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Cypermethrin Toxicity in the Liver of Developing Chick Embryo

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Abstract.- Toxicity of cypermethrin insecticide was evaluated in the liver of 16-day old chick embryo following administration of a single sub-lethal dose of cypermethrin into the eggs on day '0' of incubation. The biochemical analysis of the liver revealed that the activities of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase were significantly increased. The activities of amylase, alkaline phosphatase and acid phosphatase remained unchanged. Besides that hepatic glucose, total protein, total lipids, cholesterol, urea, uric acid and RNA contents were found to decrease, while, glycogen and DNA contents were elevated. The hepatic soluble protein and free amino acids contents, however, remained unaltered. Cypermethrin-induced histopathological changes in liver included increased sinusoidal spaces in hepatic parenchyma, cytoplasmic vacuolations in hepatocytes, hepatocytic nuclear condensation, fatty degeneration, hydropic degeneration and necrosis of hepatocytes.

Key Words: Cypermethrin, chick embryo, liver biochemistry, liver histology.

INTRODUCTION

Pyrethroid insecticides are widely used against storage pests with greater efficacy (Watters *et al.,* 1983) as well as against the pests of other agricultural-products like cotton, vegetable oils, vegetables and fruit etc (Usmani and Knowles, 2001) and there is increased risk of food being contaminated with the insecticide, which may harm domesticated animals including poultry and humans. Cypermethrin, (RS)-α-cyano-3phenoxy benzyl (IRS)-cis, trans-3-(2,2-dichlorovinyl)-2, 2-dimethyl cyclopropane carboxylate that belongs to type II pyrethroid is a photostable possessing α-cyano group with high insecticidal activity.

Cypermethrin produces drastic effects on both the non-target invertebrates (Gowlan *et al*., 2002) and vertebrates (Das and Mukherjee, 2003). In invertebrates, for example in freshwater prawn *Palaemonetes argentinus,* low doses of cypermethrin affect the survival and alter metabolic activity while high doses cause mortality (Collins and Cappello, 2006). In vertebrates, cypermethrin toxicity includes the biochemical changes both in the blood and tissues as well as histopathological changes in different organs of the body. Cypermethrin-induced changes in the activities of enzymes like aldolase, carboxypeptidase A, alpha chymotrypsin, ATPase, lactate dehydrogenase, lipase, phosphorylase a, phosphorylase b, SDH and trypsin as well as in the biochemical components like glycogen, lactate, pyruvate, protein, DNA and RNA were observed in different organs of different species of fishes *viz., Tilapia mossambica* (Reddy and Yellamma, 1991a), *Lepidocephalichthyes thermalis* (Sheela and Muniandi, 1992), Carp Fish (Simon *et al*., 1999) and *Labeo rohita* (Das and Mukherjee, 2003).

Cypermethrin induced increases in peripheral natural killer cell and antibody dependent cytotoxic activity in rats (Santoni *et al*., 1997) and increases in the number of numerical chromosome aberrations of the bone marrow cells, decreases in delayed type hypersensitivity reaction, mean cell volume of the RBCs and Ht value and white blood cell count in the peripheral blood of male Wistar rats have also been reported. (Institoris *et al.,* 1999). Haratym-Maj (2002) observed an increase in the number of leukocytes in peripheral blood and inhibition and mobilization of hemopoietic system in female mice following cypermethrin administration.

Cypermethrin is also known to affect the development of animals. Gonzalez-Doncel *et al.* (2005) have shown that the exposure of the embryos of *Oryzias latipes* to cypermethrin had resulted in visceral oedemas intimately associated to the gall bladder with subsequent pericardial oedemas in the developing organs of these embryos. In

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Physalaemus biligonigerus tadpoles (Anura: Leptodactylidae), it induces apoptosis in the telencephalon (Izaguirre *et al*., 2000).

Although a lot of work has been done on the toxicity of cypermethrin in fishes and mammals, little is known about chicks (Kapoor *et al*., 1988). Since liver is responsible for the detoxification of endogenously produced waste products or exogenously derived toxins and drugs as well as metabolism of xenobiotics is thus a frequent target of drug toxicity. In addition, the young animals are more sensitive to the toxic effects of cypermethrin than old animals because of their less developed metabolic capacity (Sheets, 2000). Therefore, the present study was designed to evaluate the toxicity of cypermethrin in the liver of developing chicks to determine the efficiency of developing liver to metabolize the toxin.

