Original Paper

Biochemical and Histopathological Changes in Liver due to Chlorpyrifos Toxicity in Albino Rats

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ABSTRACT

The present study was conducted to examine the biochemical and histopathological changes in liver of albino rats with oral sub-lethal (20 mg/kg) administration of chlorpyrifos as single, double and multiple doses with 48 hr intervals.

Protease and glutamate dehydrogenase (GDH) enzyme activities increased in a dose and time dependent manner. Chlorpyrifos-induced histopathological changes included central venous congestion, sinusoidal haemorrhages, and focal necrotic areas in liver. Diffuse haemorrhagic areas were observed in the heart. Degenerative changes in the muscle layer, hypertrophy of goblet cells, and infiltration and hyperaemic changes in blood vessels were observed in the intestine.

These results suggest that structural integrity of certain organ systems can be disrupted to a great extent from chlorpyrifos exposure.

Key Words: Chlorpyrifos; Protease; Glutamate dehydrogenase; Liver histopathology

Introduction

Modern agricultural practice involves extensive usage of pesticides. A large variety of pesticides are now being synthesized and used by different nations replacing relatively less toxic and low potency chemicals for pest control. Organophosphate compounds were first used for the eradication of pests and insects, and this was first demonstrated by Gerhard and Schrader in Germany.[1] Today they are among the most commonly used groups of pesticides all over the world.[2] Chlorpyrifos is an organophosphate insecticide with a wide range of toxicity in mammals.[3] Since chlorpyrifos is absorbed through the skin, especially through cuts and scratches, dermal contact should be avoided.[4] In addition to causing inhibition of cholinesterase, acute exposure to chlorpyrifos often causes skin irritation. The severity of poisoning with chlorpyrifos will determine the amount and range of symptoms which are experienced.

Protease is an enzyme that causes proteolysis. It begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases are known to break down proteins to small peptides and ultimately to amino acids. They are present in almost all the tissues of mammals.[5] Changes in protease activities indicate the changes in energy cycle. All the proteins under normal conditions, irrespective of their location, are continuously degraded and replaced by new ones.[6] Proteolytic activity is known to increase in various physiological and pathological conditions.[7]

Glutamate dehydrogenase (GDH) is a regulatory enzyme known to check the deamination process to minimize the ammonia level, and plays a significant role in the catabolism of amino acids. GDH catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate and ammonia. This reaction is important in the linkage of nitrogen metabolism to carbohydrate metabolism via Krebs cycle, and it is the main pathway for the transformation of ammonia to α-amino group nitrogen.[8] The gen-

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eral pattern of GDH activity correlates inversely with the decreasing ammonia concentration, suggesting the role of GDH in the production as well as ammonia detoxification.[9]

For understanding the pathological conditions induced in an animal, histological studies are essential to have a clear understanding as to how these insecticides cause injury to the tissues. Some pesticides are toxic at very low concentrations, and impair the metabolic strategy of the animal physiologically and structurally. Pesticides that enter the body via internal digestive system after oral administration are distributed to all parts of the body in their un-metabolised form. Histopathology is useful to distinguish normal cells from abnormal or diseased ones, which helps in the diagnosis of pesticide exposure.

The aim of the present study was to assess the biochemical and histopathological effects in the liver, and to some extent also in the heart and intestine of rats after chlorpyrifos exposure.

Materials and Methods

Test Chemical: Chlorpyrifos Technical (95.30%) was obtained from Nagarjuna Agri Chem Limited, Ravulapalem Mandal, East Godavari District, A.P., India.

Animals: Healthy adult albino rats of same age group (100±10 days) and weight (200±10g) were obtained from the Indian Institute of Science (IISc) Bangalore, India, and maintained at 25±2ºC, with 12 hr light, 12 hr dark cycles, and food and water ad libitum.

Experimental Design: Toxicity of chlorpyrifos was evaluated by Probit method of Finney,[10] and the LD_{50} for albino rats was found to be 200 mg/kg bw. One-tenth of the LD_{50} value (20 mg/kg bw) was selected as sub-lethal dose. The animals were divided into four groups comprising ten animals each. The second, third and fourth groups of animals were termed as experimental animals. These animals were treated 10, 20 and 30 days with 48 hr regular intervals The first group of animals was considered as controls. After the stipulated time, the animals were sacrificed and the tissues were quickly isolated under ice-cold condition, and stored in formalin for histopathological studies, and in deep freezer at -80°C for biochemical analysis.

