CHAPTER-7

ELECTRON MICROSCOPIC STUDY
Introduction:

Endosulfan produces cumulative toxic effects to the different organs such as liver, gills and kidney in many fish species. From the study of histology it was found that the tissues of liver, gills and kidney of *Channa punctatus* were badly damaged due to the effect of endosulfan. So, to support the different changes in the liver, gills and kidney, these three organs were selected for electron microscopic study to view the alteration in different cellular structure in micro anatomical level.

Review of literature:

Extensive investigation were carried out by number of workers to study the changes occurred in the liver and other organs of different species of fishes after the treatment with various insecticides. Olson *et al.*, 1973, Dua *et al.*, 1994, Acharya *et al.*, 2005, Kalele *et al.*, 2005, Muthukumaravel *et al.*, 2008 already did some works on this micro anatomical level in different fish species on different target organs and with different chemicals like heavy metals, pesticides and insecticides.

Braunbeck and Appelbaum (1999) reported enlargement of nucleolus, increase in number and size of golgi fields and R.E.R (Rough Endoplasmic reticulum) lamellae, proliferation of peroxisomes and lysosomes. Swelling of granular Endoplasmic reticulum (GER) in the hepatocytes of the arsenic exposed quail was detected by Nystrom; 1982.

Light and SEM study on the gill architecture was studied by workers like Gupta and Rajbanshi; 1995, Gupta and Dua; 2002, Gupta and Kumar; 2006.
(25-26°C) to dry). After drying, the tissues were mounted on brass stubs and coated with gold palladium of 35 nm thick with the machine fine coat ion sputter JFC-1100.

Then the tissues were placed in scanning electron microscopy plate for visualizing the outer surface of the tissues and were taken photographs using JEOL JSM 6360 SEM Japan.

**Method for Transmission Electron Microscope study (TEM):**

For TEM, the tissues of liver and kidney of control and treated groups of fishes were dissected out in normal saline solution. The sizes of the tissues were of 1-2 mm. Then the tissues were fixed in primary fixative (2.5 -3% Glutaraldehyde or Karnovsky’s Fixative (Karnovsky, 1965) for 1 hr at 4 °C. After that the tissues were washed twice in 0.1M Sodium Cacodylate Buffer 3 changes of fifteen minutes each at 4 °C. Then the tissues were dehydrated by using different concentration of acetone. Namely 30%, 50%, 70%, 80%, 90% and 100% (dry) acetone were used for dehydration for 15 minutes with 2 changes at 4 °C. Again 100% Acetone was used for 30 minutes at room temperature. Then the tissues were cleaned with Propylene Oxide at room temperature for 15 minutes with 2 changes.

Then infiltration was carried out as

i) Prop. Oxide : Embedding Medium -> 3 : 1 (over night)

ii) Prop. Oxide : Embedding Medium -> 1 : 1 (1 hour)

iii) Prop. Oxide : Embedding Medium -> 1 : 3 (1 hour in vacuum)

iv) Pure Embedding Medium -> (1 hour at 50°C)
Infiltrated samples were transferred into BEEM capsules or embedding moulds and oriented. Pure embedding medium was then poured into the capsule or mould and transferred into the Embedding Oven set at 50°C; and kept over night (12-24 hrs).

Temperature was then raised to 60°C and kept so for 24-48 hours for polymerization. Then the tissues were cut into 60-90 nm sections by using the Ultra-microtome which was floated on double distilled water. A copper grid was then immersed under the sections and lifted up scooping the sections and allowed to dry.

A double staining method was adopted using Uranyl Acetate and then Lead Citrate which would help in enhancing the contrast of biological samples.

**Results and Discussion:**

Previous studies showed that the normal and control sets revealed almost similar results in the histological, haematological and biochemical parameters, so, in this study of SEM and TEM experiments with normal sets were ignored. Only control and treated sets of fishes were considered. Experiments were performed at 72 hr and 96 hr of exposure to the endosulfan, where the changes were quite distinct.