MATERIALS AND METHODS

Fertilized eggs obtained from Government Poultry Farm at Muzaffarabad Azad Kashmir, Pakistan were injected with different concentrations of cypermethrin insecticide. Dilutions were made in acetone. LD_{50} was determined by using probit analysis. After measuring the LD_{50}, which was found to be 676 ppm, a single sub lethal dose (0.05 ml) of cypermethrin of various concentrations such as 50, 100, 200 and 400 ppm was injected into the yolk of each egg at vegetal pole by disposable tuberculin syringe at day '0' of incubation. Equal amount of acetone was injected into the controls. The eggs were incubated at 38±0.5°C in incubators and were rotated every two hours to avoid the sticking of the embryos to the shell membranes.

On day 16 of incubation, the liver from each embryo was taken out, weighed and divided into three parts. One part was used for making saline homogenate; second part for the extraction of lipid, cholesterol and nucleic acids and the third part fixed in Bouin's fluid for histological studies.

The saline homogenate was used for the estimation of alkaline phosphatase (AkP, Orthophosphoric monoester phosphohydrolase, alkaline optimum, EC: 3:1:3:1) and acid phosphatase (AcP, orthophosphoric monoester phosphohydrolase, acid optimum, EC: 3:1:3:2) activities according to Kind and King (1954); lactate dehydrogenase (LDH, L, Lactate NAD oxidoreductase (EC 1:1:1:27) activity by a method based on Cabaud and Wroblewski (1958); aspartate aminotransferase (AST; L, aspartate: 2 oxoglutarate aminotransferase, EC 2:6:1:1) and alanine aminotransferase (ALT; L, alanine, 2 oxoglutarate aminotransferase, EC 2:6:1:2) activities according to Reitman and Frankel (1957); and amylase (1, 4 a-D glucanhydrolase, EC 3:2:1:1) activity according to the procedure described by Wootton and Freeman (1964). The saline homogenate was also used for the estimation of glucose content by O-toluidine method of Hartel *et al*. (1969), soluble and total protein content by the method of Lowry *et al*. (1951), amino acid content according to the ninhydrin method of Moore and Stein (1957), urea content according to the diacetyl monoxime method as described by Natelson *et al*. (1951), and uric acid content according to the method described by Carraway (1963). Protein extract was prepared by digesting freshly prepared saline homogenate in 0.5N NaOH for 24 hours. Glycogen content in the supernatant left after centrifugation (removal of protein) was precipitated with ethanol and then dissolved in distilled water and estimated by the Anthrone method of Consolazio and Lacono (1963).

For the extraction of total lipid and cholesterol, the tissues were ground in hot ethanol (60°C) and kept for extraction overnight. After centrifugation at 5,000 rpm for 10 minutes, the supernatant was obtained and used for the estimation of total lipid by Vanillin reagent (Zöllner and Kirsch, 1962) and cholesterol content according to Liebermann and Burchardt Reaction (Henry and Henry, 1974). Nucleic acids were extracted according to the method described by Shakoori and Ahmed (1973). The pellet left after lipid extraction was used for the preparation of RNA and DNA extracts. RNA extract was prepared in 20% cold PCA and estimated according to the Orcinol method, while DNA was extracted in 10% hot perchloric acid (PCA) and estimated according to diphenylamine method. Both these estimations followed the procedure as described by Schneider (1957).

Histological studies of liver

Liver tissues fixed in Bouin's and eosin fluids were processed for wax embedding and then 6-8 μm thick sections were stained in hematoxylin. The sizes of both the hepatic cells and their nuclei were measured using ocular micrometer that was calibrated against stage micrometer.

RESULTS AND DISCUSSION

Biochemical changes in liver

Table I shows the cypermethrin-induced changes in some of the enzyme activities and biochemical constituents of liver of 16-day old chick embryo following administration of different doses of insecticide. Among enzymes, the activities of AST, ALT and LDH were significantly elevated, whereas those of amylase, AkP and AcP remained unchanged. Both the transaminases, ALT and AST showed increases at 400 ppm by 94% and 54%, respectively, whereas LDH activity increased at 100, 200 and 400 ppm by 107, 90 and 118%, respectively.

