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**Research Article**

# ULTRASTRUCTURAL PATHOLOGY OF LIVER AND KIDNEY IN INDIGENOUS CHICKEN INDUCED BY CHRONIC CHLORPYRIPHOS TOXICITY

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**ABSTRACT**

Chlorpyriphos (CPF) is a broad-spectrum organophosphate pesticide which is widely used in agriculture and in domestic use against harmful insects. To our knowledge literature on chronic low dose effects of CPF especially on ultrastructural pathology are scanty. Therefore the present study aimed to investigate the effect of oral (p.o.) administration of CPF induced ultrastructural alterations in liver and kidney of indigenous chicken. The birds were divided into two groups I and II. Group I served as control and group II was treated with CPF (0.36 mg/kg) orally daily upto 12 weeks. Renal and hepatic tissues were collected at monthly interval up to 3 months and processed for ultrastructural pathology. Ultrastructural examinations of liver and kidney indicated significant injury in chicken receiving chronic dose of chlorpyriphos. The results indicated that chronic CPF intoxication has a toxic effect on liver and kidney.



## INTRODUCTION

Chlorpyriphos ((*O*-*O*-diethyl-*O*-[3, 5, 6 trichloro-2-pyridyl]-phosphorothioate) (see Figure 1) is one of about 100 organophosphorus (OP) insecticides in the market today. It is used to kill insect pests by disrupting their nervous system. Chlorpyriphos (CPF) has an advantage over other products in that it is effective against a wide range of plant-eating insect pests (Baba *et al*, 2013).

Chlorpyrifos (CPF) induces deleterious effects primarily through acetylcholinestrase inhibition and produces symptoms characteristic of cholinergic overstimulation like salivation, nausea, vomiting, tremor and convulsions in mammalian species including human beings (Kamrin, 1997). Chronic exposure to CPF elicits a number of other toxic affects including hepatic dysfunction, immunological abnormalities, embryotoxicity, genotoxicity, teratogenicity, neurotoxicity, and neurobehaviourial changes (Rahman *et al*, 2002, Herfod *et al*, 2005, Verma *et al*, 2007, Ahmed *et al*, 2010).

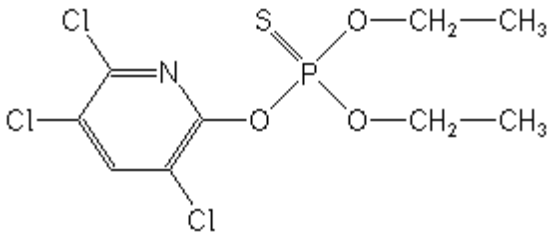


**Figure 1** Structure of Chlorpyrifos

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In veterinary practice CPF is widely used for the control of pests, mites, flies and lice affecting livestock and poultry (Loomi *et al*, 1972). It has also been used for the control of termites in chicken houses (Leidy *et al*, 1991). The residues of chlorpyriphos were detected in poultry egg, meat and cow milk and milk products (Rawat *et al*, 2003).

Chicken are more commonly affected with pesticide toxicity because poultry houses are frequently dusted with pesticides. Chemical pesticide causes health consequences to the birds culminating in great economic loss. It is also posing a potential threat to public health due to the presence of pesticide residues in poultry meat and egg. Ample evidence exists to suggest that the use of pesticides on crops, in storehouses, in poultry houses plus the nonjudicious application for spraying animals or in dipping solutions to prevent ectoparasites leaves behind its residue causing serious health effects (Pal and Kushwah, 1998). Assam is mainly an agriculture based state, where pesticides are widely used by the farmers. Most of the rural masses rear indigenous chicken in backyard poultry models. There is every possibility of picking up of toxic substances from the grain and dead insects available in the grazing field as these birds are let loose in the field during the day time. They are more prone to accidental chlorpyriphos toxicity than the commercial birds which are generally reared in closed confinement. Literature on chronic low dose effects of pesticides, especially as environmental pollutants are scanty. There are many facets of pesticides toxicity that remain unexplored and need immediate attention to safeguard the health of avian populations and livestock and also, thereby, public health. Therefore the present study has been undertaken to study the ultrastructural alterations of chronic chlorpyriphos intoxication in indigenous chicken.

