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Effect of a Single Sublethal Dose of Permethrin on the Development of Liver in Chick Embryo

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Abstract.- Present study was designed to evaluate the toxicity of single sublethal doses of various concentrations (25, 50, 100 and 200 ppm) of permethrin insecticide on the liver of 16-day old chick embryo. Permethrin was injected in to the eggs on day '0' of incubation. Liver was analysed for the estimation of a few enzyme activities and some biochemical constituents. Among enzymes, the activities of alkaline phosphatase (AkP), acid phosphatase (AcP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were significantly decreased, whereas the activity of amylase remained unchanged. From among biochemical components glucose, glycogen, total protein, soluble protein, urea and RNA contents were significantly decreased, while total lipids and FAA content were significantly elevated. However, cholesterol, uric acid and DNA contents remained unaltered with permethrin treatment. Permethrin-induced histopathological changes in liver showed increased sinusoidal spaces in hepatic parenchyma, cytoplasmic vacuolations in hepatocytes, hepatocytic nuclear condensation, fatty degeneration, hydropic degeneration and necrosis of hepatocytes. In addition, a few histological parameters like number of cells /microscopic field, number of nuclei/cell and the number of nucleoli/nucleus were also affected with permethrin treatment. The insecticide therefore had a serious toxic effects on the developing liver.

Key words: Permethrin, chick embryo, liver biochemistry, liver histology.

INTRODUCTION

Insecticides are used against pests to increase the production of food grains and other agricultural-products like cotton, vegetable oils, vegetables and fruit etc. These toxic chemicals however, have adversely affected the non-target organisms including poultry. Insecticides from hens, fed with contaminated feed are transported to young embryos through eggs and thus cause severe teratological abnormalities, biochemical changes and organ dysfunction and mortality in the young embryos. Many workers have undertaken toxicological studies of pesticides and their metabolites on chick embryos and adult chicks (Mufti and Nasim, 1987; Qadri *et al*., 1987; Lanselink *et al*., 1993). Bhattacharya *et al*. (1993) reported many post-mortem and histopathological changes in liver kidney, spleen, heart and brain of chicks due to endosulfan toxicity. Majmunder *et al*. (1994) found that fenvalerate resides in liver and induces histopathological changes such as necrosis in liver, increased GPT activity and decreases glycogen content and AcP activity in liver.

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A lot of information is available on the toxicity of insecticides, pesticides, herbicides and other toxicants on chick embryo (Scheideler, 1993; Mufti and Nasim, 1987), while,. a little data are available on the toxicity of permethrin on the liver of developing chick embryo.

Permethrin [3- (2,2-dichloro-ethenyl) - 2,2-dimethylcyclopropanecarboxylic acid-(3-phenoxy-phenyl) methylester], being a most promising pyrethroid is photostable and possess high insecticidal activity. The most common metabolites of permethrin found in rat plasma and urine are m-phenoxybenzoic acid and m-phenoxybenzoyl alcohol (Abu-Qare and Abou-Donia, 2001b). Permethrin has been found to affect non-target invertebrates (Anderson, 1983) and vertebrates (Kumaraguru and Beamish, 1981; Kapoor *et al*., 1988). Many workers studied the toxicity of permethrin in terms of mortality, absorption, distribution, metabolism and excretion in vertebrates (Glickman *et al*., 1981; Ishmael and Litchfield, 1988; Ferguson and Audserik, 1990). In fish, permethrin is known to possess residual effect in different organs of the body (Glickman *et al*., 1981). It is also known to reduce the growth rate and increase the metabolic rate in rainbow trout, *Salmo gairdneri* (Kumaraguru and Beamish, 1981). In

chicks, permethrin is known to induce microsomal protein, cytochrome P-450 and NADPH cytochrome C reductase in dose dependent manner (Kapoor *et al*., 1988). It is also known to decrease the number of neurites/neuron, neurite length and number of neurites/cell through interference with intracellular calcium regulation in chick brain (Ferguson and Audserik, 1990). Acute toxicity of permethrin to hemoglobin, red cell (RBC) count and chloride level has also been observed in chick blood (Qadri *et al*., 1987).

