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Repeated dose toxicity of alfa-cypermethrin in rats

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The present study was performed to investigate the subacute effect of α-cypermethrin (α-CP) in rats. Alfa-cypermethrin a synthetic pyrethroid insecticide, dissolved in dimethyl sulfoxide (DMSO) and oral LD_{50} was investigated after administering orally different doses in rats and was determined as 145 mg/kg. Other groups of rats were given repeated daily oral dose (1/10 LD_{50}) of α-CP for 30 days. The animals were sacrificed on 31st day. Activities of various enzymes, cytochrome P450 and b5 contents in liver, hepatic antioxidant status, tissue residue concentration, haemogram and pathological changes were studied. It increased the serum aminotransaminases (AST, ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) activities and blood glucose level significantly. α-CP decreased RBC count, PCV and Hb level significantly. It significantly decreased cytochrome P450 in liver. Residues were present in different tissues. It increased malondialdehyde (MDA) level, while decreased the activities of catalase (CAT), superoxide dismutase (SOD) and glycogen level in liver significantly. Mild to moderate histological alterations were observed in lungs, liver, stomach, kidneys, testes and cerebellum. So repeated daily oral doses of α-CP at 1/10LD_{50} altered the biochemical parameters, decreased cytochrome P450 content, antioxidant status, which correlated with histopathological changes of tissues.

Key words: α-CP, cytochrome P450, cytochrome b5, antioxidants, tissue residue concentration, histopathology, rat

Introduction

Cypermethrin is a synthetic pyrethroid with potent insecticidal property. The technical grade cypermethrin is a racemic mixture of 8 isomers (four cis and four trans isomers). Two stereoisomer is termed α-isomer of cypermethrin, which is believed to be the most active isomer, and is known as α-cypermethrin (α-CP) [20]. Alfa-cypermethrin is extensively used as an ectoparasiticide in animals, and as insecticides in crop production and public health programme [20]. Some of the toxic actions of α-CP have been reported earlier [20], but reports on tissue residue level and effects after repeated daily oral administration of α-CP on cytochrome P450, cytochrome b5, antioxidant status, blood biochemistry, and histology of some tissues in rats are not available. It has been recorded [1] that the vehicle has a great influence on the LD_{50}, probably by influencing absorption. The oral LD_{50} values for rats were 79 mg/kg (5% in corn oil) [20] and 40-80 mg/kg (10% in corn oil) [20]. But the report of LD_{50} value of α-CP for rats in presence of dimethylsulfoxide as a vehicle is not available. Therefore, the present study was undertaken to determine the oral median lethal dose of α-CP dissolved in DMSO and to investigate the subacute toxicity (30 days) of α-CP.

Materials and Methods

Materials

Alfa-cypermethrin (α-CP, >99% pure, Gharda Chemicals Ltd. Bombay).

Animals and experimental design

Ninety [90] adult Wistar rats of both sexes (equal sex ratio; weighing about 200 ± 20 g) were divided into nine equal groups (I to IX) each containing ten [10] animals. All rats were kept under controlled conditions of temperature (22 ± 1°C) and humidity (60 ± 5%). They were given pellet food (Amrut feeds Ltd., Pune, India) and drinking water *ad libitum*. A twelve hour day and night cycle was maintained in the animal house. The experimental protocol met the national guidelines on the proper care and use of animals in the laboratory research. The Institutional Animal Ethics Committee approved this experimental protocol.

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The animals were grouped as follows:

Groups	Treatment
Group-I	DMSO (1 ml) + α-CP at the dose of 100 mg/kg.b.wt.
Group-II	DMSO (1 ml) + α-CP at the dose of 125 mg/kg. b.wt.
Group-III	DMSO (1 ml) + α-CP at the dose of 150 mg/kg. b.wt.
Group-IV	DMSO (1 ml) + α-CP at the dose of 175 mg/kg. b.wt.
Group-V	DMSO (1 ml) + α-CP at the dose of 200 mg/kg. b.wt.
Group-VI	DMSO (1 ml) + α-CP at the dose of 225 mg/kg. b.wt.
Group-VII	DMSO (1 ml) (Control for Group-I to VI).
Group-VIII	DMSO (1 ml) + α-CP at the dose of 14.5 mg/kg (1/10LD_{50}) b.wt. × 30days.
Group-IX	DMSO (1 ml) × 30days (Control for Group-VIII)

