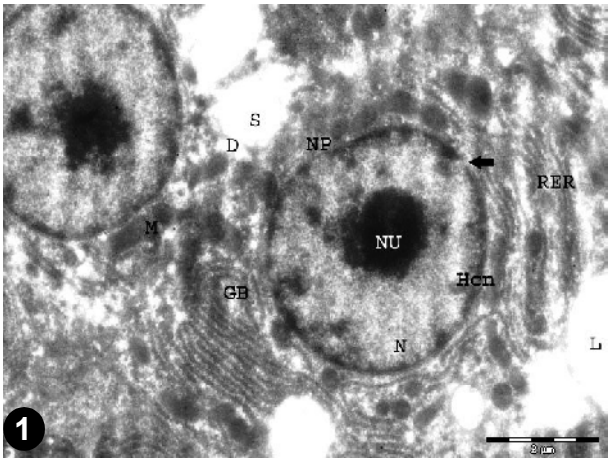

Fig.1. Control liver of *Clarias batrachus* showing prominent nucleus (N), nuclear pore (NP), heterochromatin (Hcn), Golgi vesicle (GB), mitochondria (M) and sinusoids (S). x 2,200

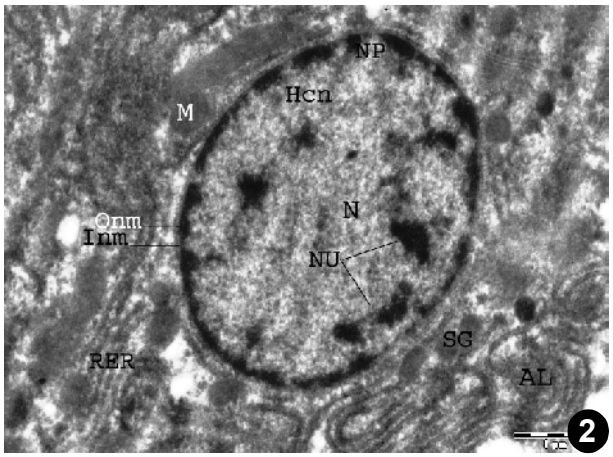

Fig. 2. Hepatic cell of normal fish showing mitochondria (M), rough endoplasmic reticulum (RER), nucleus (N) with nuclear pore (NP), patches of heterochromatin (Hcn) and nucleolus (NU) and few secretory granules (SG). x 3,500

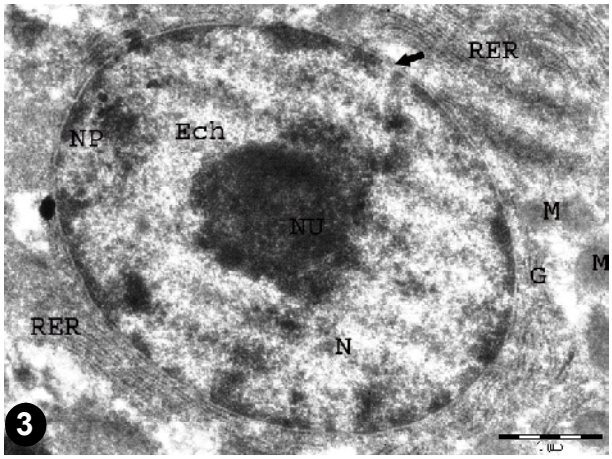

Fig. 3. Hepatocytes of treated fish (0.004 ppm) on day 7 showing enlarged nucleolus (Nu), dilated nuclear pore (arrow), few rough endoplasmic reticulum (RER) and mitochondria (M). x 4,400