## MATERIALS AND METHODS

### *Animals*

Three month old unsexed thirty two indigenous chicken were procured from All India Coordinated Research Project (AICRP) on Poultry, College of Veterinary Science, A.A.U, Khanapara Guwahati-781022 were wing banded, weighed and reared in the Department of Pathology, College of Veterinary Science, A.A.U, Khanapara Guwahati-781022 with *ad libitum* supply of feed and water. They were randomly distributed into two groups of 16 chicken each i.e. control, chlorpyriphos treated. The experimental trials were approved by the Institutional Animal Ethics Committee (No.770/ac/CPCSEA/FVSc/AAU/IAEC/11-12/128), India and conducted under its guidelines.

### *Chemical (Insecticide)*

Commercial products of chlorpyriphos (20%) used in this study was procured from Excel Crop Care Private Limited, Mumbai, India.

### *Treatment groups*

Thirty-two chicken were randomly segregated into two groups of 16 each and fasted for 6 h prior to dosing. Group I served as control and received distilled water p.o. for 90 days. Group II served as CPF group. CPF was diluted in a tenfold serial dilution with distilled water to obtain a concentration of 0.2 mg/ml (10−4). Fresh preparations were orally administered daily using oral gavage. Group II was administered 0.36 mg/kg b.w. CPF (1.8 ml of 10−4 dilution) daily up to 90 days. Doses were calculated on weekly body weight basis and administered accordingly. The birds were closely watched for the presence of clinical signs, if any, and sacrificed at weekly interval till the end of the experiment.

### *Ultrastructural examination*

### *Collection of material*

Liver and kidney tissues were collected fresh at monthly interval during post mortem examination of the sacrificed birds in the treatment groups. The affected part was cut into small pieces under stereo microscope to make the size of the tissue sample of about 0.5x× 0.5x.

### *Processing and dehydration*

After collection the tissue samples were fixed in 2.5% gluteraldehyde for 4 h at $4^oC$. Then the fixed tissue samples were placed in 0.1 M Karnovsky's buffer for 15 minutes at $4^oC$. This step was repeated 3 times or 3 changes in 0.1 M Karnovsky's buffer for 15 minutes each at $4^oC$. The samples were stored in 0.1 M sodium cacodylate buffer at $4^oC$. Transportation of the samples was done in 1.5 ml ependroff (micro centrifuge tube) in the same buffer in ice to the Sophisticated Analytical Instrumentation Facility (SAIF), North Eastern Hill University (NEHU), Shillong, Meghalaya for further processing and dehydration (Wischnitzer, 1997) .

### *Embedding*

Tissues were embedded in epoxy resins to prepare blocks for sectioning.

Sectioning

An ultra-microtome was used to get the desired sections of samples for transmission electron microscopy. The thickness of the sections was 700-800 $A^o$.

### *Staining*

In preparation for staining, thin sections were placed on grids and permitted to dry. The ultrathin sections were stained by negative staining with saturated uranyl acetate and lead citrate. Each grid was floated with the thin sections downward in 1% solution of the negative stain. After staining, the sections were washed with distilled water by floating the grid on the surface of distilled water or by gentle agitation in distilled water. The grid was allowed to dry with the sections upward on the filter paper.

### *Observation*

The stained sections were mounted on a digital transmission electron microscope (JEOL-2100) which was operated at an accelerating voltage of 120kV. The areas of interest were first observed under the viewfinder, thereafter fine focused in a high end digital monitor and the images were captured and saved electronically for further analysis.

## RESULTS

In chronic toxicity group of chicken immediately after oral dosing the chicken developed increased thirst. Gradually the symptoms disappeared, except for reduced feed intake and gradual reduction in body weight gain. After two months of

treatment several of the birds exhibited slightly staggering gait, leg weakness and tremor suffered from diarrhoea. Some of the birds developed curled toes with pale mucous membrane and prominent keel bone.

