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Hepatotoxic Effects of Low Dose Endosulfan: A Duration Dependent Histological Study

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SUMMARY

Endosulfan is a commonly used pesticide and is highly hepatotoxic. This study was designed to assess the histological changes in rat liver produced by a low toxic dose (2.5mg/kg) of endosulfan in a duration dependent manner. Eighty male Albino rats were randomly divided into control group (A) and experimental group (B). Each group was further divided into 4 sub-groups (1, 2, 3 and 4) having 10 animals each. Group B was given daily intraperitoneal injection of 2.5mg/kg endosulfan for 2,4,6 and 8 weeks. Control group was injected an equal volume of distilled water for the same duration. At the end of the experiment animals were sacrificed, liver dissected and prepared for histological study under microscope. Endosulfan at a low toxic dose (2.5mg/kg) caused negative growth rate and increased relative tissue weight index (RTWI). Histology revealed dilatation of central veins, sinusoids and portal tract, along with enlargement, cloudy swelling, vacuolation and necrosis of hepatocytes with nuclear changes. All these changes worsened with increase in duration of exposure to endosulfan.

INTRODUCTION

Endosulfan is a cyclodiene chlorinated hydrocarbon used for controlling insects, pests, mites and tse tse flies, for wood preservation, seed dressings and termite control. Endosulfan is poisonous to gastrointestinal tract (GIT), skin and nervous system. After absorption into blood it is bound to serum lipoproteins and is deposited in kidney, GIT, liver, brain, muscle, spleen, lung and heart[1]. Endosulfan undergoes metabolic transformation in the liver. Both, the original drug and the toxic metabolites can affect the structure and function of liver[2].

Small concentrations of endosulfan depress mitochondrial respiration possibly by hindering ion flux across the mitochondrial membrane. Large doses cause lysis of mitochondrial membrane, uncoupling of oxidative phosphorylation and blockade of the electron transport system[3]. It readily adheres to clay particles and persists in soil and water for several years, posing persistent threat to the environment[4].

Acceptable daily intake (ADI) as evaluated by the joint meeting of pesticide residues of FAO/WHO in 1968 is 7.5μg/kg.[5] LD_{50} through oral route for male rat is 100-160mg/kg.[6]

In this study effects of endosulfan on the histology of liver was observed in Albino rats with a low dose (2.5mg/kg) with increasing duration of exposure.

MATERIAL AND METHODS

Endosulfan solution 0.1% was prepared in distilled water from 35% thiodan solution manufactured by Hoechst Pakistan and used in this experiment.

Eighty adult, male Albino rats, obtained from Veterinary Research Institute Lahore were kept in animal house of Postgraduate Medical Institute, Lahore and fed on commercial diet and fresh water ad libitum. All the rats were kept under optimum temperature (20-30°C). After an acclimatization period of two weeks these animals were randomly divided into group A (control) and group B (experimental) each comprising 40 rats. Each group was further divided into 4 sub-groups (1, 2, 3 and

4) having 10 animals each and treated for 2, 4, 6 and 8 weeks. Animals in group B (experimental) were given 0.1% endosulfan solution, intraperitoneally as 2.5mg/kg body weight once daily for 2, 4, 6 and 8 weeks. Group A (control) animals received an equal volume of distilled water intraperitoneally daily for same duration. Animals from each group were sacrificed at the end of 2, 4, 6 and 8 weeks, 24 hours following the last injection so as to enable last dose to be metabolized completely. Body weight of all animals was taken at the start and end of each experiment and also on weekly basis. Gross appearance and weight of liver was noted. Relative tissue weight index (RTWI) and growth rate (% gain per day) were calculated. RTWI was calculated by formula:

$$\frac{\text{mean weight of liver (gms)}}{\text{mean body weight}} \times 100$$

Growth rate was calculated by formula:

$$\frac{\dfrac{\text{weight at start (gms) - weight at end (gms)}}{\text{duration of exposure (days)}}}{\text{weight at start (gms)}} \times 100$$

After routine processing liver tissue was fixed in 10% formalin, embedded in paraffin, sections made at 4-5μm and stained with eosin and hemotoxyline.[7] Stained sections of liver were studied for histological changes. Comparison was made between control and experimental groups regarding following parameters.