Biochemical Investigations: Five percent homogenate of the tissues were prepared in 0.25M ice-cold sucrose solution for glutamate dehydrogenase (GDH), and ice-cold distilled for protease, and these were centrifuged at 2500 rpm for 10 min in a refrigerated centrifuge at 4ºC to remove cell debris. Clear cell-free extracts were used as enzyme source. Protease activity was measured with the reaction mixture in a volume of 2 ml containing 100 micromoles of phosphate buffer (pH 7.4), 20 mg of heat denatured haemoglobin as substrate, and 0.5ml of the supernatant.[11] The contents were incubated at 37°C for 30 minutes and the reaction was stopped by the addition of 2 ml of 10% TCA. Zero time controls were prepared by adding 2 ml of 10% TCA prior to the addition of enzyme source. The contents of the samples were filtered and the free amino acid level was determined in the filtrates. To 0.5 ml of aliquot of the filtrate, 2 ml of ninhydrin reagent was added. The contents were heated in boiling water bath for 5 minutes and cooled. The volume was made up to 10 ml with distilled water and read at 570 nm against a reagent blank in a spectrophotometer. The proteolytic activity was expressed as micromoles of tyrosine equivalents / mg protein / hr.

Glutamate dehydrogenase (GDH) activity was measured by the method of Lee and Lardy (1965).[12] The incubation mixture contained 100 micromoles of phosphate buffer (pH 7.4), 40 micromoles of sodium glutamate, 0.1 micromoles of NAD, 4 micromoles of 2,4-iodophenyl-3-(nitrophinyl)-5-phenyltetrazolium chloride (INT), and the enzyme source. This was incubated for 30 min at 37ºC and stopped with 5.0 ml of glacial acetic acid. The colour was extracted by shaking with 5.0 ml of toluene. After keeping the tubes overnight at 4ºC, the colour extract was measured. All spectrophotometric measurements were determined using Hitachi U-2800 model spectrophotometer. The enzyme activity was expressed as micromoles of formazon formed/mg protein/hr.

Histopathological Analysis: Tissues were isolated from control and chlorpyrifos-treated rats. They were gently rinsed with a physiological saline solution (0.9% NaCl) to remove blood and debris adhering to the tissues. They were fixed in 5% formalin for 24 hrs. The fixative was removed by washing through running tapwater overnight. After dehydrating through a graded series of alcohols, the tissues were cleared in methyl benzoate, and embedded in paraffin wax. Sections were cut at 6 micron thickness and stained with haematoxylin, and counter-stained with eosin (dissolved in 95% alcohol). After dehydration and clearing, sections were mounted with DPX and observed under the microscope.

Results

The present study reveals significant variation in protease and GDH activities after exposure to chlorpyrifos in liver tissues of albino rats **(Table 1)**. Protease and GDH content in experimental animals showed significant increase ($p<0.05/0.01$). Alterations in enzyme activities were dependent upon time and sub-lethal concentrations in treated albino rats. The histological sections of control and chlorpyrifos-treated liver tissue are presented in **Plate 1**.

Histopathology of liver: Normal liver clearly demonstrates central vein, sinusoids, hepatocytes, and centrally placed prominent nucleus **(Plate 1; Fig A).**

Rats administered 10-day exposure to chlorpyrifos showed central vein congestion (CVC), cellular swelling (CS), cytoplasm porus in sub-capsular hepatocytes, sinusoidal haemorrhage (SH) and degenerative changes in hepatocytes **(Plate 1; Fig B)**.

Rats administered 20-day exposure to chlorpyrifos showed central vascular congestion (CVC) and dilated sinusoids (DS) at 10x magnification. Sinusoidal haemorrhage and focal necrotic areas (FNA) were evident at 40x magnification **(Plate 1; Fig C & D)**.

Rats administered 30-day exposure to chlorpyrifos showed severe degenerative changes in central vein (SDCV) and diffuse necrotic areas (DNA) at 10x, and clear necrotic areas (DNA) at 40x magnification **(Plate 1; Fig E & F)**.

Discussion

Protease is an enzyme which conducts proteolysis, that begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases are known to break down proteins to small peptides and ultimately to amino acids. They are present in almost all the tissues of mammals. Under proteolysis, enhanced breakdown dominates over synthesis, while in the case of anabolic process, increased synthesis dominates the protein breakdown.[13] Further, histopathological damage and hydromineral imbalance during pesticide stress has been reported to account for the elevated protease activity.[14]

Increase in protease activity observed at single, double and multiple doses of chlorpyrifos on different tissues of albino rats were clearly reflected in breakdown of proteins **(Table 1)**. Proteases have been shown in earlier studies to be activated during stress conditions, indicating a possible relation between inactivation of oxidative enzymes, reduction in energy production and acceleration of proteolysis.[15]

In this study, chlorpyrifos caused significant increases in protease activity in the treated rats; similarly, several authors have reported increased protease activity in different animal models under pesticidal toxicity, such as in fishes treated with atrazine,[16] and cypermethrin.[17,18] Increased protease activity in tissues of various experimental animals exposed to hexachlorophene, sodium selenite and aluminum acetate have also been reported.[19-21]

The elevated protease activity, in general, indicates profound loss of proteins causing structural disorganization and disassembly of structural proteins in different tissues during chlorpyrifos toxicity.