Groups I-VI were used for determination of LD_{50} of α-CP. Group VII served as control for groups I-VI. The animals were fasted overnight and α-CP was administered orally after dissolving in DMSO (1ml) as stated above. The animals were observed for respiratory and CNS symptoms, behavioral changes and death. LD_{50} was determined as per the method of Miller and Tainter (9). Group VIII was used for short-term toxicity study. Group IX served as control for Group VIII. α-CP was administered orally to the animals of group VIII at 14.5-mg/kg b.wt. and group IX animals were dosed equal volume of DMSO only (1 ml) daily for 30 days. On the 31st day group-VIII and control group (group-IX) were sacrificed under halothane anesthesia by severing the neck vessels aseptically.

Hematological analysis

Blood was collected in three sets of test tubes from the severed neck vessels of each animal. Blood smears were prepared for differential leukocyte count. One set was kept under refrigeration (4°C) for separation of serum and utilized for estimation of activities of aspartate transaminase (AST) [16], alanine transaminase (ALT) [16], lactate dehydrogenase (LDH) [1], alkaline phosphatase (ALP) [7] and total protein (TP) [3], globulin (GLB) and albumin (ALB) [17]. The blood of another set of test tubes having mixture of potassium oxalate and sodium fluoride as anticoagulant was used for estimation of glucose [18]. Blood in the 3rd set of test tubes was heparinized and used for RBC, WBC counts and measuring PCV and haemoglobin level.

Biochemical analysis

Portions of lungs, liver, stomach, kidney, stomach, testes and cerebellum were collected in 10% formalin solution for histopathology. One portion of liver was washed in physiological saline, homogenized and the homogenate was kept for estimation of catalase activity (CAT) [8], levels of reduced glutathione (GSH) [6], malondialdehyde (MDA) [15], glycogen [13] and tissue protein [10]. Another portion of liver was collected in ice-cold 1.15% KCl, homogenized within 10 min, centrifuged, microsomal pellets were separated and used for estimation of superoxide dismutase (SOD) [12], cytochrome P450 and b5 [14] contents by DB-UV-Vis spectrophotometer.

Animal was sacrificed and the liver was perfused *in situ* with homogenizing buffer A (Tris-HCL + EDTA + BHT) by single pass injection through the portal vein and dissected out, placed in ice cold KCl (1.15%). All the subsequent steps in the preparation of microsomal fraction were carried out at 0-4°C. Then the liver was minced and mixed with 4 volumes of buffer A and homogenized in a mechanically driven Teflon glass homogenizer (Remi RQ 127 A). The homogenate was centrifuged at 10000 × g in an automatic high-speed cold centrifuge (Hitachi-SCR 20B) by using the rotor RPR 20-2 for 30 min. The supernatant was recentrifuged at 105,000 × g for 1 hr in an automatic preparative ultracentrifuge (Hitachi 70 P-72) using rotor RP-65T to yield microsomal pellet. Microsomal pellet was suspended in buffer B (Pot. Pyrophosphate + EDTA + BHT) and homogenized with four passes of mechanically driven Teflon glass homogenizer (Remi RQ 127A), and again centrifuged at 104,000 × g for 1 hr. The supernatant fraction was decanted and the microsomal pellet was resuspended in a minimum volume of buffer C (Tris-Hcl + EDTA + Glycerol) and stored at –20°C till further use. The pellet was used for estimating SOD activity and cytochrome P450 and b5 levels.

Residue level determination

The tissue residue levels of α-CP in brain, lungs, liver, heart, kidney and testes were estimated by the method of Marei *et al*. [11].

Tissues (2 g) were extracted for 4 min with acetonitrile (25 ml) and anhydrous sodium sulfate (0.5 g) using a homogenizer. The extract was filtered through anhydrous sodium sulfate (0.5 g) and the tissues were re-extracted twice with acetonitrile (1st by 25 and 2ndly by 12 ml). The extract was clarified by centrifugation and filtered through anhydrous sodium sulfate. The combined acetonitrile extracts were concentrated to 20 ml and partitioned with hexane (2 × 10 ml). The hexane phases were discarded and the acetonitrile phase was evaporated to dryness using a rotary vacuum evaporator at 40°C. The volume was finally made up to 5 ml with acetone for GLC estimation.