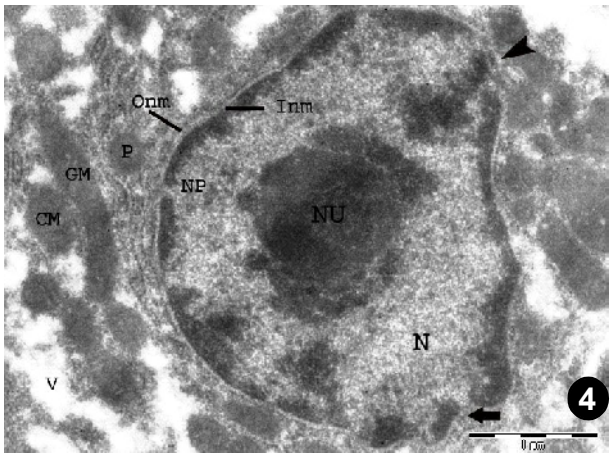

Fig. 4. Hepatocytes of treated fish (0.004 ppm) on day15 showing irregular shape of nucleus (arrow), dilation in the nuclear pore and oozing out of nuclear material into cytoplasm (arrow head), few giant mitochondria (GM) and circular mitochondria (CM). x 5,600

blood were collected from caudal vein of fish of each group using 5.0 ml graduated hypodermic syringe fitted with 26 G needle. The serum was separated by centrifuging at 5000 rpm for 10 min and stored at 4°C.

Colorimetric estimation of SGPT and SGOT. SGPT and SGOT were assessed by Reitmann and Frankel method (1957), using SGPT and SGOT kit, supplied by Crest Biosystem, Goa. The absorbance of the test sample (Abs. T) at 505 nm green filter was measured against absorbance of standard (Abs. S) and the level of SGPT and SGOT were measured from the calibration curve.

Fixation of tissue for histopathological study. After termination of each exposure day,

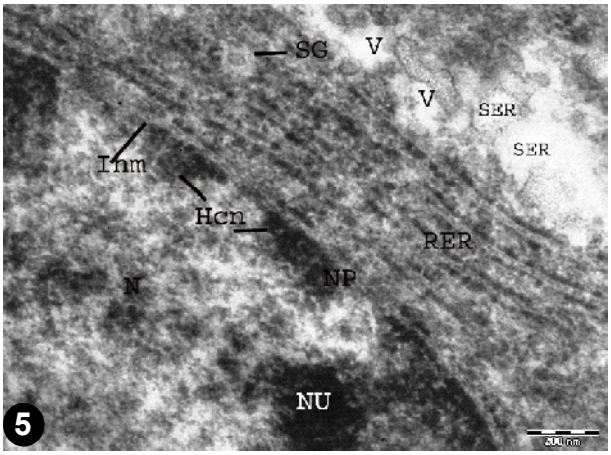

Fig. 5. Hepatocytes of treated fish (0.004 ppm) on day 30 showing dilated nuclear pore (NP), deposition of heterochromatin (Hcn) at the inner periphery of nuclear membrane, vacuoles (V) and secretory granules (SG). x 14,000

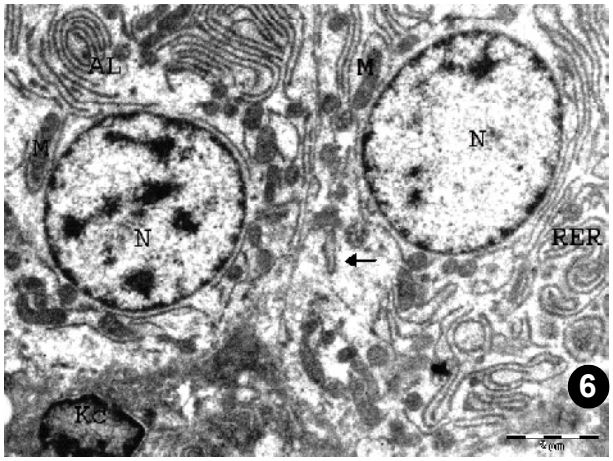

Fig. 6. Hepatocytes of treated fish (0.008 ppm) on day 7 showing vacuolated hepatoplasm (arrow), circular annulated lamellae (AL), liver macrophages (Kc) and abundance of rough endoplasmic reticulum (RER). x 1,800

