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Assessment of Biochemical and Histopathological Changes in the Liver of Chick Embryo Treated with a Commercial Deltamethrin Formulation

Research Article

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ABSTRACT

We studied the hepatotoxic effects of a deltamethrin containing formulation (Decis®) on developing embryo of *Gallus domesticus*. Fertilized eggs were immersed in three different concentrations of deltamethrin (12.5, 25 and 50 mg/L) for 60 min at 37 ˚C prior to incubation (ED 0) and on the 4th day of incubation (ED 4) and incubated till embryonic day (ED) 16. Exposure to 50 mg/L of deltamethrin resulted in a significant decrease in total protein and total glycogen content in the liver when exposed on ED 4 and ED 0 respectively, while total glutathione content was reduced at concentrations applied on both the exposure days. Among enzymes, liver alkaline phosphatase activity was significantly increased at 25 and 50 mg/L deltamethrin when embryos were exposed on ED 0 and 4, whereas the glutamate pyruvate transaminase activity showed a marked increase in animals exposed to 50 mg/L deltamethrin only at ED 4. Histophathological evaluation revealed mild to marked cellular lesions in liver sections of insecticide treated embryo. The magnitude of most of these changes appeared to be dose dependent being more severe on exposure to highest dose of deltamethrin. The observed pathological changes include degeneration and necrosis of hepatocytes, cytoplasmic vacuolization, enlarged and congested sinusoidal spaces, leucocyte infiltrations and congestion and dilation of the central vein.

KEY WORDS biochemical, chick embryo, deltamethrin, hepatotoxic, pathological, synthetic pyre-throid.

INTRODUCTION

Synthetic pyrethroids are rapidly replacing conventional pesticides like organochlorines, organophosphates and carbamates because of the worldwide concern about their safety (Datta and Kaviraj, 2003). These pyrethroids are synthetic analogues of pyrethrins extracted from *Chrysanthemum cinerariaefolium* flowers, having insecticidal properties; they came on the market in the early 1970 s for agricultural purposes. Deltamethrin, [(S)-a-cyano-3-phenoxybenzyl-(1R)-cis-3-(2, 2-dibromovinyl)-2, 2-dimethylcyclopropane carbo-xylate] type II synthetic pyrethroid, was synthesized in 1974 and since then, it has been applied for a range of commercial crops and by extension controls a variety of pests. It is used as an active ingredient in the number of commercial available insecticide formulations, which are mostly used in cotton belt and also in veterinary medicinal formulations to treat cattle. Deltamethrin is considered the most powerful and therefore the most toxic pyrethroid. Even though this chemical is broken down via UV and sun light, it is quite resistant to storage and can preserve its activity for 6 months at 40 °C. For this reason it may represent a risk to both mammals and the ecosystem as whole (Özkan and Üstüner, 2012). Deltamethrin has varying degree of toxicological impacts in different animal species, such as fish (Datta and Kaviraj, 2003; Ural and Sa-

glam, 2005; Köprücü *et al.* 2006; Velíšek *et al.* 2006; Sharma and Ansari, 2011; Amin and Hashem, 2012), Japanese quail (Martin, 1990), south American toad (Salibián, 1992) and rats (Manna *et al.* 2005; El-Maghraby *et al.* 2010).

The avian egg and developing embryo has been widely used in toxicological research due to the properties such as small size, well known embryonic development, ease of accessibility of animals during experimentation, short incubation period (21 days), minimal expenditure of time and money and possibility of experimenting on large scale for statistically valid results, which proved this animal as a promising experimental model for preliminary teratological screening of various toxicants (Jelinek, 1982; Kotwani, 1998). Therefore, the present study was undertaken to investigate the possible adverse effects of a commercial formulation of deltamethrin on the liver of the developing embryo of *Gallus domesticus*.

MATERIALS AND METHODS

Toxicant

The pesticide used in the present study was a concentrate formulation containing an emulsion of deltamethrin-(25 g deltamethrin/L; Decis®-Bayer Crop Science Limited, Gujarat, India).

Test animal

Fertilized eggs of *Gallus domesticus* (BV 300 breed) were obtained from a commercial hatchery (Kewalramani Hatcheries, Ajmer, India), cleaned and placed in an incubator at 38±1 ˚C with relative humidity of 70-80%. The eggs were turned (90 degrees) periodically during storage. Use of the chick embryos was in conformity with the policies of Institutional Animal Care and Use Committee (IACUC, 2008). All the experimental procedures conducted on the animals, including the housing, care and maintenance of experimental animals / eggs, were approved by the Animal Ethical Committee of Institute.