***Ultrastructural study***

Out of the four images analyzed, following observations were recorded.

***Liver***

There was increase in the amount of RER and SER in conjunction with dis-integrity of nuclear membrane and margination of nuclear chromatin with aggregation of smooth endoplasmic reticulam around it (Fig. 2). The RER were fragmented and distended and swollen (Fig. 3 & 4). The cytosol appeared rarefied with disintegrating organelles. Nucleus of some degenerated cell showed complete karyolysis and ribosome studded membranous pseudoinclusions which is enclosed by a disintegrated cytocavitary network and abundant free ribosomes.

Most of the mitochondria were swollen and others were dilated with peripherally placed cristae (Fig. 3) or complete cristolysis and matrix lysis, along with dilated cytocavitary networks, giving rise to vacuoles. The cells also became vacuolar in appearance as a result of lysis in the mitochondrial matrices.

There was increased chromatin content and cytoplasmic density of some hepatocyte nuclei. The nuclear membrane appeared dilated. The chromatin content of some hepatocyte nuclei and cytoplasmic density increased.

There was presence of dense myelin figures (Fig. 4) along with increase in glycogen content of the cells. Peroxisomes are evident in association with injured mitochondria (Fig. 5).


**Figure 2** TEM image of liver showing disintegrity of nuclear membrane and chromatin margination in the degenerated nucleus (N). Aggregation of smooth endoplasmic reticulum (SER) around the nucleus.duct.
scale bar: 20µm

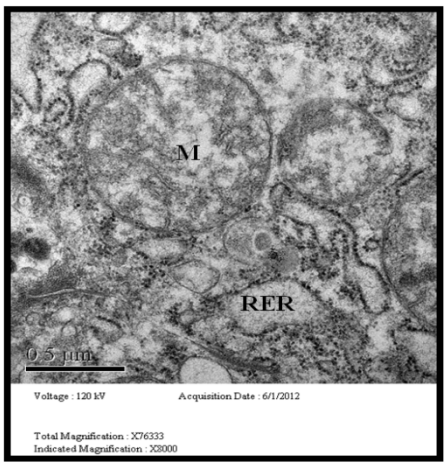


**Figure 3** TEM image of liver showing mitochondria (M) with peripherally placed cristae and fairly homogenous matrix. Swollen and dilated rough endoplasmic reticulum (RER).
scale bar: 20µm

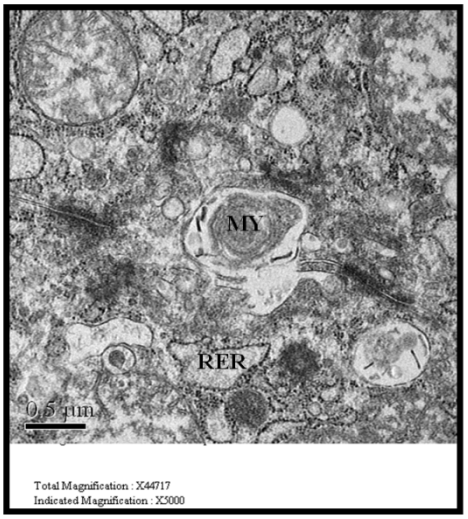


**Figure 4** TEM image of liver showing formation of myelin figures (MY) along with swollen mitochondria and dilated rough endoplasmic reticulum (RER).
scale bar: 20µm

