RESEARCH ARTICLE

STUDY OF THE TOXIC EFFECTS OF CYPERMETHRIN IN EXPERIMENTAL ANIMALS

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ABSTRACT
This study focuses on the toxic effects of a commercially available pesticide, cypermethrin (CM), on animals. This pesticide was administered in the form of aerosol spray through a nebulizer. The study was performed in four different groups and a constant dose of the pesticide was administered once, twice, thrice and four times a day to the respective group for a period of 30 days. The animals were then dissected to study the pesticide effects on different organs. The organs were preserved in 10% formalin. The tissues were processed by basic histopathological method and the slides were prepared for observation. The results were recorded on a performa and were quantified by a unique scoring system. It is concluded that the injurious effects to the mentioned organs were dose and frequency dependent.

Keywords: Cypermethrin, aerosol, histopathological effects.

1. INTRODUCTION
Pakistan is an agricultural country and according to a report from World Bank1, more than 60% population lives in rural area and among them majority of the population is occupied in agricultural sector. In order to enhance and improve the production of crops, farmers rely on synthetic insecticides in most of the agricultural societies. The indiscriminate use of insecticides result in serious health implications to the community because of their ignorant behavior and illiteracy. The pesticides acting against insects are labeled as insecticides, others acting on fungi are known as fungicides and those active against herbs are classified as herbicides. Some other types have also been known for their specific action such as nematicides, avicides and rodenticides that are active against nematodes, birds and rodents, respectively2.

Pyrethroids are synthetically produced molecules which are chemically analogous to pyrethrins and are used for vector control. However, they are deteriorated quickly in the presence of sunlight and are completely dissociated in few days. Most pyrethroids are also used in conjunction with piperonyl butoxide. Some of these new compounds may not break down as readily in sunlight like pyrethrins2. The most primitive pyrethroids are similar to pyrethrin I and II. They are formed by changing the ester of chrysanthemic acid resulting in altered and improved pesticidal activity. The other improved types of commercially important esters consist of tetramethrin, allethrin, phenothrin, barthrin, dimethrin and bioresmethrin. An additional family of pyrethroids have altered acid portion with modified alcohol components and involve more complex organic synthesis. The constituents of this broad class include the dichlorovinyl and dibromovinyl derivatives. The other examples include tefluthrin, fenpropathrin, bioethanomethrin, malathion, aldrin, dieldrin and cypermethrin3.

Cypermethrin (CM) is a synthetic insecticide that belongs to pyrethroid group of insecticides and is known to have excellent insecticidal activity. The data received by the continuous experimentation of various pesticides focusing on the metabolism of CM is of interest. It is believed that the metabolic pathways are more toxic to insects than mammals because the metabolic rate is relatively slower in
insects as compared to animals. CM is a contact poison that affects the nervous system of vertebrates and invertebrates. The mechanism of poisoning involves an effect on voltage-dependent sodium channels and inhibiting an energy generating enzyme known as ATPase. Pyrethrins and pyrethroids are widely used nowadays to control adult mosquito population in North America and are among the most common pesticides used in public health perspective. Its use has now surpassed the conventional synthetic pesticides including organochlorines and organophosphates, which were utilized previously.

CM exhibits relatively low toxicity to mammalian and avian life with sufficient stability in air and light. In third world countries, CM is utilized to manage several pests. It is most commonly active against lepidopterous pests of cotton, fruit, and vegetable crops. It is commercially available and marketed as an emulsifiable concentrate or wettable powder. CM is not considered to be carcinogenic in rabbits, mice and rats when given in high doses. However, rats showed signs of intoxication and pathological changes of nervous system in high doses. In one of the study, rats showed neurotoxicity and demonstration of histopathological changes in the tibial and sciatic nerves with axon degeneration. An increase in leukocytes in peripheral blood was observed in male and female mice with sub-acute poisoning of CM.

2. MATERIALS AND METHODS
2.1. Experimental Animals
Healthy male and female rabbits (locally breed) of one year age, weighing 1300-1500 g with no sign of illness, were selected for the study. The reasons for choosing rabbit in this study were predominantly their docile and non-aggressive nature and easy handling. They are widely bred and are economical as compared to other larger animals and also have short vital cycles (gestation, lactation and puberty). The male and female rabbits were kept in separate wire cages in healthy and fresh environment and were fed ad libitum. They were usually provided with normal diet consisting of fresh water, grains, beans and carrots. Fresh grass and green fodder were given in the morning and evening whereas fresh drinking water was provided round the clock. Temperature of the rabbits was recorded daily in the morning and evening before and after the administration of the pesticide. The experimental animals were acclimatized for five days and a total of 10 animals were used in the experiment series. Five groups were made and two rabbits were placed in each group. Group one served as control group.

2.2. Selection of Pesticide
The commercially available CM solution (11%) was used in order to simulate the histopathological model of exposure in different organs of living beings.

2.3. Experimental Design
The pesticide was administered in the form of aerosol spray in order to examine the effects of CM on various organs of the body. Conventional nebulizer was used to produce vapors and the cylinder was charged with 0.5 cc CM solution. In order to retain the vapors, the cages were covered with filter papers during the period of exposure. Four frequencies of exposure with a constant dose were planned on alternate days for 30 days. In the control group normal saline was used in the vaporizer. The frequency of dosing in all groups is given in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosing Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (control)</td>
<td>No exposure</td>
</tr>
<tr>
<td>2</td>
<td>Once a day</td>
</tr>
<tr>
<td>3</td>
<td>Twice a day (12 hourly)</td>
</tr>
<tr>
<td>4</td>
<td>Thrice a day (8 hourly)</td>
</tr>
<tr>
<td>5</td>
<td>Four times a day (6 hourly)</td>
</tr>
</tbody>
</table>

2.4. Post Exposure Observations
The animals in each group were observed twice
daily for any clinical signs and behavioral alterations. After 30 days of pesticide exposure, the changes in blood cells were observed using automated hematology analyzer (Sysmex KX-21N, USA). The blood was obtained from cardiac puncture and collected in tubes containing an anticoagulant. The observations of increase or decrease in hematology parameters were recorded in the performa. The body weight of all animals was recorded twice weekly. The animals from each group were slaughtered on the 30th day. After slaughtering of rabbits, the visceral organs were examined for gross lesions including skin, lungs, liver, kidneys, stomach and brain and were stored in 10% buffered formalin.