1. General architecture of hepatic lobule.
2. Morphology of hepatocyte for change in size, vacuolation, necrosis and change in nuclei.
3. Portal triad was seen for bile duct hyperplasia and inflammatory changes.
4. Central vein and sinusoids were noted for change in size, endothelial and Kupffer cell morphology, congestion and haemorrhage.
5. Liver capsule was examined for any thickening, atrophy and rupture.

Morphometric analysis was performed with the help of an objective micrometer at magnification x 400. Following were measured:

1. Diameter of central vein.
2. Size of hepatocyte in a field taken at random.
3. Size of nuclei selected at random.
4. Number of nucleoli in nuclei of cells in a given field taken at random.
5. Number of necrotic foci per high power field.

Mean of all variables was expressed as mean $\pm$ standard deviation (S.D). Difference in mean values of control and experimental group was analyzed using two tailed student's "t" test. Significance was established at 5% level.[8] All the data was analyzed using EP-Info-6 (Epidemiological Package-6 Information) software.

RESULTS

Throughout study, control group (A) remained healthy and active having normal intake of food and water without any mortality and morbidity. Animals in experimental group (B) treated for 2 weeks became less active while those treated for 4 and 6 weeks became irritable also. Most of the animals treated for 8 weeks, showed aggressiveness, abnormal posturing (head between legs) and ataxic gait. Bodies of most animals were dirty bruised and lacerated. Skin showed marked hair loss and dryness. Food and water requirement gradually reduced with increase in duration of exposure. No mortality was seen in experimental group. Animals in control group showed continuous gain in body weight while those in experimental group showed negative growth rate (Table 1). Decrease in body weight was statistically insignificant ($p > .05$) in B1, just significant in B2 ($p < .05$) and highly significant ($p < .001$) in B3 and B4 (Table 1). Liver weight was significantly increased and body weight considerably reduced in all experimental groups (B) except in B1. This resulted in significant increase in relative tissue weight index (RTWI) in all experimental groups except B1 (Table 2). All experimental groups showed larger congested liver covered with thin capsule showing no adhesion to surrounding structures. Reddish brown colour of liver became increasingly darker in animals with increasing duration of experiment. In group B4 few areas of pale discolouration were seen which corresponded to areas of hepatic necrosis. Histologically capsule of liver showed no change in thickness. Hepatic lobular architecture remained

Table 1: Effectof 2.5mg/kg dose of Endosulfan on the mean body weights at start and end of experiment and on growth rate (% gain/ day)of Albino rats at 2,4,6 and 8 weeks.

Groups	*Weight at start of experiment (gms)*	*Weight at end of experiment (gms)*	*Mean growth rate (% gain/day)*
Control group (A) (n=40)			
A1 2 weeks (n=10)	180.94±7.30	184.61±8.20	+.14
A2 4 weeks (n=10)	178.96±6.97	188.96±11.91	+.19
A3 6 weeks (n=10)	181+10.73	195.13±9.92	+.18
A4 8 weeks (n=10)	181.61±11.90	198.86±9.43	+.16
Experiment group (B) (n=40)			
B1 2 weeks (n=10)	187.1±8.56	185.92±9.23^{+}	-.04
B2 4 weeks (n=10)	189.12±10.8	186±8.00*	-.05
B3 6 weeks (n=10)	191.2±8.46	184.61±12.20***	-.08
B4 8 weeks (n=10)	187±7.60	178.23±10.87***	-.08

+ P>.05 A1 vs B1
* P<.05 A2 vs B2
*** P<.001 A3 vs B3
*** P<.001 A4 vs B4
Based on independent sample `t' test.
All values reported as Mean ± SD
+values indicate, increase while -values indicate decrease in mean growth rate.