**Scanning Electron Microscope study of liver:**

From the study of SEM, it was found that the cellular architecture of liver of *C. punctatus*, in the control group of fishes was observed to be in normal texture and well arranged condition. The cells were intact in the control group of fishes (Plate No-7.1, 7.5 and 7.6). Again no breakage of cell membrane was
PLATE 7.1: Photomicrograph of liver (Control)
A - Hepatocyte

PLATE 7.2: Photomicrograph of liver (72 hr. treated with endosulfan)
A - Hole formation
PLATE 7.3: Photomicrograph of liver (72 hr. treated with endosulfan)
A - Hole formation

PLATE 7.4: Photomicrograph of liver (96 hr. treated with endosulfan)
A - Wrinkle of epithelial cell. B - Even Cell Sheet. C - Damaged hepatocyte
PLATE 7.5: Photomicrograph of liver (Control)
A - Hepatocyte

PLATE 7.6: Photomicrograph of liver (Control)
A - Hepatocyte
PLATE 7.7: Photomicrograph of liver (72 hr. treated with endosulfan)
A - Damaged cellular architecture
B - Hole formation

PLATE 7.8: Photomicrograph of liver (96 hr. treated with endosulfan)
A - Hole formation. B - Cellular necrosis. C - Rupture of Cell Membrane
observed. But in case of treated group of fishes wrinkle and shrinkage of epithelial cells with loose connection between the two cells was observed in liver. The destruction and necrosis of epithelial cells were resulted in the formation of an uneven cell sheet.

In 72 hr of treatment, the hepatocytes were damaged and loose blood cells were observed, which indicated the haemorrhage of the liver due to endosulafan exposure (Plate No-7.2, 7.3 and 7.7). Thus the cellular architecture was disturbed with necrosis and cell damage in treated group of fishes at 72 hr of exposure.

Again, at 96 hr of exposure to endosulfan, damaged cell membrane was observed and further damage in liver architecture was observed (Plate No-7.4, 7.8). Uniformity of cell membrane was lost. Due to damage of membrane, no distinct cellular architecture was found.

**Transmission Electron Microscope study of liver:**

TEM revealed that in case of control set of fishes; the nucleus was uniform with nucleolus. Though, hexagonal shaped hepatocyte was observed but few different shaped hepatic cells were also noticed. The hepatocytes were observed rich with mitochondria and these were round and oval shaped (Plate No-4.13, 4.16). Mitochondria and Endoplasmic reticulum were observed in normal condition.

Severe alterations were noticed in the treated fishes at 72 hr on the treatment with endosulfan. Large number of vacuoles was observed (Plate No- 4.14, 4.17). The shaped of the nucleus was changed and nucleolus observed in
PLATE 7.13: Photomicrograph of liver (Control) TEM, 14x
A - Mitochondria with mitochondrial wall.  B - Nucleus
C - Rough Endoplasmic Reticulum

PLATE 7.14: Photomicrograph of liver (72 hr. treated with endosulfan) TEM, 14x
A - Mitochondria with broken wall, B - Broken plasma membrane, C - Lysosome, D - Vacuole,
E - Free ribosomes, F - Microvilli, G - Rough Endoplasmic Reticulum
PLATE 7.15: Photomicrograph of liver (96 hr. treated with endosulfan) TEM, 14x
A - Mitochondria with broken wall, B- Broken RER wall, C-Damaged nucleus, D-Free Ribosome

PLATE 7.16: Photomicrograph of liver (Control), TEM, 40x
A - Nucleolus, B-Nucleus, C-Mitochondria, D-Vacuole, E-RER.
PLATE 7.17: Photomicrograph of liver (72 hr. treated with endosulfan) TEM, 40x  
A - Nucleus, B-Vacuole, C-Mitochondria, D-Lysosome

PLATE 7.18: Photomicrograph of liver (96 hr. treated with endosulfan) TEM, 40x  
A - Nucleus, B-large vacuole, C-Mitochondria, D-Free Ribosome
decreasing size. Mitochondria were observed in increasing number and fragmentation of mitochondrial wall was observed where cristae were difficult to recognize. Large numbers of lipid droplets and lysosomes were observed. Again broken cell wall was observed.

At 96 hr of experiment with the endosulfan, the changes in micro-anatomical level of hepatocytes were very prominent. Large vacuole was observed. Damaged nucleus and nucleolus were observed (Plate No- 7.15, 7.18). Mitochondrial wall and cristae were damaged and observed in increasing numbers. Rough ER was also observed in broken condition and in increasing numbers.

The study of electron microscope revealed that liver was badly affected due to the treatment of endosulfan. In this study mitochondria were degenerated and cristae were difficult to be seen. Again fragmentation of ER was observed, which were also reported earlier by El-Elamy et al., 1993. He also reported the presence of lipid droplets in the neopybuthrin exposed Tilapia nilotica.

In the present findings increase in lipid droplets were noticed, which might be due to reduced fatty acid oxidation resulting from impairment of mitochondrial function. Similar opinion was also put forwarded by Diazani, 1957 and Ei-Elaimy et al., 1993.