Experimental design

The eggs were immersed in different aqueous emulsions of insecticide prepared in distilled water (vehicle) for 60 min at 37 °C temperature, at two moments: prior to incubation (ED 0) and on embryonic day 4 (stage 24, Hamburger and Hamilton, 1951) (ED 4). The insecticides were used at the concentrations 12.5, 25 and 50 mg/L of deltamethrin, based on the recommended dose (25 mg/L) for crop protection. Two other groups represented the control group I (Untreated eggs) and II (eggs immersed in vehicle, i.e. distilled water). Twenty eggs were assigned for each treatment groups.

All the eggs were kept for incubation and candled daily for breakdown examination of clear and dead embryos. At the end of experiment, the liver was excised from all of the sacrificed chick embryos on embryonic day 16 (stage 42, Hamburger and Hamilton, 1951) and submitted to biochemical and histopathological analyses.

Biochemical study

Dissected liver from each of sacrificed animal was washed with saline solution, weighed and cut into two parts. One liver fragment was homogenized (10%) with a known volume of ice-cold phosphate buffer saline (PBS, pH 7.4) for estimating the total protein content, total GSH content and the enzymatic activity. The other fragment of the liver was used for analysis of total cholesterol and total glycogen content. Total protein content was estimated according to the method of Lowry *et al.* (1951). The Liebermann and Burchardt reaction (Henry and Henry, 1974) was used for estimating total cholesterol content. Glycogen content was extracted and estimated according to Montgomery procedure (1957). The reduced form of glutathione (GSH) was determined according to Moron *et al.* (1979). The activities of phosphatases (Alkaline Phosphatase- ALP; orthophosphoric monoester phosphohydrolase, EC 3:1:3:1 and Acid Phosphatase -ACP; monoester phosphohydrolase, EC 3:1:3:2) were estimated according to the method of Kind and King (1954), while amino transaminases activities (Glutamate pyruvate transaminase-GPT; L-alanine 2-oxoglutarate aminotransferase; EC 2.6.1.2 and Glutamate oxaloacetate transaminase-GOT; L-aspartate, 2-oxoglutarate aminotransferase; EC 2.6.1.1) were estimated according to King's method (1965).

Histopathological study

Samples of Bouin's fixed liver tissue were cut into smaller pieces after 24 hours of fixation. Tissues were processed in a series of graded ethanol and xylene and embedded in a mixture of paraffin and bee wax (3:1); liver sections were cut at 5 µm and stained with haematoxylin and eosin. The stained sections were examined and scoring of histopathological changes was done as follow: (-) absent; (+) mild; (++) moderate; (+++) severe and (++++) extremely severe (Bancroft *et al.* 1996).

Statistical analysis

All the obtained values of biochemical parameters were presented as mean ±Standard error (SE) and statistical significance was performed by IBM SPSS Statistics 17 analytic software using student "t" test. The value of p less than (≤) 0.05, 0.01 and 0.001 were considered to be significant, highly significant and very highly significant against control values.

RESULTS AND DISCUSSION

Biochemical changes

The biochemical profiles of the total hepatic protein content was significantly decreased (P≤0.05) in embryos treated with 50 mg/L of deltamethrin on the 4th day of incubation, whereas the total glycogen content showed a marked decrease (P≤0.05) in the animals treated with 50 mg/L of deltamethrin prior to incubation. The total cholesterol content of liver resulted unaffected following deltamethrin treatments on either embryonic days. Liver GSH content was significantly (P≤0.05), highly significantly (P≤0.01) and very highly significantly (P≤0.001) decreased after exposure to 12.5, 25 and 50 mg/L deltamethrin treatment respectively on embryonic day 0, while when the exposure occurred on the 4th day of incubation, the liver GSH content was significantly depleted at 12.5 mg/L deltamethrin concentration and highly significantly deplected at 25 and 50 mg/L of deltamethrin, compared with their respective control values (Figure 1). With regards to enzymes, a statistically significant (P≤0.05) and a highly significant (P≤0.01) increase in liver ALP activity was observed in animals recovered from eggs treated prior to incubation with 25 and 50 mg/L of deltamethrin respectively, while it was significantly increased (P≤0.05) following treatment with 50 mg/L of deltamethrin on embryonic day 4. ACP activity remained unaltered. Among liver transaminases, GPT activity showed a highly significant increase (P≤0.01) at 50 mg/L deltamethrin concentration only when eggs were exposed at the 4th day of incubation, while the activity of GOT remained unchanged (Figure 2).