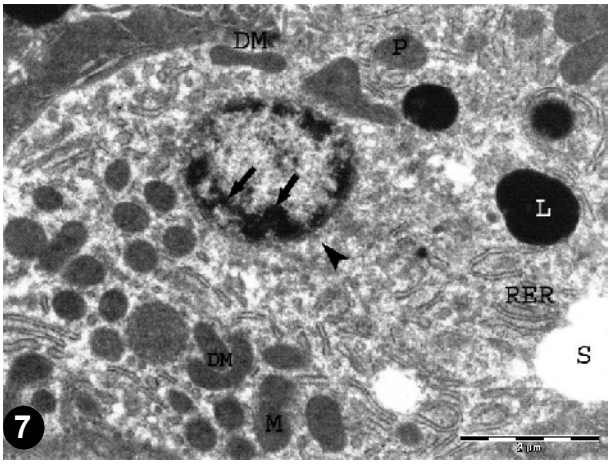

Fig. 7. Hepatocytes of treated fish (0.008 ppm) on day 15 showing dilated margin of nuclear membrane (arrow head), autolyzed chromatin (arrow), giant lysosome (L), sausage shaped mitochondria (M) and dumbbell shaped mitochondria (DM). x 2,800

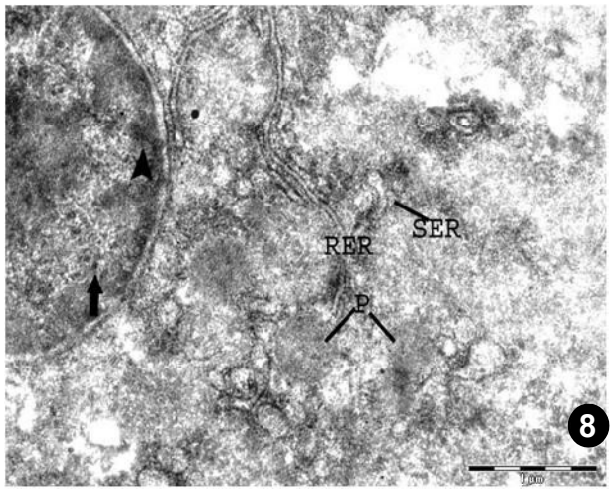

Fig. 8. Hepatocytes of treated fish (0.008 ppm) on day 30 showing extensive vacuolation, reduced array of smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER) and few peroxisomes (P). x 5,600

liver tissues of six fish of different experimental groups were fixed in 2.5% gluteraldehyde at 0.1 M phosphate buffer at pH 7.4 (4°C). The sample preparation and photography of transmission electron microscopy were done on ''Morgaginy" transmission electron microscope at SAIF-EM (Sophisticated Analytical Instrument Facility, Electron Microscope) Unit, AIIMS, New Delhi.

Statistical Analysis. The data obtained for SGPT and SGOT in each group were subjected to statistical analysis (Snedecor and Cochran, 1989). They were represented as mean $\pm$ SD and subjected to paired 't' test. The significance level was tested at 5% and 1%.

Results and Discussion

The activity of serum enzymes of fish exposed to endosulfan are shown in Table1. At sublethal concentration (0.004 ppm), 140.0% increase in SGPT was found on day 7 exposure to endosulfan while 73.0% and 43.0% increase were recorded on day 15 and 30 respectively.At higher dose (0.008 ppm) a significant rise of 26.6% was recorded on day 15.

At sublethal exposure, SGOT showed a significant ($P<0.01$) increase of 150 %, 112% and 81.25% on day7, 15 and 30 respectively, while at higher dose of endosulfan, a non significant decline of 6.25% and 12.5% were recorded on day 7and 30.

Transaminases, SGPT and SGOT are the key enzymes which take active part in transmination reactions of the body and utilization of carbohydrate and protein. The activities of these enzymes have been measured in the fish under exposure to various chemical pollutants. They are sensitive indicator of liver damage and injury in different types of tissues. Both these enzymes may be elevated in a variety of infiltrative diseases of liver and biliary obstruction. SGPT is released into the blood stream as a result of liver injury and degree of its elevation roughly parallels the extent of liver damage. In the present investigation, endosulfan sets in a wave of biochemical imbalance leading to fluctuation in SGPT and SGOT. Similar kind of elevation in SGPT and SGOT of fish have also been reported after exposure to different pesticides as in - *Channa gachua* (Koul *et al*., 2007), *Clarias batrachus* (Mukhopadhyay and Dehadrai, 1980), *Tilapia mossambica* (Rao and Rao, 1984) and *Channa striatus* (Sadhu *et al*., 1985).