Table 2: Effect of 2.5mg/kg dose of Endosulfan on relative tissue weight index (RTWI) of Albino rats at 2, 4, 6 and 8 weeks.

Groups	*Mean body weight at the end of experiment (gms)*	*Mean liver weight (gms)*	*Relative tissue weight index (RTWI) %*
Control group (A) (n=40)			
A1 2 weeks (n=10)	184.61±8.20	7.76±0.19	4.20±0.9
A2 4 weeks (n=10)	188.96±11.91	7.98±0.17	4.22±.11
A3 6 weeks (n=10)	195.13±9.92	8.27±0.13	2.23±.12
A4 8 weeks (n=10)	198.86±9.43	8.59±0.08	4.31±.14
Experiment group (B) (n=40)			
B1 2 weeks (n=10)	185.92±9.23	8.59±0.16	4.60±.12^{+}
B2 4 weeks (n=10)	186±8.00	11.92±0.09	6.40±.13***
B3 6 weeks (n=10)	184.61±12.20	13.97±0.17	7.56±.11***
B4 8 weeks (n=10)	178.23±10.87	16.31±0.10	9.15±.14***

+ p>.05 A1 vs B1
*** p<.001 A2 vs B2
*** p<.001 A3 vs B3
*** p<.001 A4 vs B4
Based on independent sample `t' test.
All values reported as Mean ± SD

Table 3: Effect of 2.5mg/kg dose of Endosulfan on different parameters of morphometric analysis in Albino rats at 2, 4, 6 and 8 weeks.

Groups	*Daimeter of central vein (μm) (n=50)*	*Size of hepatocyte (μm) (n=100)*	*Size of nucleus (μm) (100)*	*No. of nucleoli/ nucleus (100)*	*No. of necrotic foci/field*
Control group (A) (n=40)					
A1 2 weeks (n=10)	47.76±1.91	16.90±0.29	7.19±.11	1.18±0.02	0
A2 4 weeks (n=10)	47.1±1.62	16.71±0.23	7.73±.12	1.14±0.02	0
A3 6 weeks (n=10)	48.21±3.70	16.36±0.20	7.12±.1	1.2±0.03	0
A4 8 weeks (n=10)	47.52±2.83	16.48±0.26	7.0±.11	1.2±0.04	0
Experiment group (B) (n=40)					
B1 2 weeks (n=10)	48.30±1.26*	17.1±0.3*	7.2±.13+	1.23±0.03*	0
B2 4 weeks (n=10)	47.85±1.25**	16.90±.27**	7.85±.14**	1.26±0.05**	# 1-2minute
B3 6 weeks (n=10)	52.14±2.37***	16.55±0.26**	7.25±.13**	1.39±0.06***	@ 1-3 small
B4 8 weeks (n=10)	56.70±2.96***	19.14±.21***	7.31±.12***	1.51±0.05***	#3-4 fairly large

+ $p > .05$
* $p < .05$ #Minute foci $< 200\mu m2$
** $p < .01$ @Small foci $200\text{-}500\mu m2$
*** $p < .001$ #Fairly large foci $> 500\mu m2$
Based on independent sample `t' test.
All values reported as Mean ± SD

generally preserved in all the experimental sub-groups.

At 2 weeks (B1) (Fig. No. 1, Table 3) mild congestion and dilatation of sinusoids and central vein was noted. Portal tract showed very mild dilatation of vessels with hardly any inflammatory cells in it. Hepatocytes showed enlargement, cloudy swelling, and early vacuolation, with nuclear degenerative changes like pyknosis and karryorrhexis. Histological changes in group B1 were graded mild (Table 4).

At 4 weeks (B2) (Table 3) sinusoids showed moderate congestion, dilatation and prominence of kupffer cells. Central vein was significantly ($p < .05$) dilated. Portal tract showed dilatation of vessels and bile duct hyperplasia alongwith few inflammatory cells. Hepatocytes were considerably increased in size with variable degree of swelling, vacuolation and necrosis. Nuclear changes like karryolysis, karryorrhexis and pyknosis were also seen. Size of nuclei was increased containing 1-3 nucleoli. 1-3 minute areas of necrosis measuring upto $200\mu m^2$ were seen/HPF. Histological changes in group B2 were graded as moderate (Table 4).