Histopathological changes

The appearance of liver sections of untreated and vehicle treated control chick embryo was normal with clearly defined liver parenchyma formed of hepatic cords and hepatic sinusoids, arranged radially around the central vein (Table 1 and Figure 3). The hepatocytes were polygonal, with centrally located round vesicular nuclei. Blood sinusoids were narrow, followed their branching and anastomosed with each other. However, some mild alterations under the standard con-trol range were observed in few liver sections of both the controls and control II treated on ED 0 and ED 4, respec-tively. The liver sections of chick embryos treated with 12.5, 25 and 50 mg/L of deltamethrin on embryonic day 0 revealed congestion and dilation of the central vein, degen-eration of hepatocytes that showed pyknotic nuclei, enlarged blood sinusoids and dense inflammatory leukocyte infiltration. Activations of Kupffer cells were also observed in a few animals treated with 25 mg/L of deltamethrin. Hya-linization and necrosis of hepatocytes were more severe in the group exposed to 50 mg/L of deltamethrin.

The charac-teristic radial arrangement of hepatocytes was lost in this group. Exposure to deltamethrin at 12.5 mg/L on embry-onic day 4 revealed a moderate increase in sinusoidal spaces with some degenerative changes in hepatocytes and leucocyte infiltration. Clear round areas in hepatic paren-chyma indicated fatty changes. Increased blood sinusoidal spaces with large vacuoles in the hepatic parenchyma, dis-turbed hepatic architecture due to necrosis of hepatocytes and dilation of central vein were more prominent in liver sections from the 25 and 50 mg/L of deltamethrin treated groups. In addition, clumping of hepatocytes in rounded masses in mid-zonal area was also observed in liver sec-tions of 2 chick embryos treated with 25 mg/L of deltame-thrin. Pesticides are generally known to interfere with im-portant biochemical and enzymatic processes such as car-bohydrates, lipids and protein metabolism that regulate the normal physiology of animals. Any type of alteration in these processes can alter the homeostasis of the organism and also affect normal functioning of their organs (Ksheerasagar *et al.* 2011). The toxic responses occur frequently in the liver as it is a predominant organ for xenobiotic metabolism and detoxifi-cation, as well as the first organ to encounter ingested toxi-cants that enter portal circulation (Levi, 1987; Treinen-Moslen, 2001). In our study, insecticide mediated bio-chemical alterations indicate hepatic damage, which was well supported by observing pathological lesions in the liver section of developing chick embryo.

Biochemical changes

The results of the present study exhibited a decrease in total liver protein content in chick embryo exposed to 50 mg/L of deltamethrin on the 4th day of incubation. This depletion in protein content may be attributed to degradation and possi-ble use of these proteins in metabolic processes during the stress condition induced by the insecticide. Indeed it is pos-sible that animals may need more energy to detoxify the toxicant and to overcome the stress. Omotuyi *et al.* (2006) and Okechukwo and Auta (2007) reported that the reduc-tion in total protein level is likely due to proteolysis of structural proteins by using their amino acid for translation of detoxifying enzyme synthesis and / or conjugation of insecticide metabolite. Impaired incorporation of the amino acids into polypeptide chains may also affect the quantity of protein (Okechukwo and Auta, 2007). Moid *et al.* (2012) observed a decline in the protein content in different organs such as the liver, spleen and gastrocnemius muscles of mice after deltamethrin treatment, suggesting that low protein level may indicate liver dysfunction resulting from a change in enzymatic activity which may either cause re-duced protein synthesis or their increased breakdown.