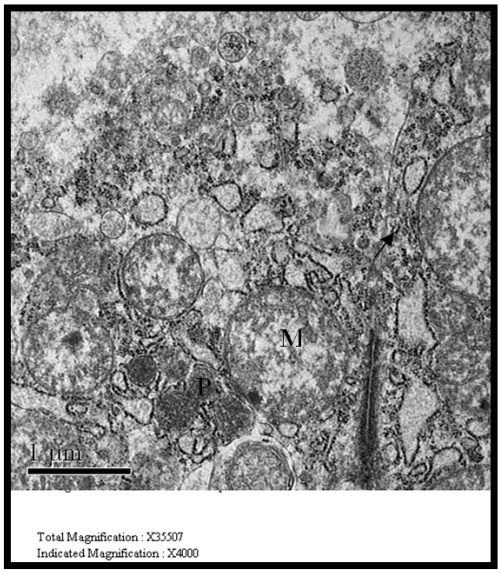


**Figure 5** TEM image of liver showing accumulation of peroxisomes (P) near degenerated mitochondria and dilated rough endoplasmic reticulum and presence of free ribosomes (arrow).
scale bar: 20µm

***Kidneys***

The glomerular structure was affected markedly in chlorpyriphos treated birds. Moreover a slight increase in the thickness of the glomerular basement membrane with deposition of electron dense material was focally seen.

There was reduplication of SER in early stages of poisoning and chronic toxicity was often accompanied by massive development of SER.

Furthermore, there was fusion of foot processes, vacuolization and lighting of cytoplasm of podocytes with deposition of electron dense material in the podocytes**.** In addition; there was presence of scattered electron dense fibrils.

Furthermore, there were swollen and pleomorphic mitochondria along with swollen and dilated RER (Fig. 6). The distal convoluted tubule cells showed increase in cytoplasmic density.

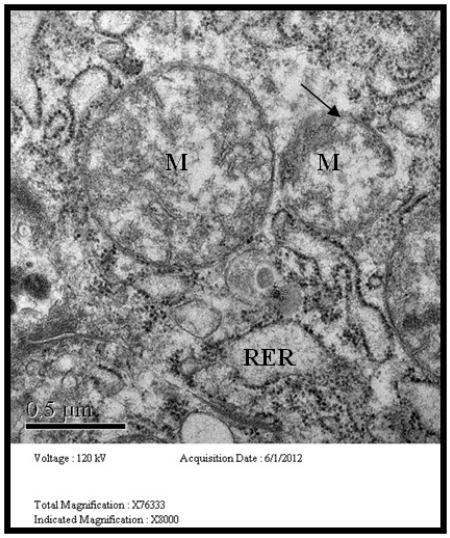


**Figure 6** TEM image of kidney showing dissolution of mitochondrial membrane (arrow), loss of mitochondrial (M) cristae, swollen rough endoplasmic reticulam (RER)
scale bar: 20µm

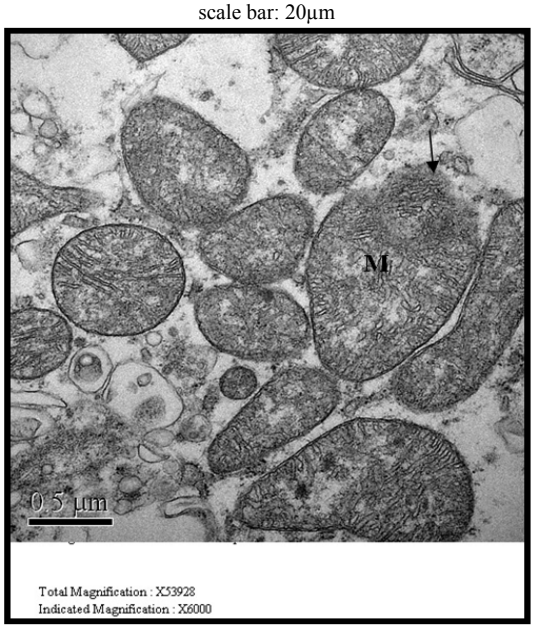


**Figure 7** TEM image of kidney showing rupture of wall of mitochondria (arrow) with cristae undergoing degenerative changes. scale bar: 20µm

Most of the mitochondria were swollen and others were dilated with complete cristolysis and matrix lysis, along with dilated cytocavitary networks, giving rise to vacuoles. Additionally, there was lysis of mitochondrial wall in some places (Fig. 7).