A stock solution of 1 mg per litre of α-CP (analytical grade > 99%) was prepared as an external standard. The retention times of α-CP was 13.5 min. The data were

recorded in a HP 3392A integrator.

A Hewlett Packard (USA) model 5890A gas chromatograph coupled with a 3392 A (HP) integrator and equipped with a ^{63}Ni electron capture detector was used for analysis of α-CP. Operational parameters were:

Injector temperature- 275°C
Oven temperature- 255°C,
Detector temperature- 275°C,
Flow rate of carrier gas N_2 - 70 ml per minute.

Column: An 1.8 × 2 mm I.D. glass column packed with 3% OV-101 on chromosorb W.H.P. (80-100 mesh) was used.

With 10 µl Cap. Hamilton Syringe 2 µl of standard and samples were injected into gas liquid chromatograph.

Histopathological examination

Small pieces of lungs, liver, stomach, kidneys and cerebellum were fixed in 10% neutral buffered formalin and testis in Bouin's fluid. Sections of 3-5 µ thicknesses were cut and stained with haematoxylin and eosin (H & E) for observation under light microscope.

Statistical analysis

All values were expressed as mean ± S.E.M. Statistical analysis was done by using SPSS 10.1. Statistical significance between two means was assessed by Student's t-test at $p < 0.05$.

Results

Clinical signs

α-CP did not produce any gross effect at 100 mg/kg. However, at higher doses ranging from 125 to 225 mg/kg, it produced signs of CNS stimulation followed by prolonged depression. Initially the intoxicated animals exhibited chewing, licking and salivation, which was followed by CNS depression. A variable sequence of motor symptoms developed that involved occasional pawing, or burrowing, coarse whole body tremor associated with movement, gradual development of hind limb extensor tone and an increase in startle response. Finally, choreoathetosis (sinuous writhing) developed, and the animals exhibited slow twisting or writhing movement of neck and tail. When symptoms progressed, choreoathetosis became continuous and the righting reflex was gradually lost. Violently twisting movements sometimes lifted the body from the floor in severely affected animals and these animals were cases of severe athetosis. At the terminal stage, animals showed laboured breathing, gasping and death. The mortality data during determination of LD_{50} were 0, 4, 6, 6, 9 and 10 against the doses were 100, 125, 150, 175, 200 and 225 mg/kg b.wt respectively (Table 1). The acute oral LD_{50} value was calculated as 145 mg/kg body weights.

Biochemical and hematological profiles

Effect of α-CP on certain blood and liver biochemical and antioxidants parameters are summarized in Table 2 and 3, respectively. α-CP significantly ($p < 0.05$) increased the activities of serum AST, ALT, ALP, and LDH. In liver cytochrome P450 content and activities of CAT and SOD were decreased while MDA level was increased significantly ($P < 0.05$) without any significant alteration of GSH level and cytochrome b5 content in liver. Blood glucose level was significantly ($p < 0.05$) increased, and liver glycogen was significantly ($p < 0.05$) decreased. Serum GLB and total protein levels were significantly decreased. α-CP decreased PCV, Hb level, and counts of RBC,

Table 1. Acute toxicity of α-CP in rats

Dose (mg/kg)	Mortality
100	0/10
125	4/10
150	6/10
175	6/10
200	9/10
250	10/10
LD_{50}	145mg/kg

Table 2. Effects of α-CP on certain biochemical parameters in serum and blood of rats after daily oral administration at 14.5 mg/kg for 30 days (Values are mean ± SE, n = 10)

Parameters	Control	α-CP treated
ALP activity (IU/L)	78.03 ± 2.58	161.53 ± 6.60*
AST activity (IU/L)	59.45 ± 3.52	72.00 ± 4.97*
ALT activity (IU/L)	12.00 ± 1.43	26.50 ± 1.67*
LDH activity (IU/L)	49.41 ± 2.58	64.80 ± 2.01*
TP (gm/dl)	8.12 ± .022	6.41 ± 0.17*
ALB (gm/dl)	4.53 ± 0.29	4.50 ± 0.26
GLB (gm/dl)	3.81 ± 0.21	2.16 ± 0.49*
Blood Glucose mmol/L	3.70 ± 0.48	6.22 ± 0.85*

*$p < 0.05$ in comparison with control
ALP: Alkaline Phosphatase, AST: Aspartate transaminase, ALT: Alanine transaminase; LDH: Lactate dehydrogenase, TP: Total protein; ALB: Albumin; GLB: Globulin.