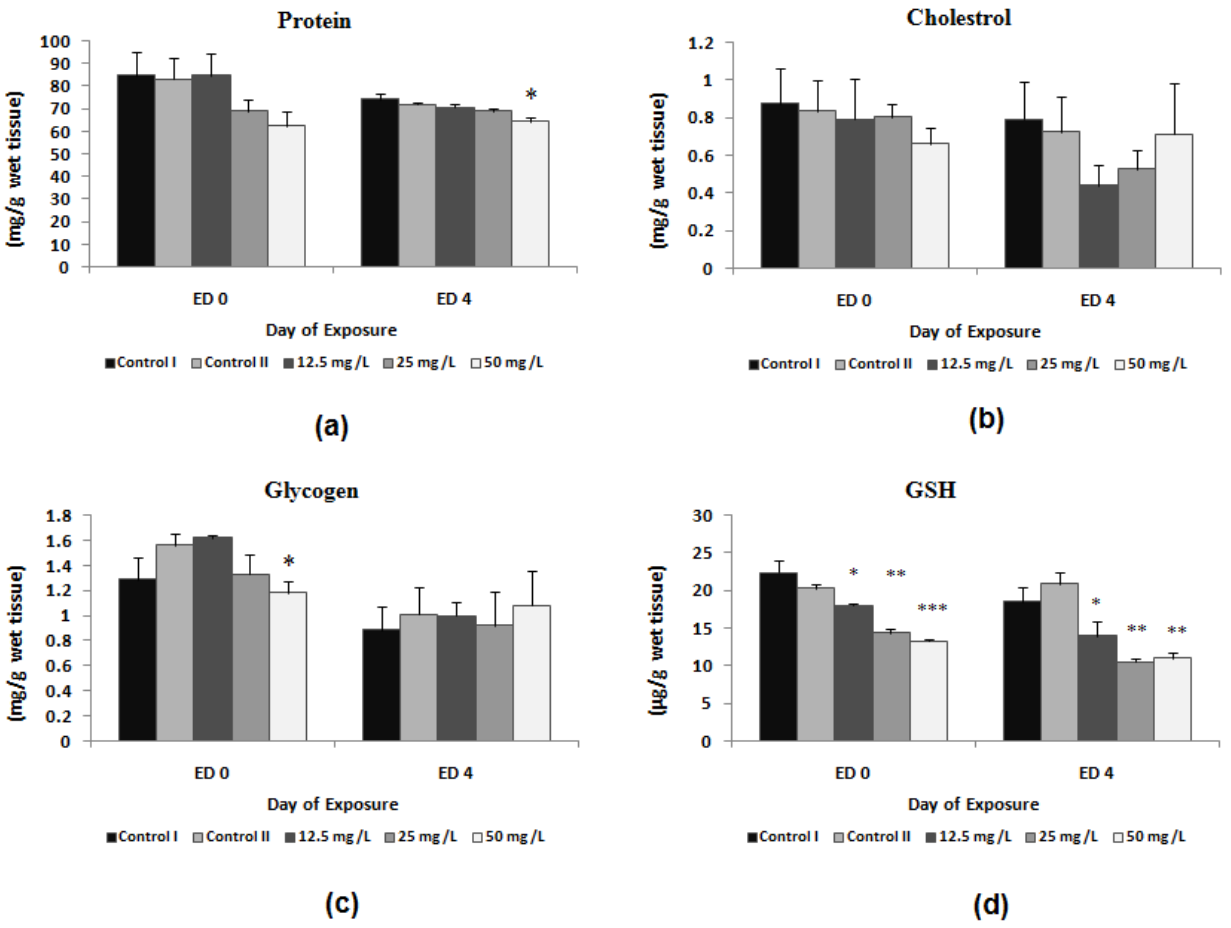

Figure 1 Deltamethrin effect on: (a) the total protein content; (b) the total cholesterol content; (c) the total glycogen content and (d) the total GSH content in the liver of 16 day old chick embryo recovered from eggs treated with deltamethrin (Decis) on embryonic day 0 and embryonic day 4
Each value represents Mean±SE
N= 5-6 animals per group
Statistical difference from the controls: * significant at (P≤0.05); ** highly significant at (P≤0.01) and *** very highly significant using student "t" test
ED: embryonic day and GSH: reduced glutathione

The present results are in agreement with those reported by other investigators who studied the effect of deltamethrin in various models, like *Cyprinus carpio* (Velíšek *et al.* 2006), rats (Yousef *et al.* 2006; El-Maghraby *et al.* 2010), *Danio rerio* (Sharma and Ansari, 2011) and *Clarias gariepinus* (Amin and Hashem, 2012) and found that this insecticide causes a significant reduction in total protein content. Presently, a marked decrease in the total liver glycogen content was observed in embryos exposed to the highest dose concentration (50 mg/L) of deltamethrin prior to incubation. Our results are in accordance with the finding of Anwar *et al.* (2004) who also observed a decrease in liver glycogen content of 16-day old chick embryo treated with permethrin (50, 100 and 200 ppm) on day "0" of incubation. According to them, the significant reduction in total glycogen content might have occurred as a result of its use to detoxify the insecticide or its metabolites through the glucuronidation process, a process by which toxic metabolites combine with glucuronic acid component of UDP - glucuronic acid and are excreted from liver through bile. Furthermore, they also attributed this decrease to an increase in activity of the phosphorylase enzyme (phosphorylase-a and phosphorylase-b) involved in glycogen breakdown (glycogenolysis) for the glucuronidation process or to provide glucose into the circulatory system to cope with the energy requirement of cells which are under stress. Present findings are comparable to findings of Muthukumaravel *et al.* (2013) and Bhusan *et al.* (2013) who studied the toxic effect of λ-cyfluthrin in *Oreochromis mossambicus* and that of cypermethrin and beta cyfluthrin in the Wistar rats respectively, reporting a significant depletion in total hepatic glycogen content.