In mammals, for example in rats, permethrin causes liver hypertrophy, change in microsomal enzyme activity and proliferation of smooth endoplasmic reticulum (Ishmael and Litchfield, 1988). Kostka *et al.* (1997) observed the induction of CYP 2B and slight increase in CYP1A in the liver of rats treated with 620mg/Kg of permethrin. Abdel-Rehman *et al.* (2001) observed the toxic effects of permethrin on the nervous system in mammals. They found that permethrin causes diffuse neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and hippocampus, and Purkinje neuron loss in the cerebellum in rats. Karen *et al.* (2001) also observed the permethrin-induced neurotoxicity in mammals through interfering with dopaminergic transmission. Sheets (2000) observed that young rats are more sensitive than old rats at lethal dose to pyrethroids and this greater susceptibility of the neonates to pyrethroids appears to be due to the limited metabolic capacity. Permethrin has been found to induce fetal changes in rats (Spencer and Berhane, 1982).

As permethrin is commercially used in large quantity, the studies of its secondary effects in developing chicks are of great toxicological importance. A little information is available about the toxicity of permethrin in the liver of developing chick. Liver is responsible for the detoxification of endogenously produced waste products or exogenously derived toxins and drugs and plays a central role in metabolism of xenobiotics and thus is a frequent target of drug toxicity. Therefore, the present study was designed to evaluate the toxicity of permethrin in the liver of 16-day old chick embryo.

MATERIALS AND METHODS

Fertilized eggs were obtained from Government Poultry Farm at Muzaffarabad, Azad Kashmir, Pakistan. Eggs were injected with different concentrations of permethrin insecticide. Dilutions were made in acetone. LD_{50} was obtained using probit analysis. After measuring the LD_{50}, which was found 676 ppm, a single sublethal dose (0.05 ml) of the insecticide of various concentrations; 25 ppm, 50 ppm, 100 ppm and 200 ppm was injected into the yolk of each egg at vegetal pole by disposable tuberculin syringe on day '0' of incubation. Equal amount of acetone was injected into the controls. The eggs were incubated at 38±0.5°C in incubators. The eggs were rotated every two hours to avoid the sticking of the embryos to the shell membranes.

On 16th day of incubation the liver from each embryo was taken out, weighed and divided into three parts. One part was used for making saline homogenate, while the other part was used for extraction of lipid, cholesterol and nucleic acids. The third part was fixed in bouin's fluid for light microscopical 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 amino transferase (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 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. Total protein was estimated according to Lowry *et al*. (1951). 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 Iacono (1963).

For extraction of the total lipid and cholesterol contents the tissues were boiled in the ethanol and then kept overnight. After centrifugation the supernatant was used for the estimation of total lipid by Vanillin reagent according to Zöllner and Kirsch (1962) and cholesterol content according to Liebermann and Burchardt Reaction described by Henry and Henry (1974).

The nucleic acids were extracted according to the method described by Shakoori and Ahmad (1973). The pellet left during lipid extraction was used for preparation of DNA and RNA extracts. DNA was extracted in hot PCA and estimated according to diphenylamine method. RNA extract was made in cold PCA and estimated according to the Orcinol method. The methods used for nucleic acid estimation were according to Schneider (1957).

RESULTS

Liver weight

A significant decrease of 10.8 and 10.4% in the embryo/liver weight ratio was observed, respectively, at a dose of 100 and 200 ppm of permethrin. At the lower two doses (25 and 50 ppm) no change was observed in the embryo/liver weight ratio when compared to that of control embryos.