Of the biochemical components hepatic glucose and total protein contents decreased, while, glycogen and DNA contents increased. All other components *viz*., soluble protein, free amino acids, total lipids, cholesterol, urea, uric acid and RNA contents remained unaltered. Glucose and glycogen showed significant changes at 400 ppm. Glucose content showed 33% decrease whereas glycogen showed 278% increase in the developing liver. Total protein contents decreased at 200 and 400 ppm by 33 and 40%, respectively. The DNA content also increased at all the doses 102% at 50ppm, 33% at 100ppm, 35% at 200ppm and 29% at 400 ppm while RNA remained unchanged.

Cypermethrin treatment resulted in the significant decrease in glucose and an increase in glycogen contents of the liver of 16-day-old chick embryo at the dose of 400 ppm. Decrease in hepatic glucose might have occurred as a result of increased demand of energy under stress or its utilization in glycogen synthesis; as glycogen content was increased in the present study. These results are in accordance with the results of Manna *et al.* (2004) who also observed increase in hepatic glycogen content in rats treated with cypermethrin. Langslow and Hales (1971) and Hazelwood (1972) reported that the avian pancreas is richly endowed with glucagon and that the plasma levels of glucagon in birds are higher than in man. In the light of these observations it can be speculated that cypermethrin might have interacted with glucagon the hormone responsible for glycogenolysis and thus resulted in increased hepatic glycogen content. Decrease in hepatic total protein content as observed in the present study significantly correlates with the necrotic changes in the liver. Increase in hepatic DNA content with cypermethrin is in agreement with the work of Das and Mukherjee (2003) who also reported the similar results in Indian major carp, *Labeo rohita*, following cypermethrin treatment.

Increase in hepatic ALT, AST and LDH activities as observed in the present study are in agreement with the work of Reddy and Yellamma (1991b) and Khan *et al*. (2009) who noticed cypermethrin-induced increases in ALT and AST activities in liver of the fish *Tilapia mossambica* and in male dwarf goats *Capra hircus,* respectively. Increases in hepatic ALT and ASI activities were also observed in Freshwater fish *Cirrhinus mrigala* exposed sublethal concentration (1 μg/L) for 1, 7, 14, 21 days of cypermethrin (Prashanth and Neelagund, 2008). ALT catalyzes the interconversion of alanine and pyruvic acid. Accumulation of pyruvic acid in the cells occurs when enzymes of the citric acid cycle and the enzymes which hydrolyses ATP *i.e.,* ATPase to generate energy are inhibited. The activities of the citric acid cycle enzymes like succinate dehydrogenase, isocitrate dehydrogenase and the activity of cytochrome c oxidase the enzyme of the electron transport chain is known to be inhibited by cypermethrin (Reddy and Yellamma, 1991a). Cypermethrin-induced inhibition of succinate dehydrogenase at mitochondrial level has also been observed in various tissues of common carp, *Cyprinus carpio* (Kamalaveni *et al.,* 2001). Das and Mukherjee (2003) observed the inhibition of succinate dehydrogenase and ATPase activities in brain, kidney and liver of the Indian major carp, *Labeo rohita,* while the inhibition of Na, K and Mg dependent ATPase activity was observed in the liver of albino rats following cypermethrin treatment (El-Toukhy and Girgis, 1993).

Table I.- Effects of cypermethrin treatment on the activities of some of the enzymes biochemical constituents of the liver of 16-day old Chick embryo developed from eggs administered with (0.05ml) of cypermethrin of various concentrations (50, 100, 200 and 400ppm). Cypermethrin was dissolved in acetone. Control eggs received acetone only.