Reduced glutathione (GSH) is a major endogenous antioxidant and also a redox regulator playing an important protective role in detoxifying both endogenous molecules (free radicals) and xenobiotics.

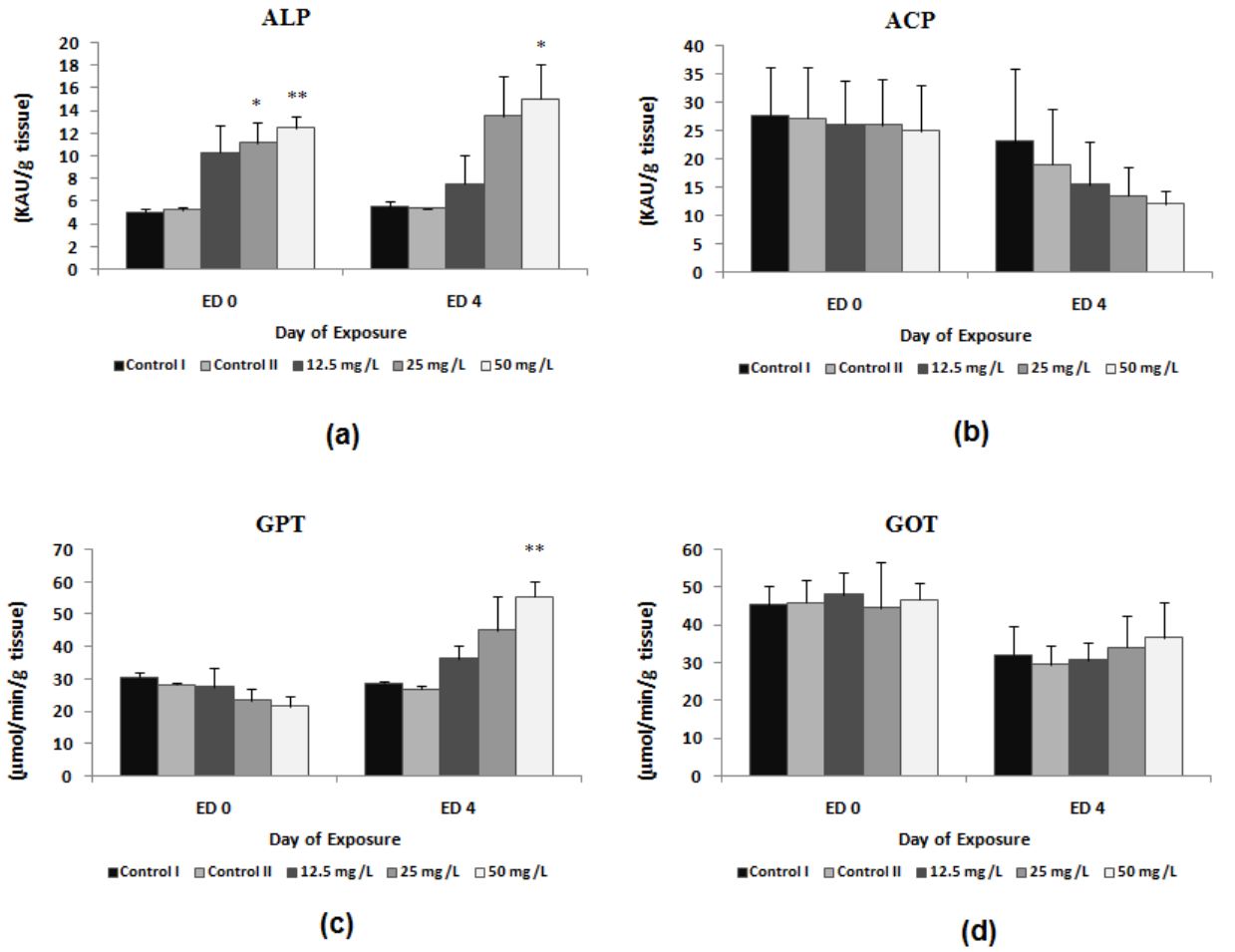

Figure 2 Effect of deltamethrin treatments on: (a) ALP activity; (b) ACP activity; (c) GPT activity and (d) GOT activity in the liver of 16 day old chick embryo recovered from eggs treated with deltamethrin (Decis) on embryonic day 0 and embryonic day 4
Each value represents Mean±SE
N= 5-6 animals per group
Statistical difference from the controls: * significant at (P≤0.05) and ** highly significant at (P≤0.01) using student "t" test
ED: embryonic day; ALP: alkaline phosphatase; ACP: acid phosphatase; GPT: glutamate pyruvate transaminase; GOT: glutamate oxaloacetate transaminase; KAU: king armstrong unit: the amount of enzymes that transforms one mg of phenol in 15 minutes and unit of GPT and GOT acitivity: µmol of pyruvate formed/min/g tissue