Rupture of nuclear membrane was observed and nuclear membrane was seen protruded at one or more region, which was also reported by Herzberg;1986. Kotak et al.,1996 and Braunbeck et al.,1989,1999. Severe necrotic cells were observed by irregularity in shape and few cytoplasmic organelles. Again
extensive vacuolization was observed in cytoplasm, which ultimately affected the entire cytoplasm.

Workers like Woodward and Hagan (1947) reported the effect of insecticide on liver by treating dogs with α–BHC. Mathur (1962) observed liver cells with migration hypertrophy, vacuolation and necrosis in fishes exposed to BHC. Bhattacharya et al., 1975, Shastry and Sharma; 1978, Mandal and Kulashrestha; 1980 noticed liver cord disarray, vacuolation, breaking down of cell boundaries and necrosis of liver due to the treatment of pesticides and different chemicals. Again, BHC produced various types of liver damage and caused hepato-cellular carcinogenesis in mammals, reported by Hitachi et al., 1975. Ito et al., 1975, Baros and Saliba, 1978.

**Scanning Electron microscopic study of Gill:**

In control group of gill of *C. punctatus*, primary gill lamellae were observed in normal condition with free of mucous and the uniformity of secondary gill lamellae was noticed, arising from primary lamellae. Presence of uniform gill racker with denticular structure was observed in control group of fishes (Plate No-7.9).

In 72 hr of exposure to endosulfan, severe erosion of epithelial layer was observed (Plate No-7.10). Roughness of gill racker was noticed. Again the denticular structures were damaged and fusions of gill lamellae were observed in the experiment.

At 96 hour of exposure to endosulfan, damaged and fusion of gill lamellae were observed. Clumping of gill lamellae was observed. Swellings, fusion of
PLATE 7.9: Photomicrograph of gill (Control)
C - Well arranged lamellae with gill rackers

PLATE 7.10: Photomicrograph of gill (72 hr. treated with endosulfan)
A - Erosion of epithelial layer. B - Roughness of gill racker.
C - Damaged denticular structure. D - Fusion of gill lamellae
PLATE 7.11: Photomicrograph of gill (96 hr. treated with endosulfan)
C - Breakage of epithelial layer. D - Swelling of gill lamellae.
E - Damaged denticular structure.

PLATE 7.12: Photomicrograph of gill (96 hr. treated with endosulfan)
A - Damaged gill racker. B - Fusion of gill lamellae.
lamellae, destruction of denticular structure were also observed in treated group of fishes (Plate No-7.11 and 7.12).

It is universally known that the gills of fish are the major route for the entry of any chemical substances present in the water body. Any changes in the respiratory epithelium of the gill normally affect the respiratory and osmoregulatory capacity of the gills. Physical damage of the gills may inhibit the $O_2$ uptake in the fish species, which later on cause suffocation to the fish and ultimately lead to die.

On the treatment with endosulfan, fusion and clumping of secondary gill lamellae were noticed. This result was confirmed by the findings of Skidmore and Tovell;1972, Roy and Datta Munshi;1991 and Gupta and Rajbanshi;1995. Again erosion of epithelial cells, destruction of denticular structure, deformity of gill racker, fusion of lamellae were observed in the present experiment, which are in good agreement with the observation of Dua and Johal;1994, Gupta and Dua;2002 and Acharya et al., 2005.

Transmission Electron Microscopic Study of kidney:

In the control group of fishes, the kidney cells were found in normal condition. The cell membrane, nuclear membrane was observed in uniform pattern. Again tubular cells were observed hexagonal shape. Mitochondria are surrounding by interdigitations. Near mitochondria RER were observed clearly. Basement membrane was observed with endothelial cell (Plate No-7.19, 7.20, 7.21).
PLATE 7.19: Photomicrograph of Kidney (Control) TEM, 4x
A - Nucleus, B-Plasma membrane, C-Mitochondria, D-RER, E-Lysosome

PLATE 7.20: Photomicrograph of kidney (Control) TEM, 8x
A - Nucleus, B-Lysosome, C- Mitochondria, D-Plasma-membrane.
PLATE 7.21: Photomicrograph of Kidney (Control) TEM, 40x
A - Mitochondria, B - RER

PLATE 7.22: Photomicrograph of Kidney (72 hr. treated with endosulfan) TEM, 8x
A - Tubular cell with deformed nucleus, B - Free ribosome, C - Vacuole, D - Mitochondria,
E - Lysosome, F - Enlarge space of lumen of tubule
In 72 hr experiment with endosulfan, the kidney cells were observed in irregular shaped. Mitochondria and Endoplasmic reticulum were observed in damaged condition with broken membrane (Plate No-7.22, 7.23, 7.24, 7.25). Cristae were also indistinct and mitochondria were not covered with interdigitations. Nucleus was also observed in irregular shape and numbers of electron dense hyaline droplets were observed in tubular cell cytoplasm. Autophagic vacuoles and lots of free ribosome were noticed in the lumen of the tubular cell cytoplasm.