Glutamate dehydrogenase (GDH) catalyzes the reversible reaction of oxidative deamination of glutamate to α-ketoglutarate and ammonia, and plays an important role in the catabolism and biosynthesis of amino acids.[13] GDH activity levels were increased in the tissues of rats exposed to sublethal doses of chlorpyrifos in this study **(Table 1)**. GDH occurs with high activity in the mitochondrial matrix, and is commonly used as a marker for matrix space.[22] It has great importance in neurotransmitter balance in brain tissue and maintenance of nitrogen in liver tissue. GDH also plays an important role in detoxification of ammonia.[23]

Glutamate dehydrogenase (GDH) is also known to play a crucial role in protein metabolism in the cells affected by a variety of effectors.[24] This enzyme has several metabolic functions with great physiological significance. It is closely associated with the detoxification mechanisms of tissues. GDH in extra-hepatic tissues could be utilized for channeling of ammonia released during proteolysis for its detoxification into urea in the liver. Hence, the activity of GDH is considered as a sensitive indicator of stress.[25] In this study, chlorpyrifos caused significant increases in GDH activity in the treated rats. Similar results have been reported in other animal models due to pesticide toxicity, such as in fishes treated with atrazine,[16] or cypermethrin,[26] Suhasini et al reported increased GDH activity in liver tissue of albino rat exposed to hexachlorophene,[19] while John Sushma et al reported increased GDH activity in tissues of mice exposed to aluminium acetate.[21]

Plate I

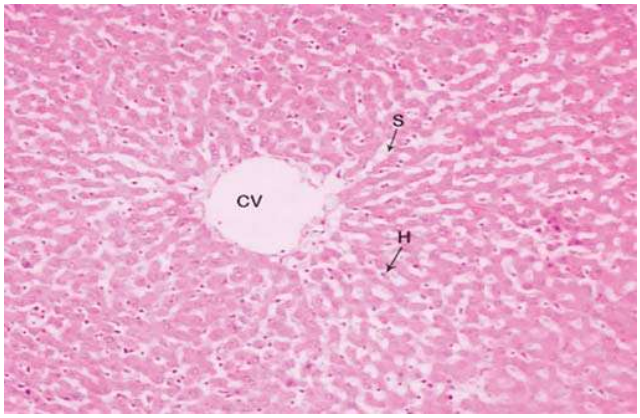


Fig A: Control rat liver showing hepatocytes (H) with centrally placed prominent nucleus (N) with sinusoids (S) and central vein (CV). H&E 10x.

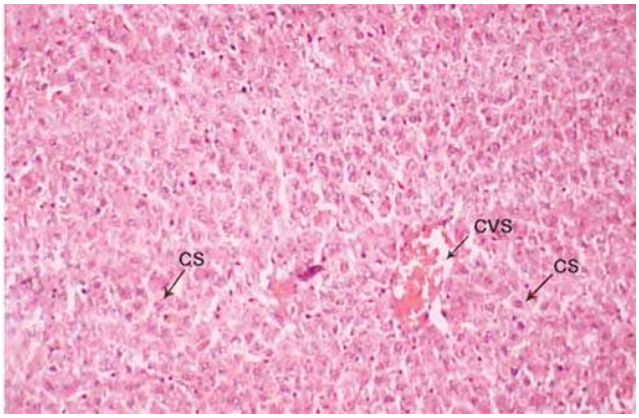
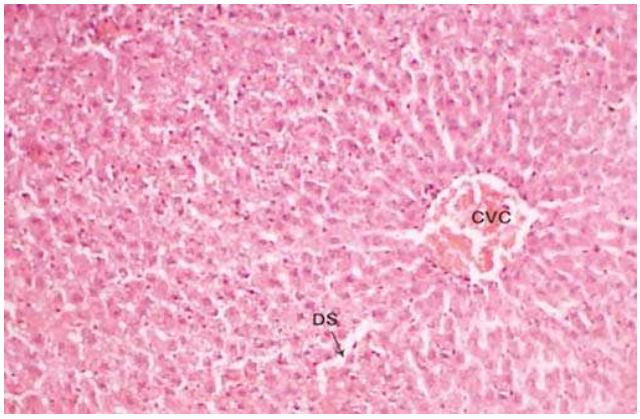


Fig B: 10-day chlorpyrifos-administered rat liver showing central vein congestion (CVC), cellular swelling (CS) cytoplasm porus in sub-capsular hepatocytes, sinusoidal haemorrhage (SH) and degenerative changes in hepatocytes. H&E 10x.