At 6 weeks (B3) (Table 3) changes were similar to those seen in group B2 but of more severity with 1-3 small areas of hepatic necrosis measuring $200\text{-}500\mu m^2$/HPF. Histological changes in group B3 were graded as moderate (Table 4).

At 8 weeks (B4) (Fig. 2, Table 3). All changes as mentioned in group B2 and B3 were found at the maximum degree. Hepatocytes maintained their normal polyhedral shape however the cell boundries were indistinct at places. All stages of cellular swelling, necrosis and fatty change were evident. Areas of haemorrhage were noted in hepatic parenchyma along with lymphocytic infiltrate. Nuclei were considerably enlarged showing pyknosis, karryorrhexis and karryolysis. 1-4 fairly large areas of necrosis measuring $> 500\mu m^2$/HPF were seen. Histological changes were graded as marked (Table 4).

DISCUSSION

This study was designed to evaluate effects of a tolerable low dose (2.5mg/kg) of endosulfan on rat liver with increasing duration. Experimental animals

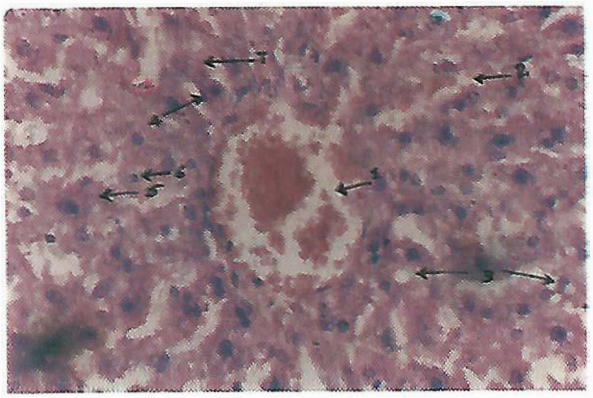

Fig. 1: **A histological section of rat liver experimental group B1.**
Note: **1. Congested and dilated central vein**
2. Congested and dilated sinusoid
3. Hepatocyte showing vacuoles
4. Prominent kupffer cell
5. Hepatocyte showing early cloudy swelling.
6. Lymphocyte
7. Karryolysis and karryorrhexis.
Staining H & E Mangification x 700.

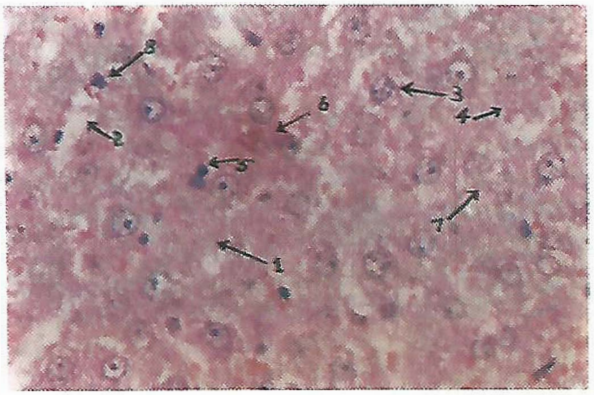

Fig. 2: **A histological section of rat liver experimental group B4.**
Note: **1. Hepatocyte swelling and balooning.**
2. Dilated and congested sinusoid.
3. Enlarged nucleus containing prominent neucleoli.
4. Marked necrosis.
5. Lymphocyte.
6. Haemorrhage into parenchyma
7. Binucleate hepatocyte
8. Prominent kupffer cell.
Staining H & E Mangification x 1400.