Biochemical analysis

Table I shows the permethrin-induced changes in some of the enzyme activities and biochemical constituents of liver of 16-day old chick embryo following administration on day '0' of incubation. Among enzymes, the activities of AkP, AcP, ALT, AST and LDH were significantly decreased, whereas, the activity of amylase remained unchanged. AkP activity showed decrease of 44, 46 and 23% at 25, 50 and 100 ppm, respectively, while AcP activity decreased only at 200 ppm and the decrease was 55%. Like phosphatases, both the transaminases, AST and ALT were decreased with permethrin treatment. AST activity decreased by 96, 36 and 65% at 50, 100 and 200 ppm, respectively, while, ALT activity decreased at all the doses, it was decreased by 43, 46, 56 and 42% at 25, 50, 100 and 200 ppm, respectively. LDH activity also showed decrease at all the doses, it was decreased by 36, 64, 70 and 51% at 25, 50, 100 and 200 ppm, respectively.

From among biochemical components hepatic glucose, glycogen, total protein, soluble protein, urea and RNA contents were decreased, whereas, total lipids and FAA content were elevated. However, cholesterol, uric acid and DNA contents remained unaltered. Among carbohydrates, glucose was decreased 28, 39, 55 and 46% at 25, 50, 100 and 200 ppm, respectively, whereas, glycogen was decreased 76, 76 and 73% at 50, 100 and 200 ppm, respectively. Total and soluble protein contents were decreased at all the doses. Total protein was decreased 36, 30, 38 and 37% at 25, 50, 100 and 200 ppm, respectively, whereas, soluble protein was decreased 48, 35, 21 and 31% at 25, 50, 100 and 200 ppm, respectively. In contrast, free amino acid contents showed a substantial increase of 443, 409 and 297% at 50, 100 and 200 ppm, respectively. Total lipid contents increased at all the doses, whereas cholesterol content remained unaffected. Total lipid content was increased 65, 127, 125 and 116% at 25, 50, 100 and 200 ppm, respectively. Urea content decreased at all the dose levels; it was decreased 27, 44, 43 and 44% at 25, 50, 100 and 200 ppm, respectively. Uric acid content was not affected with permethrin treatment. Among nucleic acids, RNA content showed a decrease of 24% at 200 ppm, whereas DNA remained unaltered.

Histological Changes in liver

Figures 1-3 show the histological structure of liver of 16-day old chick embryo, whereas other Figures show the effect of permethrin administered at a dose of 25 ppm (Figs. 4-6), 50 ppm (Figs. 7-9), 100 ppm (Figs. 10-12 and 200 ppm (Figs. 13-15. The histological sections of liver of the vehicle-

Table I.- Effect of permethrin on the activities of some of the enzyme and biochemical constituents of the liver of 16-day old Chick Embryo Permethrin of various concentrations (25, 50, 100 and 200 ppm) dissolved in acetone were administered into the eggs at day '0' of incubation. Control eggs received acetone only. Livers were taken out from the embryos on day '16' of incubation.