High levels of toxicants that are detoxified by GSH can cause its severe depletion, which is an important factor in determining cellular redox status in cells (Han *et al.* 2006). The GSH depletion in liver (major reservoir of GSH) can potentiate covalent binding of xenobiotics and its metabolites to DNA, protein and other biomolecules and cause hepatotoxicity (Levi, 1987). The present results demonstrated that exposure to insecticide deltamethrin depleted hepatic GSH content in chick embryos in a dose dependent manner. This reduction in total reduced glutathione content was either due to its decreased synthesis (Parthasarathy and Joseph, 2011) or to its inclusion in the detoxification process of reactive metabolites caused by the insecticide (Aouacheri *et al.* 2005). In poultry birds, a decrease in liver GSH content has been observed following treatment with permethrin for a period of 30 days (Ezeji *et al.* 2012).

The present results are also in line with the work reported by Rehman *et al.* (2006) who noted a reduction of the GSH content in kidney and liver of mice treated with deltamethrin.

Alkaline phosphatases (ALP) are found mainly in parenchymal cells of the liver and are responsible for mediating the active transport of metabolites across the membrane. These enzymes are also associated with protein synthesis and glycogen metabolism (Pilo *et al.* 1972; Ksheerasagar *et al.* 2011).

In the current study, administration of deltamethrin on embryonic day 0 and 4 resulted in a significant elevation of liver alkaline phosphatase activity.

The increased level of ALP activity might be due to its increased synthesis in the presence of increased biliary pressure (Gabriel *et al.* 2009).

Table 1 Semi-quantitative scoring of histophathological findings in the liver of 16 day old chick embryo recovered from eggs treated with deltamethrin on embryonic day 0 and 4

Histopathological findings	Embryonic day 0					Embryonic day 4				
	Control		Deltamethrin			Control		Deltamethrin		
	I	II	12.5 mg/L	25 mg/L	50 mg/L	I	II	12.5 mg/L	25 mg/L	50 mg/L
Degeneration and necrosis of hepatocytes	-	-	++	++	+++	-	+	++	+++	+++
Cytoplasmic vacuolation and enlarged blood sinusoids	-	-	+++	++	++	-	+	++	++	+++
Leucocyte infiltration	+	+	+++	+++	+++	-	-	+	++	+
Fatty infiltration	-	-	-	-	-	-	+	++	++	+++
Hepatocytes with pycnotic nuclei	-	+	+	++	++	-	-	-	-	-
Kuffer cell activation	-	-	-	+	-	-	-	-	-	-
Congestion and/or dilation of central vein	+	+	++	++	+++	-	-	+	++	++

(–): indicates normal; (+): indicates mild; (++): indicates moderate and (+++): indicates severe.