became progressively lethargic in group B1 and irritable in B2. Those in B3 and B4 showed aggressiveness, abnormal posturing and ataxia, verifying observations made by Gilbert.[9] Other studies[3,10] noted generalized reduction in food and water intake as also observed in this experiment. Dry skin with marked hair loss in group B4 also seen by Dikshith[11] is attributed to decreased blood supply to the skin. Duration dependent decrease in mean body weight and negative growth rate (Table 1) in experimental group as also noted in other studies[10,12] may be attributed to disturbance in satiety center of brain reducing food consumption[9,13], disturbance of digestion and absorption due to endosulfan induced gut oedema[3] and enhanced degradative process and reduced utilization of food at cellular level as a result of various metabolic and biochemical disturbances[14,15]. Increase in RTWI is due to significant increase in liver weight and a greater decrease in body weight. Increase in liver weight could be explained on the basis of congestion and cloudy swelling of hepatocytes[3,10,16]. Darkeninng of colour could be due to increasing congestion and deoxygenation of blood[3]. Areas of pale discolouration seen in B4 were due to hepatic necrosis. Dilatation and congestion of vessels in experimental group as also noted by Hatch[3] may be result of impaired venous drainage due to cellular injury and swelling. Congestion and stasis leads to increased deoxygenated haemoglobin, making liver appear darker in colour. Swelling and necrosis of hepatocytes with increase in size of both cytoplasm and nuclei with no signs of cell division indicated DNA replication without cell division as observed by Ferrando et al.[17] Early vacuolation, cloudy swelling, focal areas of cellular necrosis with nuclear degenerative changes like pyknosis, karryorrhexis, karryolysis and absence of nuclei were also noted by Gyorkos et al.[18] Endosulfan by causing fragmentation of endoplasmic reticulum[19] and lysis of mitochondrial membrane[3] bring about perturberations in Ca^{++} homeostasis resulting in increase in cytosolic Ca^{++} frequently associated with development of cell injury.[20] Disturbed ion

flux across mitochondrial membrane with low dose of endosulfan[3] cause depression of cellular respiration and result in hypoxia initiating anaerobic glycolysis and impairment of sodium pump mechanism with retention of sodium and water[21] causing cloudy swelling, vacuolation, hydropic degeneration, necrosis and nuclear changes associated with necrosis[17,18]. Endosulfan also causes disturbance in fat metabolism leading to fatty change. Inflammatory cells around portal tract produce proteolytic enzymes to remove the necrotic debris, thus helping heterolysis[21]. Kupffer cells also became prominent due to same reason. Billiary hyperplasia resulted from obstruction at the level of billiary canaliculi caused by hepatocyte swelling.

This study confirms the existing findings from other studies[12,19] and suggests that endosulfan at low doses of 2.5mg/kg is hepatotoxic and its toxicity increases with increase in duration of exposure.

Table 4: Histological grading in liver of Albino rats exposed to 2.5mg/kg dose of endosulfan at 2, 4, 6 and 8 weeks.

Groups		*Histological grading*
Control group (A) (n=40)		
A1	2 weeks (n=10)	0
A2	4 weeks (n=10)	0
A3	6 weeks (n=10)	0
A4	8 weeks (n=10)	0
Experiment group (B) (n=40)		
B1	2 weeks (n=10)	+
B2	4 weeks (n=10)	++
B3	6 weeks (n=10)	++
B4	8 weeks (n=10)	+++

0 Normal

\+ Mild congestion of central vein and sinusoid. Mild degree of early vacuolation, early swelling, degeneration of hepatocytes and mild focal necrosis.

++ Mild to moderate congestion of central vein and sinusoid. Moderate vacuolation, minute to small areas of focal hepatic necrosis.

+++ Marked congestion of central vein and sinusoids with distorted and disrupted endothelium, well established vacuolation, fatty change and fairly large areas of focal hepatic necrosis.

CONCLUSION

Endosulfan by causing hepatic dysfunction causes a definite health hazard particularly when exposed for longer duration of time. Thus for practical purposes repeated exposure of endosulfan to humans should be avoided for longer duration.

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