Biochemical component[b]	Control n=6	25 ppm n=4	50 ppm n=4	100 ppm n=4	200 ppm n=5
Amylase (So U/g)	156.53±6.61[a]	160.31±15.0	147.02±6.67	198.02±38.36	137.58±23.35
AkP (KAU/g)	1.14±0.09	0.64±0.07*	0.61±0.08**	0.88±0.13	1.12±0.24
AcP (KAU/g)	2.51±0.28	1.68±0.36	1.74±0.26	2.32±0.34	1.14±0.48*
AST (IU/g)	32.56±2.4	23.32±4.09	18.35±2.44**	18.43±2.37**	11.33±4.97**
ALT (IU/g)	5.89±0.33	3.36±0.19***	3.19±0.44**	2.62±0.46***	3.42±0.42***
LDH IU/g)	55.53±6.05	35.49±4.05**	19.98±3.62***	16.63±0.86***	27.2±2.06***
Glucose (mg/g))	2.6±0.1	1.9±0.16**	1.6±0.18**	1.2±0.05***	1.4±0.2***
Glycogen (mg/g)	3.3±0.4	2.44±0.11	0.79±0.21**	0.78±0.16**	0.88±0.13***
Total Protein (mg/g)	227.3±8.3	146.4±6.6***	158.7±10.9***	140.0±11.9***	142.2±15.8***
Soluble Protein (mg/g)	71.3±3.5	37.4±4.6***	46.5±2.7***	46.9±6.0**	49.4±2.45***
Free Amino acids (mg/g)	3.1±0.1	11.8±4.9	16.7±2.2***	15.6±3.8**	12.2±2.3**
Total Lipids (mg/g)	30.7±3.7	50.5±7.3*	69.7±3.9***	69.1±7.1**	73.4±11.2***
Cholesterol (mg/g)	3.4±0.4	2.9±0.25	3.5±0.34	4.1±0.66	4.4±0.61
Urea (mg/g)	1.1±0.1	0.78±0.03*	0.6±0.07**	0.61±0.06**	0.6±0.05**
Uric Acid (mg/g)	1.3±0.2	0.92±0.06	1.2±0.13	1.4±0.16	1.1±0.11
DNA (mg/g)	3.5±0.3	3.3±0.31	3.0±0.24	3.0±0.16	2.9±0.14
RNA (mg/g)	7.5±0.3	8.1±1.05	7.3±0.33	7.5±0.59	5.5±0.35**

[a], Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, using students 't' test.
[b]Abbreviations used: IU: International unit, the amount of enzyme which under defined assay conditions will catalyse the conversion of one micro mole of substrate per minute, SoU: Somogyi Unit: The amount of enzyme that catalyses 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.

treated control chick embryos were normal in appearance. At the dose of 25 ppm, a mild increase in sinusoidal spaces and some localised degenerative changes were noticed. Some clear areas were present which probably indicated fatty change. Prominent and well-defined hypertrophied hepatocytes with clear vacuolated areas and prominent cell boundaries and condensed nuclei were also seen. Some degenerative patches were also present (Figs. 4-6). Increased sinusoidal spaces, disturbed lobule pattern and hepatic cord arrangement was also seen at higher doses of 50, 100 and 200 ppm. Necrosis of hepatocytes was seen only at the higher doses of 100 and 200 ppm (Figs. 7-15).

Morphometric studies of liver

Table II shows the effect of single treatment of permethrin on the histological parameters of liver of 16-day old chick embryo. Permethrin treatment has resulted in the decrease in number of cells/ microscopic field and increase in the size of the

Figs. 1-9. Histological structure of vehicle-treated 16 day-old chick embryo and liver of permethrin treated 16 day old chick embryo at a concentration of 25 ppm (4-6) and 50 ppm (7-9). The control histological section showed the normal architecture of hepatic parenchyma. All the hepatocytes look normal. The permethrin treatment at 25 pm showed hypertrophied hepatocytes are common. A few necrotic hepatocytes and anucleated hepatocytes are also visible. In addition, blood cell infiltration can also be seen in these sections. The permethrin treatment at 50 ppm showed disturbed hepatic architecture. Clumping of hepatocytes is seen. Fig. 9 shows necrotic and hypertrophied hepatocytes. Hepatocytes with nuclear condensation are also visible.
Magnifications: 1,4,7, 200X; 2, 5, 8, 400X; 3, 6, 9, 1000X. Stain: Hematoxylin and eosine.