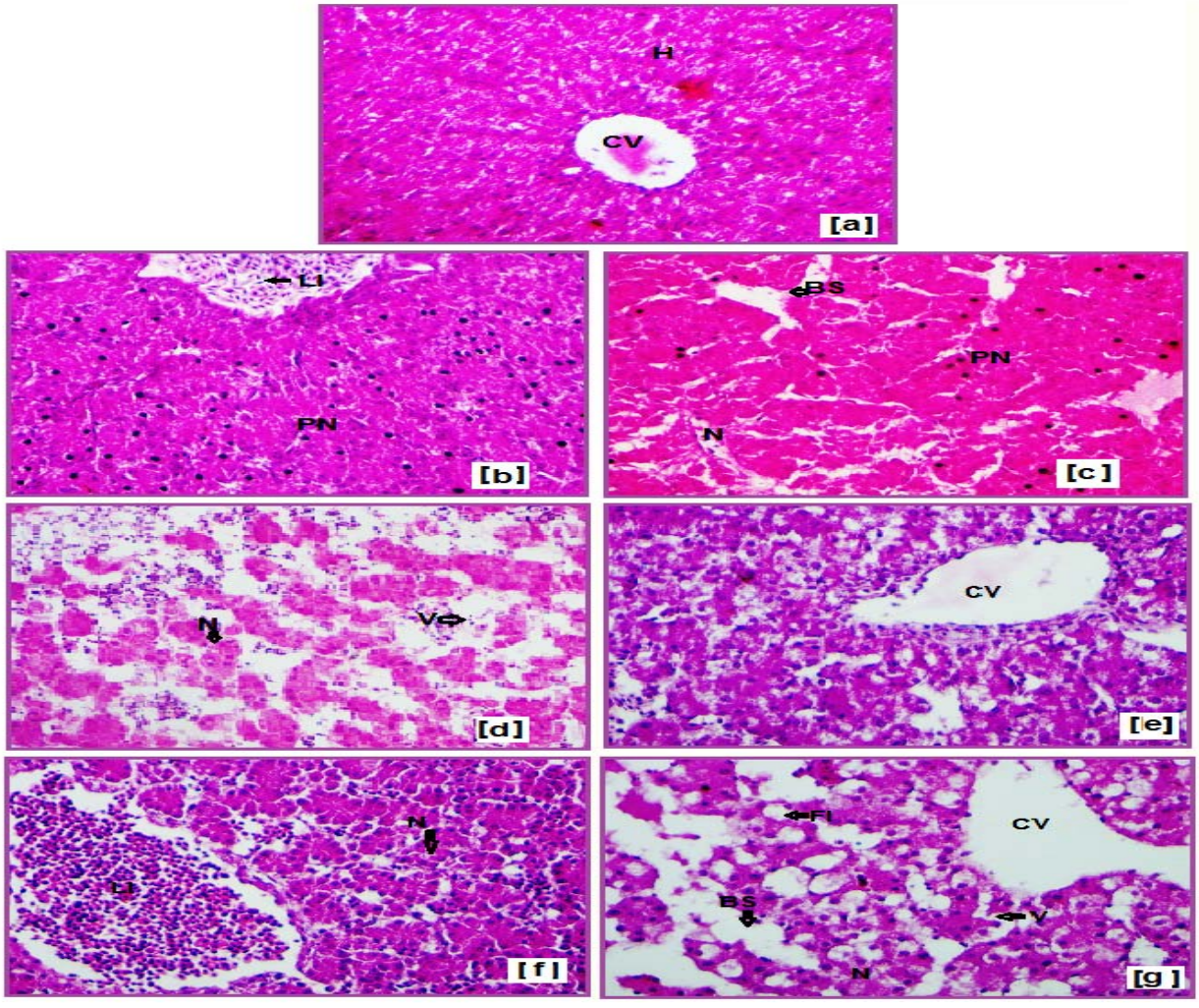

Figure 3 Photomicrographs of liver sections from 16 day old chick embryo (Haematoxylin and Eosin, magnification of 400x). [a] liver section of untreated control chick embryo showing normal histological structure of radially arranged hepatocytes (H) around central vein (CV). [b] section of the liver from treated with 12.5 mg/L of deltamethrin on ED 0 showing hepatocytes with pycnotic nuclei (PN) and leucocyte infiltrations (LI). [c] section of the liver from treated with 25 mg/L of deltamethrin on ED 0 showing necrotic hepatocytes (N) with pycnotic nuclei (PN) leading to enlarged blood sinusoids (BS). [d] section of the liver from treated with 50 mg/L of deltamethrin on ED 0 showing severe hypertrophy of hepatocytes leading to necrosis (N) and increased sinusoidal space with congestion and vacuolization (V). [e] section of the liver from treated with 12.5 mg/L of deltamethrin on ED 4 showing degenerating hepatocytes (DH) around dilated central vein (CV). [f] section of the liver from treated with 25 mg/L of deltamethrin on ED 4 showing clumping of necrotic hepatocytes (N) and leucocyte infiltrations (LI) in central vein (CV). [g] section of the liver from treated with 50 mg/L of deltamethrin on ED 4 showing vacuolization (V) and necrotic hepatocytes with enlarged blood sinusoidal space (BS) and fatty infiltration (FI) leads to loss of radial arrangement of hepatic cells.