Parameters	Control (n=6)	50ppm (n=6)	100ppm (n=6)	200ppm (n=6)	400ppm (n=6)
Amylase (So U/g)	122.12±42.84[a]	65.65±32.37	138.81±80.07	129.21±25.01	108.92±0.9
AkP (KAU/g)	0.45±0.08	0.76±0.19	0.29±0.56	0.65±0.1	0.81±0.27
AcP (KAU/g)	2.52±0.41	2.87±0.58	1.83±0.48	2.09±0.12	1.98±0.07
AST (IU/g)	38.3±7.3	42.73±10.44	43.98±18.47	53.21±4.71	58.82±5.85* 54
ALT (IU/g)	1.44±0.25	1.45±0.62	2.34±0.76	2.26±0.62	2.8±0.52*
LDH (IU/g)	52.41±5.9	75.01±21.44	108.35±22.87*	99.54±6.88**	114.09±14.55**
Glucose (mg/g)	18.56±1.95 [a]	17.7±4.3	20.66±4.18	21.98±2.3	12.38±1.48*
Glycogen (mg/g)	3.81±0.83	4.05±1.35	5.97±2.25	4.46±1.1	14.42±4.16*
Total protein (mg/g)	124.53±13.31	113.17±17.92	97.06±11.94	83.3±6.13*	75.08±5.32**
Soluble protein (mg/g)	46.83±5.8	57.96±6.56	39.2±10.47	47.19±1.68	45.98±3.67
Free amino acids (mg/g)	14.66±3.88	10.37±2.85	12.57±5.11	10.41±1.04	11.11±1.8
Total lipids (mg/g)	66.28±3.24	63.28±2.18	89.54±10.58	85.37±11.48	72.71±5.7
Cholesterol (mg/g)	19.3±2.87	18.49±1.05	22.09±1.72	23.72±4.34	17.05±1.23
Urea (mg/g)	1.12±0.48	1.08±0.31	0.47±0.12	0.47±0.07	1.04±0.49
Uric acid (mg/g)	0.64±0.14	0.46±0.1	0.85±0.34	0.77±0.15	0.7±0.11
DNA (mg/g)	1.82±0.06	3.67±0.65*	2.42±0.2*	2.46±0.22*	2.34±0.13**
RNA (mg/g)	8.45±0.57	9.16±0.64	9.18±1.18	8.39±0.84	7.67±0.69

[a] , Mean ± SEM; *, P < 0.05; **, P < 0.01; using student 't' test.

[b] , Abbreviations used: IU: International unit, the amount of enzyme which under defined assay conditions catalyzes the conversion of one micro mole of substrate per minute, SoU: Somogyi Unit: the amount of enzyme that catalyses the digestion of 5mg of starch under the experimental condition, KAU; King Armstrong Unit: The amount of enzyme that transforms one mg of phenol in 15 minutes.

Table II.- Cypermethrin-induced changes in some of the morphometric Parameters of H&E stained sections of the liver of 16th day old chick embryo developed from eggs administered with (0.05ml) of cypermethrin of various concentrations (50, 100, 200 and 400ppm dissolved in acetone). Control eggs received acetone only.

Parameters	Control	50ppm	100ppm	200ppm	400ppm
Number of cells/ Microscopic field (n=90)	582.6±33.5	303.3±22.2***	328.9±33.7***	316.8±20.0***	234.7±22.1***
No. of nuclei/cell (n=90)	1.0±00	1.0±00	1.0±00	1.0±00	1.0±00
No. of nucleoli/nucleus (n=90)	2.8±0.06	1.98±0.19***	1.46±0.09***	1.6±0.12***	1.69±0.02***
Size of cell $(\mu m)^2$ (n=90)	83.2±2.6	120.8±5.9***	184.8±10.4***	242.1±14.5***	242.2±14.9***
Size of nucleus $(\mu m)^2$ (n=90)	35.4±0.68	30.64±1.27**	32.85±0.67*	38.17±1.02**	40.33±1.46**

[a] , Mean ± SEM;

*, P < 0.05; **, P < 0.01; ***, P < 0.001, using student 't' test.

Damage to mitochondria and the inhibition of the enzymes of citric acid cycle, electron transport chain and ATP hydrolyzing enzyme with cypermethrin can result in the increased pyruvate level. This increased pyruvate level may lead to the induction of the enzymes which convert pyruvic acid either back to lactic acid by lactate dehydrogenase or to alanine by ALT. In the present study, in addition to increase in ALT activity, LDH activity was also increased. Increase in ALT and LDH activities might have occurred as a result of their induction due to increased pyruvate level. Disturbance in metabolism with cypermethrin thus can result in the changes in the biochemistry of liver

and hence lead to dysfunctioning of various organs of the body.