Degenerative and necrotic cell damage was observed in the kidney at 96 hr exposure to endosulfan. Damage of cell and nuclear membrane were noticed (Plate No-7.26, 7.27, 7.28). Electron dense hyaline droplets were observed in large numbers. Mitochondria were totally damaged and cristae was very difficult to separate from matrix. Numbers of vacuoles and lysosomes were observed in compare to control and 72 hr of treatment with endosulfan.

The kidney is a fused organ in *C. punctatus*, which is lying in a retroperitoneal location just ventral to the spinal column. This is the main organ of excretion and any chemicals are excreted from the body through kidney. So, it is immediately affected in the toxic environment.

In the present study, loss of cell interdigitations, misshaped mitochondria, decrease in RER were observed, which were also recorded by Bravo *et al.*, 2005. He also recorded the presence of autophagic vacuoles and primary lysosomes those were also noticed in the present study. Misshaped
PLATE 7.23: Photomicrograph of Kidney (72 hr. treated with endosulfan) TEM, 20x
A - Nucleus, B - Broken membrane, C - Vacuole, D - Broken Wall of Mitochondria

PLATE 7.24: Photomicrograph of Kidney (72 hr. treated with endosulfan) TEM, 40x
A - Deformed Mitochondria, B - Broken RER
PLATE 7.25: Photomicrograph of Kidney (72 hr. treated with endosulfan) TEM, 4x
A - Vacuole, B - Lipid droplet, C - Ribosome with increase in number

PLATE 7.26: Photomicrograph of Kidney (96 hr. treated with endosulfan) TEM, 4x
A - Nucleus, B - Lipid droplet, C - Mitochondria
PLATE 7.27: Photomicrograph of Kidney (96 hr. treated with endosulfan) TEM, 4x
A - Nucleus, B-Vacuole, C-Dilation of Membrane, D-Lysosome, E-Mitochondria.

PLATE 7.28: Photomicrograph of Kidney (96 hr. treated with endosulfan), TEM, 40x
A - Deformed mitochondria with indistinct cristae
mitochondria and an increase numbers of lysosomes in the proximal tubules of the kidney were also observed by Wester et al., 1990.

In kidney of fish, tubular lesions and tubular degenerations were also noticed earlier by Gomez et al., 1999. He also reported the hyaline droplets in tubular cell cytoplasm, which were also observed in the present experiment. The electron dense hyaline droplets contained protein, possibly due to the high glomerular filtration rate resulting from extensive damage to the filtration barrier. Protein was subsequently reabsorbed at tubular level, giving rise to these inclusions. As the epithelial cells contained large numbers of such droplets, it was and evidence of degeneration of cytoplasmic organelles (Gomez et al., 1990). Robergh et al., 1991 reported degenerative changes in the epithelial cells of the tubules, glomeruli and interstitial tissue of the kidney in carp exposed to mycosystin treatment. Kotak et al., 1996 reported that tubular lumina of kidney was filled with cell debris and even cellular casts.

Degenerative and necrotic changes in tubular epithelial cells were observed with shrunken glomeruli by Sahoo et al., 2003 in aflatoxin treated L. rohita.

In this study, mitochondria were observed in changing constituents in the epithelial cells of tubules. The same opinion was forwarded by Ghadially 1982 a,b). Changes in the constituents of mitochondria in the epithelial cells of proximal tubules along with presence of intracytoplasmic desmosomes in basement membrane of epithelial cells were an indication of damage in kidney due to the toxic substances (Ghadially 1982 a,b). Again, large numbers of autophagosomes were observed in this study, which might be due to defect in
the digestive apparatus of the lysosomes. This was also reported by Tandler and Rossi, 1977. Ashley, 1965 and Jantrarotai et al., 1990 observed the hyperplasia of mesangial cells and shrunken glomeruli in the fishes due to toxic effects of chemicals.

From the experiment the alteration of different cellular injury were observed by endosulfan intoxication, which indicates the quality of fish adversely affected resulting adverse impact to its ecological system.