Figs. 10-15. Permethrin treated 16 day-old chick embryo developed from eggs treated with 100 ppm (10-12), and 200 ppm (13-15) of permethrin on day '0' of incubation. In 100 ppm treated liver moderate haemorrhagic necrosis is seen. Hydropic degeneration of the hepatocytes is common. Hypertrophied hepatocytes, anucleated hepatocytes and hepatocytes with nuclear condensation are also visible (Fig. 12). Patches of blood cell infiltration are visible in Figs. 10-11. Permethrin treated 16 day-old chick embryo at a concentration of 200 ppm show the disturbed architecture of hepatic parenchyma. Moderate haemorrhagic necrosis is seen. Hydropic degeneration of the hepatocytes is common. Necrotic, hypertrophied and anucleated hepatocytes are common. Hepatocytes with fragmented nuclei are also visible. A few blood cells are also seen. Magnifications: 10, 13, 200X; 11, 14, 400X; 12, 5, 1000X. Stain: Eosine and hematoxylin.

Table II.- Toxicological effects of a single treatment of permethrin on some morphometric parameters of liver of 16-day old chick embryo

Parameters	Control (n=6)	25 ppm (n=4)	50 ppm (n=4)	100 ppm (n=4)	200 ppm (n=5)
Number of cells/ Microscopic field (n=90)	582.6 ± 33.48[a]	447 ± 17.0**	447.9 ±14.24*	452.9 ± 12.0**	439.1±12.7**
No. of nuclei/cell (n=90)	1 ± 00	1 ± 00	1 ± 00	1 ± 00	1±00
No. of nucleoli/nucleus (n=90)	2.8 ± 0.06	2.7 ± 0.1	1.2 ± 0.08***	1.1 ± 0.04***	1.1±0.0***
Size of cell $(\mu m)^2$ (n=90)	83.2 ± 2.55	82.4 ± 0.46	99.9 ± 0.63***	96.1 ± 0.33***	90.2±0.4***
Size of nucleus $(\mu m)^2$ (n=90)	35.4 ± 0.68	30.3 ± 0.14***	31.2 ± 1.14**	30.4 ±1.33**	31.2±1.1**
Size of nucleolus $(\mu m)^2$ (n=90)	1.4 ± 0.	1.4 ± 1.00	3.7 ± 0.24***	2.9 ± 0.13***	3.0±0.1***

a, Mean ± SEM

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

cells. The size of the nuclei was reduced at all the doses. The number of nucleoli/nucleus was decreased at the doses of 50, 100 and 200 ppm, whereas, the size of the nucleoli was increased at all the doses. The number of nuclei/cell remained unchanged,

DISCUSSION

Permethrin treatment has resulted in the significant decrease in the hepatic AkP, AcP, ALT, AST and LDH activities. AkP is membrane bound enzyme, it is found on all cell membranes where active transport occurs and is hydrolase and transphosphorylase in function. This decrease in AkP activity may be taken as an index of parenchymal damage (Onikienko, 1963). In the present study, decrease in AkP activity seems to occur as a result of hepatic parenchymal damage rather than its inhibition by the insecticide as the total protein content was also decreased, which is also an indicator of tissue damage. Decrease in AcP activity could be due to the release of lysosomal enzymes from the damaged hepatocytes in to the blood stream as a result of permethrin toxicity. Decreased AcP activity was also observed in the rat liver following treatment with fenvalerate (Majmunder *et al*., 1994). The activities of both the transaminases ALT and AST were significantly decreased at various dose levels and this decrease also reflects the damage to the hepatic tissue. In contrast, Anadon *et al*. (1988) reported the increased ALT activity in rat liver as a result of permethrin toxicity. Decrease in LDH activity also reflects the hepatocytic damage as the LDH is leaked out from damaged hepatocytes into the blood stream.