Histological changes in liver

Hematoxylin and eosin stained liver sections from the vehicle-treated control chick embryos were normal in appearance (Fig. 1). Treatment of embryos with cypermethrin at the dose of 50 ppm resulted in the change in hepatic architecture. Hepatocytes within parenchyma, particularly in midzonal area were clumped together in a way that they formed a masses having rounded appearance. Nuclei in the hepatocytes of the rounded masses seem to be pushed towards periphery. Nucleus/cytoplasm ratio was reduced from 1:4 to 1:1 in some of the hepatocytes, while in others remnants of the nuclei with clear cytoplasm were seen. A few actively dividing hepatocytes were also observed. Because of this clumping of hepatocytes together, there was an increase in the sinusoidal spaces. Some hypertrophied hepatocytes bordering central vein had clear cytoplasm and possessed two or more nuclei of distorted shape. Some foci of basophilic hepatocytes having actively dividing cells were also seen. Nuclear polymorphism was common. A few hepatocytes bordering central vein were necrotic. Neutrophil and blood cell infiltration was also seen. These features indicate some degenerative changes in the hepatocytes (Figs. 1BC). At 100 ppm, hypertrophied hepatocytes and some necrotic areas are visible. Rounded masses like structures were much more prominent as compared to those observed in the liver sections from 50 ppm cypermethrin-treated embryos (Figs 1 D, E). At 200 ppm, the histopathological changes were diverse and involved whole of the liver lobule. Rounded structures were so close to one another that they had completely occupied the sinusoidal spaces within the parenchyma. Hepatocytes bordering the blood vessels were elongated and compressed (Fig. 1F). At 400 ppm, the loss of hepatocytes in the hepatic parenchyma is visible. In addition, an outgrowth like structure was seen bordering the liver parenchyma and this outgrowth represent some classical features of tumors ie. increased number of hepatocytes and increased nucleus/cytoplasm ratio in the perivenal zone (Fig. 2).

Table II shows the effect of single treatment of cypermethrin on the histological parameters of liver of 16-day old chick embryo. Cypermethrin treatment resulted in the decrease in number of cells/microscopic field and increase in the size of cell at all the doses. The number of nucleoli/ nucleus was decreased at all the dose levels. The size of the nuclei was decreased at 50 and 100 ppm and increased at 200 and 400 ppm. The number of nuclei/cell remained unchanged with cypermethrin treatment.

Hepatic sections from the embryos of the cypermethrin-treated group revealed two types of changes; a) increased sinusoidal spaces, fatty change in hepatocytes, hypertrophy of the hepatocytes, hydropic degeneration and some necrotic changes in the hepatocytes and b) clumping of hepatocytes to form rounded mass like structures, which showed a progressive change with dose. For example, at 50 ppm, these structures were confined only to midzonal area of the liver lobule. At 100 ppm, these structures were much more prominent as compared to those found at the dose of 50 ppm. At 200 ppm, these structures involved whole of the liver lobule. The cells bordering the central vein were smaller in size and looked compressed because of active growth, which significantly correlates with the increased hepatic DNA content. Nucleus/ cytoplasm ratio was decreased from 1:4 to 1:1 or 1:2. Increase in sinusoidal spaces occurs as a result of toxic insult and its purpose is to increase the blood flow in the liver lobule in order to increase the oxygen and nutrient supply to meet the energy requirements of the hepatocytes under stress. Necrotic changes in the hepatocytes have also been observed in the liver of male dwarf goats (*Capra hircus*) following cypermethrin administration (Khan *et al.,* 2009)

Fatty changes in hepatocytes significantly correlate with the increased total lipid content in the liver. Hydropic degeneration and necrosis occurs as a result of disturbance in Ca^{+2} homeostasis.

Histopathological changes in the liver sections from treated animals show necrotic areas. Cypermethrin is reported to induce free radicals in the liver of male New Zealand white rabbits (El-Demerdash *et al.,* 2003) as well as lipid peroxidation in rat erythrocytes (Gabbianelli *et al.,* 2002) thus induce oxidative stress. Necrotic changes