Table 3. Effects of α-CP on certain biochemical parameters in liver of rats after daily oral administration at 14.5 mg/kg for 30 days (Values are mean ± SE, n = 10)

Parameters	Control	α-CP treated
CAT activity (U/mg protein)	0.39 ± 0.04	0.07 ± 0.01*
SOD (U/mg protein)	0.48 ± 0.02	0.13 ± 0.01*
MDA (nmol/mg protein)	0.24 ± 0.02	2.85 ± 0.18*
GSH (µmol/mg protein)	1.41 ± 0.16	1.30 ± 0.05
Glycogen (mg%)	7.94 ± 0.24	5.15 ± 0.34*
P450 (nmol/mg microsomal protein)	2.91 ± 0.02	2.74 ± 0.04*
b5 (nmol/ mg microsomal protein)	1.16 ± 0.07	1.28 ± 0.05

*$p < 0.05$ in comparison with control
CAT: Catalase, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Reduced glutathione.

Table 4. Effects of α-CP on haemogram in rats after daily oral administration at 14.5 mg/kg for 30 days (Values are mean ± SE, n = 10)

Parameters	Control	α-CP treated
RBC (Million/cmm)	9.31 ± 0.88	6.33 ± 0.56*
WBC (Thousand/cmm)	10.45 ± 1.02	9.70 ± 0.48
Neutrophils (%)	28.85 ± 0.93	33.80 ± 0.94*
Lymphocytes (%)	62.88 ± 1.13	55.60 ± 0.71*
Monocytes (%)	4.21 ± 0.39	2.50 ± 0.42*
Eosinophils (%)	1.21 ± 0.19	1.16 ± 0.16
Basophils (%)	0.39 ± 0.02	0.51 ± 0.02
Packed Cell Volume (%)	38.95 ± 0.89	35.33 ± 0.33*
Haemoglobin (gm/dl)	10.96 ± 0.96	8.28 ± 0.10*

*$p < 0.05$ in comparison with control.

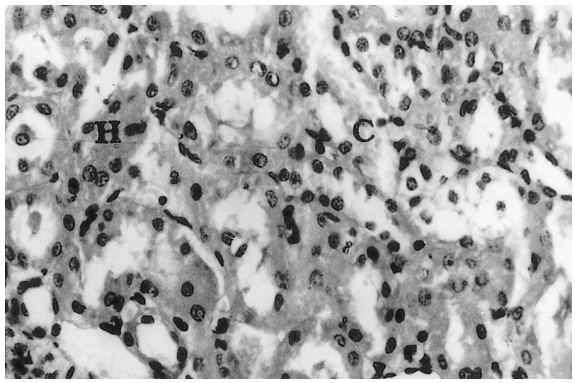

Fig. 2. Photomicrograph of rat kidney showing congestion (C) & hemorrhages (H) between the tubules after daily oral administration of α-CP at 14.5 mg/kg for 30 days, (H & E, 400×).

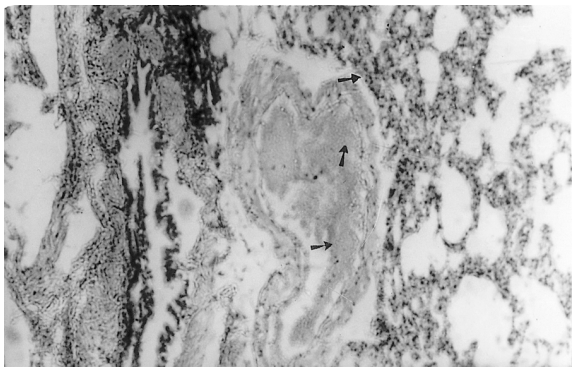

Fig. 1. Photomicrograph of rat lungs showing hemorrhages, and thickened inter-alveolar septae with infiltration of mononuclear cells (arrows) after daily oral administration of α-CP at 14.5 mg/kg for 30 days, (H & E, 450×).