There was increase in the number of electron dense bodies. Degenerative changes of small intensity were observed in some cells of proximal convoluted tubules. These changes consisted of empty spaces and partial vacuolization of the cytoplasm.

## DISCUSSION

All the clinical symptoms disappeared subsequently towards the end of the experiment. The subsequent disappearance of leg weakness and unsteady gait, seen in the present study, might be due to development of tolerance to the behavioral effects of repeated exposure to chlorpyriphos. This tolerance has been attributed to an attenuation of cholinergic responsiveness, observed in exposure to a variety of anticholinesterase compounds (Richardson *et al*, 1993).

The clinical signs observed in the present study conform to the findings of several earlier workers in different animals and birds (Srebocan *et al*, 2003, Al-Badrany and Mohammad, 2007).

Chlorpyrifos act through their active oxon metabolites and inhibits the target choline esterase (CHE). Plasma and other tissue CHE are important for assessing the extent of poisoning induced by organophosphates. Plasma cholinesterase inhibition by 20–30% usually indicates exposure to organophosphate, whereas 50% inhibition or more is associated with serious poisoning and adverse effects (Wilson, 1998). Many factors may influence the extent of CHE inhibition in OP poisoning. These include the type of OP, its dose, route and duration of exposure, species involved, toxicokinetic aspects of the insecticide, tissues examined, or sampling time. There were reports of significant inhibition of CHE activity in chlorpyriphos intoxicated chicken (Mohammad *et al*, 2008, Kammon *et al*, 2010).

A significant increase in the amount of RER together with the hypertrophy of the Golgi complexes indicated a stimulation of hepatic protein synthesis, which, in turn, most likely provides the basis for the proliferation of the SER. The cells also became vacuolar in appearance as a result of lysis in the mitochondrial matrices.

Mitochondrial changes in liver of chlorpyrifos-intoxicated animals were indicative of the increased energy requirements of the cells in an effort to overcome the toxic effects of the organophosphorus pesticide (Goel and Dhawan, 2001). Mitochondrial swelling, together with swelling and vesiculation of rough endoplasmic reticulum, observed in liver of CPF intoxicated birds in the present study constitutes the changes called cloudy swelling by phagocytosis (Ghadially, 1982). Dilatation of hepatic mitochondria and endoplasmic reticulam associated with vacuolation in the cytoplasm observed in the present study was also reported by Samanta *et al* (2017).

Presence of dense myelin figures, increase in glycogen content of the cells along with peroxisomes and injured mitochondria observed in liver in the present study were also recorded (Braunbeck and Appelbaum, 1999).

Degeneration in glomerulus observed in the present study after chronic exposure to chlorpyriphos may be related with the interaction of the biological membrane with chlorpyriphos during its passage through the filtration barrier (Mossalam *et al*, 2011).

Massive proliferation in SER in the kidney observed in the present study corroborate with the finding of earlier worker (Cheville, 1983).

The effect of chlorpyriphos on the proximal convoluted tubule (PCT) revealed swelling of the mitochondrial membrane. It may be due to the effect of chlorpyriphos metabolites that can produce oxygen free radicals and affect configuration and active transport of the cell membrane. Moreover, the lipophilic metabolites of chlorpyriphos may impair the membrane of the mitochondria and play a role in the mitochondrial dysfunction (Mossalam *et al*, 2011).

There was fusion of foot processes of podocytes. Cheville (2009) suggested that deformation and fusion of foot processes of podocytes is the critical consequence of toxic injury to the podocytes.

Nephro-ultrastructural alterations like Degenerative changes of mitochondria, dilatation of endoplasmic reticulam associated with vacuolation in the cytoplasm observed in the present study were also reported by Samanta *et al*. (2016).

## CONCLUSION

From the present investigation, it can be inferred that chronic exposure to CPF produces ultrastructural alterations in liver and kidney of indigenous chicken. However, the exact mechanism that caused cell damage leading to ultrastructural alterations needs to be elucidated

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