Histopathology provides a rapid method to detect the effect of irritants in various organs (Johnson *et al*., 1993). It is most sensitive indicator of adverse effects of dietary xenobiotics (Braunbeck and Appelbaum, 1999). Transmission electron microscopy of the liver cell of normal fish showed prominent nucleus with distinct outer and inner nuclear membrane, well developed nucleolus surrounded by heterochromatin, rough endoplasmic reticulum, Golgi body and numerous mitochondria (Fig.1, 2). Major ultrastructural alterations in hepatocytes incurred at sublethal exposure of endosulfan. The nuclear pore was highly dilated irregular margin of nuclear membrane (Fig. 3), deposition of heterochromatin on inner nuclear membrane, oozing out of nuclear material into cytoplasm, swelling of organelles and fragmented array of cisternae of rough endoplasmic reticulum (Fig. 4, 5).

At higher concentration of endosulfan, immense increase in number of irregular shaped mitochondria and circular annulated lamellae were marked at shorter duration (Fig. 6, 7). At longer duration different histopathological anomalies were observed i.e. immense vacuolation, reduced number of array of rough endoplasmic reticulum and clustering of chromatin material into small vesicles (Fig. 8).

Similar kind of hepatocellular anomalies have been reported after endosulfan and disulfoton treatment in the hepatocytes of male rainbow trout *Oncorhynchus mykiss* (Arnold *et al.,* 1995). In the present study, the rough endoplasmic reticulum underwent progressive loss of structural integrity with increasing pesticide concentration showing nearly three folds increase in the absolute and relative volume of the rough endoplasmic reticulum per hepatocyte The circular array of smooth endoplasmic reticulum cisternae form annulated lamellae. These annulated lamellae are specialized form of smooth endoplasmic reticulum, articulating from the nuclear membrane and form concentric lamellae. They are usually prevalent in cells with a high membrane turn over. Smooth endoplasmic reticulum proliferation may be regarded to be

indicative of the induction of biotransformation process (Hinton *et al.,* 1978; Braunbeck *et al.,* 1989). Rough endoplasmic reticulum proliferation and fenestration as well as circular endoplasmic reticulum arrays are indicative of MFO (mixed function oxidase) induction (Hawkes, 1980). The increase in perichromatin granules probably represents aberrations in protein synthesis. Increased incidence of heterogeneity in the lysosomal matrix of hepatocytes can be correlated with increased phospholipidosis (Arnold *et al.,* 1995). Mitochondrial swelling and degradation observed in the present investigation ultimately led to apoptosis of hepatocytes.

(Kreps *et al.,* 1987; Panda and Kar, 2003).

In conclusion, it is affirmed that endosulfan sets in a wave of biochemical imbalance followed by histopathological lesion in liver cell leading to hepatic failure in fish and other negative physiological changes.

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Table 1. SGPT and SGOT profile of *Clarias batrachus* exposed to different concentrations of endosulfan.

Biochemical parameter (U/ml)	Control	Experimental groups (Endosulfan treated)					
		0.004 ppm			0.008 ppm		
		7 days	15 days	30 days	7 days	15 days	30 days
SGPT	30±3.03	72±4.73** (+ 140.0)	52±4.56** (+73.0)	43±4.89** (+43.0)	30±1.89 (0)	38±2.72* (+26.6)	30±3.52 (0)
SGOT	16±1.67	40±4.56** (+150.0)	34±4.19** (+112.5)	29±2.36* (+81.25)	15±2.0 (-6.25)	17±2.52 (+6.25)	14±1.78 (-12.5)

Values are expressed in mean ± SD of six replicates in each group.

Significant response: * P<0.05, **P<0.01.Values in parenthesis are % reduction (–) or elevation (+) over control.

In the present investigation, distortion of plasma membrane of hepatocytes is very intense after endosulfan treatment. The probable kinetics of endosulfan toxicity lies in its potential to generate reactive oxygen species (ROS) and hydroxyl radical (OH-) that attack membrane fatty acid promoting lipid peroxidation. The balance between pro- and antioxidant system of hepatic tissue seems to be disrupted and as such normal antioxidant product could not quench the excess of free radicals and led to various damages to plasma membrane and other membrane bound organelles like mitochondria, Golgi body and lysosomes

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