Permethrin is metabolised by cytochrome P450s into its reactive metabolites, which are removed from the liver through various detoxification processes *i.e*. glucuronidation, sulfation and glutathione conjugation, etc. So decrease in hepatic glucose content as a result of permethrin toxicity might have resulted from the increased detoxification process of glucuronidation. The decrease in glycogen content in the liver is an early sign of hepatic damage. Glycogen decreases as a result of increased glycogen phosphorylase activity to supplement glucose either for increased glucuronidation process or to provide energy to the cells under stress. Soluble protein content was decreased in the present study. Decreased in soluble protein might have occurred as a result of parenchymal damage. Hepatic total protein content was decreased at all dose levels. The decrease in total protein content could be either due to damage to the hepatocytes or due to decreased protein synthesis. Shakoori *et al*. (1988) observed the depletion in protein in albino rats with another pyrethroid insecticide, cypermethrin. Free amino acid content was increased in the present study. The increased free amino acids content either indicates the degradation of tissue proteins or decreased protein synthesis. Vontas *et al*. (2001) observed the induction of both protein and lipid peroxides as a result of permethrin toxicity in insects. Protein oxides can be formed as a result of attack of reactive oxygen species (generated from permethrin metabolism) on the protein content of the hepatic

tissues. In the present study, the degradation of hepatic protein content might have occurred as a result of protein oxides formation. Total lipid content in the liver was increased. Increase in hepatic total lipid content could be due to the accumulation of fats in the liver that strictly correlates with the fatty change in the hepatocytes as revealed by light microscopic study of the liver. Decreased in RNA content may be either due to tissue damage or due to its decreased synthesis. Decreased hepatic urea content also indicates the parenchymal damage.

Morphometric and Histopathological Changes

Morphometric analysis of liver sections from chick embryos developed from eggs treated with permethrin shows the decrease in the number of cells/microscopic field, increase in the size of cells and reduction in the size of nuclei. Decrease in the number of cells/microscopic field indicates the loss of cells which might have occurred as a result of necrotic damage to hepatocytes caused by permethrin. Increase in the size of cells indicates hypertrophied hepatocytes. Decrease in the size of nuclei indicates nuclear condensation.

Liver sections from chick embryos developed from eggs treated with permethrin showed various histological changes like, increased sinusoidal spaces, fatty changes in hepatocytes, hypertrophied hepatocytes, hydropic degeneration and some necrotic changes in the hepatocytes. 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. Fatty changes in hepatocytes significantly correlate with the increased total lipid content in the hepatocytes. Hydropic degeneration and necrotic changes occur as a result of disturbance in Ca^{+2} homeostasis. In the present study, hepatic glycogen content was decreased. Glycogen content decreases as a result of increased glycogen phosphorylase a activity, which in turn is dependent on Ca^{2+} activity (Lehninger, 1982). Diez-Fernandez *et al*. (1996) in a time course study with thioacetamide in rats observed that increase in basal level of Ca^{2+} paralleled glycogen phosphorylase activity. Calcium dysregulation and calcium overload has a pivotal role in the pathology of toxic cell death. So in the present study hepatocyte necrosis might have resulted from disturbance in Ca^{+2} homeostasis as evidenced by the decreased hepatic glycogen content.

Hepatocyte nuclear condensation was also observed in the liver sections from the treated embryos. Hepatocyte nuclear condensation is a hallmark of another kind of cell death 'the programmed cell death' or apoptosis (Ray *et al.,* 1996). Abu-Qare and Abou-Donia (2001b) observed that permethrin induces apoptosis in rat brain through the release of cytochrome c from rat brain mitochondria. Cytochrome c is known to induce apoptosis (Reed, 1997). Recently, permethrin-induced apoptosis has been observed in thymocytes of C57BL/6N mice (Prater *et al*., 2002). Ray *et al.,* (1996) demonstrated that paracetamol-induced apoptosis is preceded by massive elevation in serum ALT activity coupled with rapid loss of genomic DNA, nuclear condensation, DNA fragmentation and loss of glycogen in liver. Decrease in hepatic ALT activity (as observed in present study) parallels the increased serum ALT activity. Of these parameters, hepatocytic nuclear condensation, decreased hepatic ALT activity and decreased hepatic glycogen content have been observed in the present study and these parameters may indicate apoptotic damage to liver in addition to necrosis caused by permethrin. Changes in various biochemical components, enzyme activities and histological parameters indicate that permethrin is highly toxic embryonic liver.

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