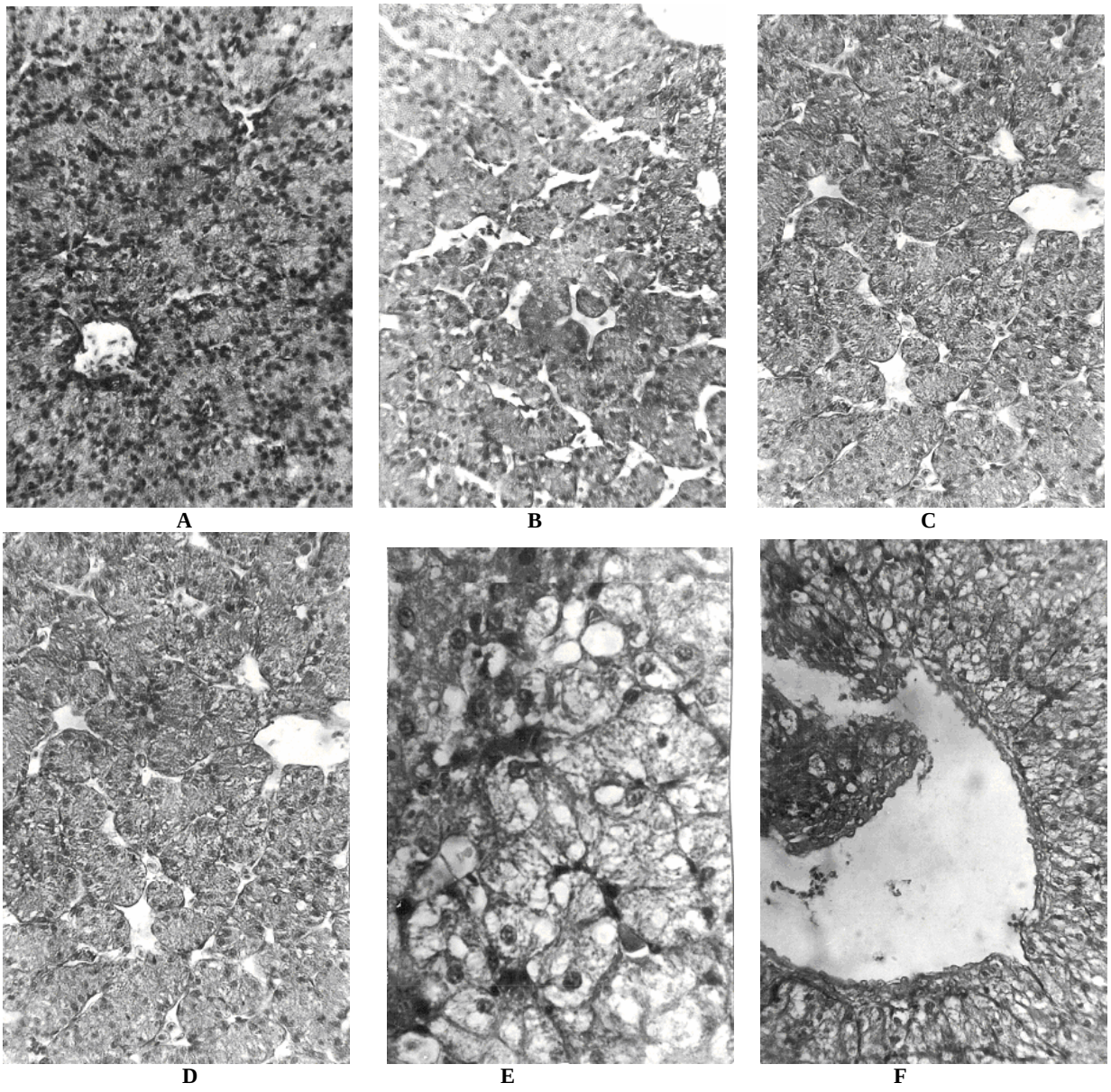

Fig. I. Histological Structure of liver of control and cypermethrin treated 16 days old chick embryo.
Stain: Hematoxylin and eosin
Magnification: 40X/100X
A. Liver sections from the control embryo showing normal appearance of the hepatocytes.
B and C. liver sections from the 50ppm cypermethrin treated 16th day old chick embryo: B, showing the necrotic hepatocytes in centrilobular zone and; C, Hepatocytes have been clumped together to form a rounded mass like structures in centrilobular zone that has resulted in increased sinusoidal spaces .
D and E. Liver sections from the100ppm cypermethrin treated 16th day old chick embryo. D) Clumping of hepatocytes together to form a rounded mass like structures is much more common E) Showing the necrotic hepatocytes in centrilobular zone, anucleated hepatocytes, hypertrophied hepatocytes and hepatocytes with nuclear condensation are also seen. Cytoplasmic vacuolations are common.
F.Liver sections from the200ppm cypermethrin treated 16th day old chick embryo. Number of hepatocytes in the centrilobular zone is much increased which may be due to increased cell division. A few necrotic hepatocytes and the hepatocytes with pyknotic nuclei are also seen.