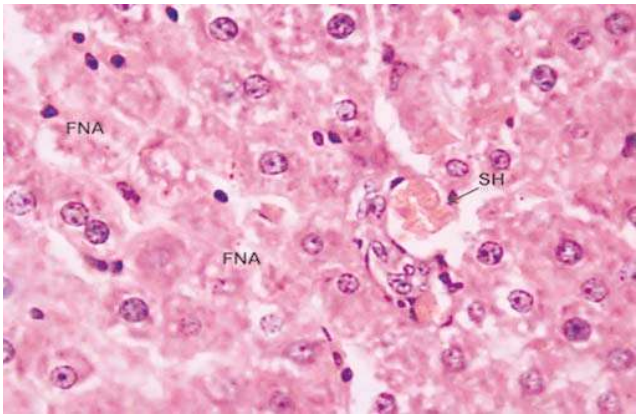
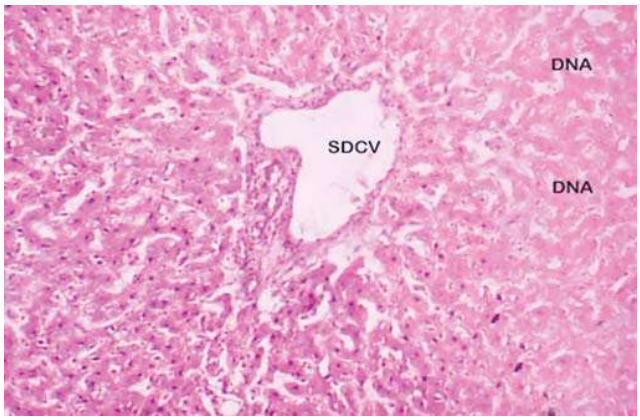


Fig C & D: 20-day chlorpyrifos-administered rat liver showing central vein congestion (CVC) and dilated sinusoids (DS). H&E 10x and 40x respectively



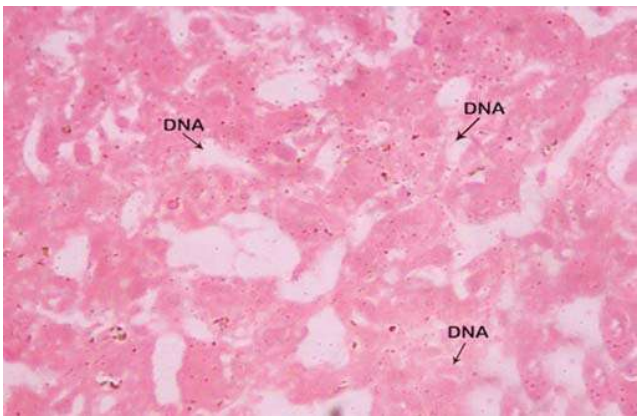


Fig E & F: 30-day chlorpyrifos-administered rat liver showing severe degenerative changes in central vein (SDCV) and diffuse necrotic areas (DNA). H&E 10x and 40x respectively.

Table 1 Changes in protease and glutamate dehydrogenase activities in liver tissues of control and chlorpyrifos-treated albino rats

Parameters	Control	10 days	20 days	30 days
Protease (micromoles tyrosine/ mg/protein/hr)	1.252 ± 0.549	1.292 ± 0.051 (3.218)	1.486 ± 0.060 (18.688)	1.667 ± 0.0573 (18.688)
GDH (micromoles formazon/ mg/protein/hr)	0.238 ± 0.010	0.259 ± 0.010 (9.6)	0.277 ± 0.006 (18.606)	0.308 ± 0.006 (31.52)

Since liver is the major metabolic centre for detoxifying pesticides, it was also adversely affected with central vascular congestion, cellular swelling of sub-capsular hepatocytes, sinusoidal haemorrhages, and degenerative changes in hepatocytes. All these changes were more pronounced in multiple-dose administered animals, which clearly indicates that repeated administration may result in severe damage of vital organs and make them less fit **(Plate 1; Fig A-F)**. Several authors have reported histopathological changes in liver in different animal models due to pesticide toxicity. Luty et al reported small lymphocytic infiltrations in the areas of blood vessels, and accumulation of nuclei of hepatocytes in the sub-capsular layer in liver tissues of rats exposed to paraquat.[27] Latuszynska reported a few small infiltrations consisting of lymphocytes and histiocytes, and signs of parenchymal degeneration of hepatocytes in the liver of rats exposed to chlorpyrifos and cypermethrin.[28] Tos-Luty et al observed degenerative changes of hepatocytes with infiltration of lymphocytes, Kupffer cell proliferation, and nuclear anisocytosis in hepatocytes in the liver of rats exposed to fenvalerate.[29] Manna et al reported congestion and fatty changes in livers of rats exposed to deltamethrin.[30] Sinusoidal congestion and parenchymatous degeneration of hepatic cells and fibrosis in hepatic portal areas have been observed in the liver of white leghorn cockerels exposed to acephate.[31]

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