Similarly, Shakoori *et al.* (1996) and Panati *et al.* (2012) observed elevation in above parameters in the liver of freshwater fish *Ctenopharyngodon idella* and in CNS and muscle of crab *Oziotelphusa senex senex* exposed to synthetic pyrethroid fenvalerate. The data from the present study revealed that the liver GPT activity of chick embryo was elevated after exposure to deltamethrin on the 4th day of incubation. The elevation in GPT activity indicates tissue damage due to formation of reactive oxygen species (ROS) and reactive intermediate after insecticide treatment (Ksheerasagar *et al.* 2011). It also indicates enhanced mobilization of amino acids towards gluconeogenesis and effective transamination to provide keto acids to serve as precursors in both the glycolytic pathway and tricarboxylic acid (TCA) cycle to the synthesis of essential constituents and also to cope with energy demands during insecticide induced toxicity (Tiwari and Singh, 2004; Savithri *et al.* 2010). An elevation of the above mentioned parameter is in concurrence with the reports of several investigators who found increased GPT activity in the liver of *Ctenopharyngodon idella* exposed with fenvalerate (Shakoori *et al.* 1996) and in the liver of rat exposed with flumethrin (Mishra *et al.* 2012) and cypermethrin and β-cyfluthrin (Bhusan *et al.* 2013) respectively.

Histopathological changes

The histopathological alterations observed herein on liver of chick embryo revealed mild to marked cellular changes upon the administration of different doses of the insecticide deltamethrin. However, some of these changes were also observed in both the control groups, which were considered to be within the standard normal range and might have occurred due to experimental and / or laboratory conditions. The most prevalent and severe types of lesions encountered included the degeneration and necrosis of hepatocytes, cytoplasmic vacuolization, enlarged and congested sinusoidal spaces, leucocyte infiltrations and congestion and / or dilation of the central vein. Sahu and Ghatak (2002) also observed these hepatic cellular changes such as cytoplasmic vacuolization with disintegration of liver cells in the chick embryo treated with dimecron prior to incubation. They suggested that vacuolated cells might have resulted from strong toxic effect of insecticide on cell membrane, which gets ruptured, and on nuclear degeneration, causing cytoplasmic vacuolization. Anwar *et al.* (2004) and Anwar and Shakoori (2010) reported that increased sinusoidal spaces are required to increase the blood flow in the liver lobule so to increase oxygen and nutrient supply to fulfill the requirements of hepatocytes under stress. The degenerative and necrotic changes evidenced in the hepatocytes might have occurred as a result of insecticide-mediated liver injury, associated with the formation of reactive metabolites through hepatic cytochrome P-450 catalyzed oxidation (Jain *et al.* 2011). These reactive metabolites bind to cellular macromolecules such as protein and unsaturated lipids present on plasma membrane and change their biological properties ultimately resulting in the membrane destruction (Levi, 1987). Furthermore, the leucocyte infiltrations observed were considered as prominent responses of body tissues suggestive of irritability, inflammation and hypersensitivity toward the insecticide mediated toxic insult (Khogali *et al.* 2005; Sakr, 2007).

Moreover, the exposure of developing chick embryo to deltamethrin prior to incubation resulted in mild to moderate occurrence of darkly stained pycnotic nuclei in hepatocytes, which were also described by Anwar *et al.* (2004) in liver sections of chick embryo treated with permethrin, whom consider this type of nuclei indicative of irreversible nuclear condensation and DNA fragmentation, which are a hallmark of programmed cell death (apoptosis) resulting from insecticide toxicity. In the present study, fatty infiltrations were observed in the liver of chick embryo exposed to deltamethrin on 4th day of incubation. Moon *et al.* (2007) reported that fatty infiltrations in the liver could be due to accumulation of high levels of triglycerides in parenchymal cells, caused by the defect in any steps from synthesis to release of triglyceride conjugated apoproteins mediated by hepatotoxic substances. Furthermore, an excess of lipid content can also result from an oversupply of free fatty acid from adipose tissue due to its impaired oxidation (Levi, 1987; Treinen-Moslen, 2001). The pathological changes observed in the present study are also similar to those reported by other researchers in different experimental animal models under the influence of deltamethrin (Tos-Luty *et al.* 2001; Manna *et al.* 2005; Yildirim *et al.* 2006; Staicu *et al.* 2007).

CONCLUSION

The results of the present investigation clearly allow to conclude that the observed biochemical and histopathological changes in the liver of developing chick embryo are due to the toxic effect of the deltamethrin containing formulation (Decis®) under the used experimental conditions. Although these results cannot be directly extrapolated to mammals, they may be useful for comprehensively predict mechanisms of potential teratogenicity caused by these insecticides at preliminary screening level. Further studies with technical grade deltamethrin on mammals would no doubt aid in better understanding of the potential health effects of this insecticide. But notwithstanding with this concept, it can be recommended that the deltamethrin containing formulations should be used with cautions in the environment where pregnant animal or woman live as even

low concentration of such insecticides may interfere with the normal foetal development.

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