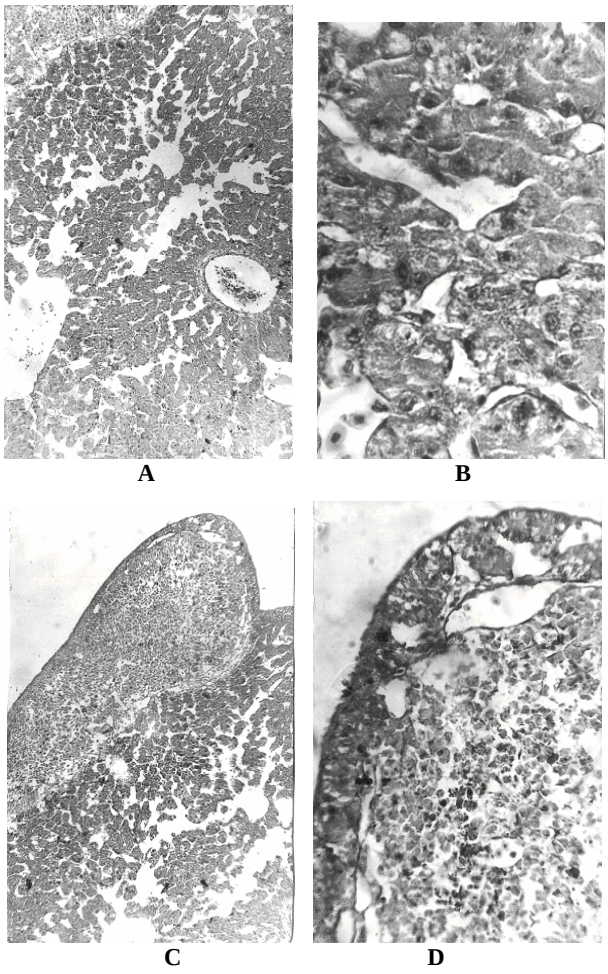

Fig. 2. Histological Structure of liver of 400ppm cypermethrin-treated 16 days old chick embryo.
Stain: Hematoxylin and Eosin
Magnification: 40X/100X
A and B. showing the loss of hepatocytes from the parenchyma that has resulted in increased sinusoidal spaces. A few necrotic hepatocytes and the hepatocytes with nuclear condensation are also seen.
C and D: An outgrowth like structure is seen bordering the liver section. Part of the outgrowth away from the hepatic parenchyma is bound by a layer formed of hepatocytes. However, the part of this outgrowth adjacent to hepatic parenchyma is encircled by multinucleated elongated structure that separated it from the hepatic parenchyma. Inside this outgrowth, diverse types of structures are present. Some of these structures are small-sized cells while some structures are distorted hepatocytes that looked like debris.

in hepatocytes as observed in the present study might have occurred as a result of cypermethrin-induced free radicals formation as well as lipid peroxidation. Lipid peroxidation is a chain reaction resulting from the spread of highly reactive polyunsaturated fatty acids radicals, initiated by the attack of hydroxyl radicals on the unsaturated bonds of membrane phospholipids (Tribble *et al.,* 1987). Cypermethrin-induced oxidative damage is also evident from the findings of Manna *et al.* (2004) who observed increase in malondialdehyde level and decrease in the activities of catalase, superoxide dismutase in liver of rat.

At 400 ppm an outgrowth like structure was observed and this structure could be tumor like outgrowth as the cells within this outgrowth were not differentiated. Increased hepatic DNA content as observed in the present study (Table I) also support the tumor formation. Tumors result when cells are transformed as a result of exposure of normal cells either to non-genotoxic or to genotoxic carcinogens. Evidence for the carcinogenicity of cypermethrin comes from the findings of Slamenova *et al*. (1992) who found that cypermethrin induces anchorage independent growth of BHK21 cells and morphological transformation of Syrian hamster embryo cells. Other evidence for the carcinogenic activity of cypermethrin comes from the findings of Kornuta *et al.* (1996) indicating that cypermethrin exerts its carcinogenic activity by interacting directly with DNA thus damaging its structure. Shukla *et al.* (2002) also observed cypermethrin-induced tumor formation in both male and female Swiss albino mice. Cypermethrin is also known to induce MCF-7 cell proliferation in vitro (Chen *et al.,* 2002). Evidence for the genotoxicity of cypermethrin comes from the findings of Giri *et al*. (2003) who observed that cypermethrin (5, 10 and 20 mg/kg) induces significant increases in the frequency of sister chromatid exchanges in mice and from the findings of Shukla and Taneja (2002) who observed the mutagenic potential of cypermethrin (induction of dominant lethal mutations in male germ cells) using the dominant lethal assay in male Swiss albino mice. Finding of these authors suggest that cypermethrin can act both as genotoxic and non-genotoxic carcinogenic agent

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