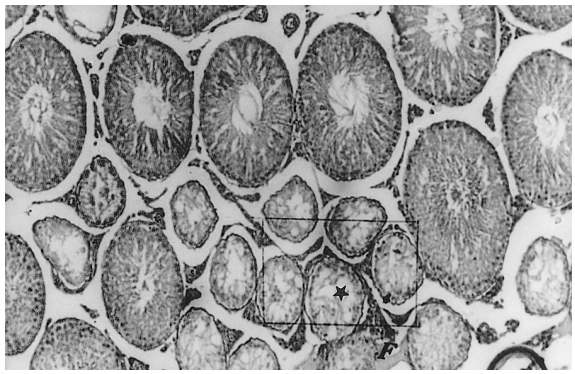

Fig. 3. Photomicrograph of rat testis showing edematous fluid accumulation between the tubules (F) and vacuolation (*) within the tubule after daily oral administration of α-CP at 14.5 mg/kg for 30 days, (H & E, 100×).

leukocyte and monocytes, whereas neutrophil count was increased significantly (Table 4).

Residue level of α-CP

The levels of α-CP following daily oral administration for 30 days were 0.07 ± 0.01, 0.08 ± 0.02, 0.12 ± 0.10, 0.58 ± 0.11, 1.02 ± 0.21 and 0.21 ± 0.01 ppm in liver, brain, testis, kidney, lung, and heart, respectively. Concentration of α-CP was maximum in the lungs.

Pathological findings

At postmortem, rats showed bloated stomach with severe hemorrhages in both stomach and intestine. Hemorrhages were also seen in lungs. No changes were discernable in other visceral organs.

α-CP produced oedema and emphysema in lungs (Fig. 1). Congestion, hemorrhages and disruption of sinusoids were found in liver. In stomach, it produced desquamation and necrosis of the epithelium. Kidneys showed congestion with accumulation of red blood cells (Fig. 2). The section of testis revealed oedema between seminiferous tubules and vacuolation within the tubules (Fig. 3). Congestion and hemorrhages were apparent in meningeal vessels of the cerebellum.

Discussion

The pattern of the motor signs after α-CP administration is strongly suggestive of central nervous system involvement. The acute oral LD_{50} value of α-CP in DMSO was 145 mg/kg, which is higher than the LD_{50} values of alfa-cypermethrin determined using other vehicles like corn oil. This suggests that the vehicle DMSO reduced the toxicity of α-CP in rats, which may be due to an antioxidant effect of DMSO. Not only activities of SOD and CAT but also levels of GSH and MDA levels in the liver reflect the oxidative status and the serum enzymes like AST, ALT and ALP represent the functional status of the liver [19]. Increase of transaminase activity along with the decreased of content of free radical ($O_2^{\cdot-}$) scavengers are probably the consequence of α-CP induced pathological changes in liver. Increased catecholamine release [2] causes glycogenolysis and this may be a reason for significant decrease in liver glycogen

leading to hyperglycemia. Decreased in RBC count, PCV and Hb indicate depressed erythropoiesis and increase of neutrophils represents inflammation in visceral organs. The decreased CAT and SOD activities and increased MDA level in liver as well as increased serum AST, ALT and ALP levels suggest that α-CP causes hepatic damage. The pathogenesis may be through free radical ($O_2^{-\cdot}$) formation α-CP undergoes metabolism in the liver via hydrolytic ester cleavage and oxidative pathways by the cytochrome P450 microsomal enzyme system [4] which probably decreased the P450 contents in liver that may causes in oxidative stress producing depletion of activity of CAT, SOD and glycogen level and increased the level of MDA leading to hepatic degeneration and necrosis. The present antioxidant status and biochemical changes correlated with histopathological changes of tissues corroborated with the findings of Giray *et al*. [5]. In conclusion oral LD_{50} of α-CP dissolved in DMSO was determined as 145 mg/kg in rats. In repeated short-term toxicity study at 1/10 LD_{50} dose for 30 days increased was observed in liver MDA, serum AST, ALT, ALP, LDH, and glucose but the activities of SOD and CAT, glycogen level and cytochrome P450 content decreased. Residue levels of α-CP were observed in different tissues. It produced moderate cytotoxic effects in lungs, liver, stomach and testis, and least effect in cerebellum. The pathological changes correlated with the altered enzyme activities.

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