2.5. Scoring System
In order to quantify the appearance of histopathological damage and lesions in the viscera recovered, a unique scoring system was prepared to award the pathological changes a specific number (Table 2). Low score indicates small histologically identifiable damage whereas a high cumulative number exhibits marked damage and injury. Zero score was given when no remarkable change was noticed in the skin lesion. 1 score was given to the presence of focal edema or swelling observed in the skin. 2 score was given to a mild edema along with mild inflammatory change, further 3 scoring was given to moderate inflammation, 4 score to abscess formation and 5 to necrotic change in the skin, last but not the least any other pathology observed was described as 6 score.

2.6. Histopathological Study
The tissue samples for histopathological evaluation were taken from the skin, lung, liver, brain and kidneys. The tissue sections were fixed in 10% buffered formalin and processed for the histopathological studies using routine method of dehydration and embedding in paraffin as per standard technique. The sections of 4-5 µ thickness were cut using Thermo Shandon microtome machine and were examined for histopathological studies after staining with hematoxylin and eosin (H & E) method. The inclusion criteria of tissues were based on the following points:

i. Only those sections were included in the study which were of appropriate thickness i.e. 4 µ.
ii. The size of section on the slide was representative of the lesion.
iii. The H & E slides were properly stained.
iv. Significant lesion was identified for quantitative scoring scheme as described in the previous section.

3. RESULTS
3.1. Inhalation of CM
The administration of commercially available pesticide, CM, through respiratory system produced different pathological changes in the various organs which are summarized in Table 3.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total Score*</th>
<th>Key</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-10</td>
<td>No significant histopathological damage</td>
</tr>
<tr>
<td>2</td>
<td>11-20</td>
<td>Mild histopathological damage</td>
</tr>
<tr>
<td>3</td>
<td>21-30</td>
<td>Moderate histopathological damage</td>
</tr>
<tr>
<td>4</td>
<td>31-40</td>
<td>Marked histopathological damage</td>
</tr>
</tbody>
</table>

*Lowest possible score = 0; maximum possible score = 40 (under high power magnification).
Table 3. Scores of each animal in different groups after 1 month exposure with CM.

<table>
<thead>
<tr>
<th>Animal Code</th>
<th>Group</th>
<th>BC*</th>
<th>BP*</th>
<th>Skin</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>GIT</th>
<th>Brain</th>
<th>Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4/40</td>
</tr>
<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
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<tr>
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<td>0</td>
<td>0</td>
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<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>8/40</td>
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<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>10/40</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>12/40</td>
</tr>
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<td>1</td>
<td>3</td>
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<td>2</td>
<td>1</td>
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<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>19/40</td>
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<tr>
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<td>5</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>25/40</td>
</tr>
</tbody>
</table>

*BC = Behavior change; BP = Blood parameters.

3.2. Group 1: Control
The animals of control group were exposed to fumes of normal saline by nebulizer once a day. These animals showed mild lung, liver and kidney edema with mild changes in behavior. Hematological parameters were within normal limits.

3.3. Group 2: Once Per Day Dose
The second group with per day inhalation of CM demonstrated normal skin histology. Only edema was seen in the lung alveoli. The liver sinusoids showed congestion and mild inflammatory reaction. The sections of kidney revealed interstitial edema, inflammation and mild tubular damage while the gastrointestinal tract and brain showed little changes.

3.4. Group 3: Twice Per Day Dose
The third group with 12 hourly administration of CM demonstrated increased irritability of the animal, increase in blood parameters and features of edema with mild inflammation of the upper dermal layers of skin, edema of lung alveoli with moderate inflammation, interstitial edema, tubular damage and congestion of the kidney parenchyma. The section of brain was unremarkable.

3.5. Group 4: Three Times Per Day Dose
The group with 8 hourly exposure of the CM showed marked irritably of the animal with increased hematological parameters. Remarkable edema and inflammatory reaction were seen in lungs and liver. The sections of kidneys revealed moderate tubular damage with inflammation of interstitial tissue. The gastrointestinal section showed focal edema, hemorrhage and inflammation of the lamina properia. The brain tissues also revealed edema with inflammatory reaction.

3.6. Group 5: Four Times Per Day Dose
The group with 6 hourly exposure of the CM showed marked irritably of the animals with increased hematological parameters. Remarkable edema and inflammatory reactions were observed in lung (Figs. 1 and 2). The histological section of liver also showed congestion and inflammatory infiltration. The sections of kidney revealed moderate to marked tubular damage with inflammation of interstitial tissue. The GIT section showed focal edema, hemorrhage and inflammation of the lamina properia whereas the brain tissues also revealed edema with inflammatory reaction.
**Fig. 1.** The histopathological section of lung (100x magnification) revealing thickened alveoli with disrupted architecture, hemorrhage and mild inflammation.

**Fig. 2.** The histopathological section of lung (400x magnification) revealing thickened alveoli with disrupted architecture, hemorrhage and mild inflammation of the septa.
3.7. Interpretation of Scoring of CM Exposure
The culminate scores of the various parameters including behavior change, blood parameters, histopathological sections of skin, lung, liver, kidneys, gastrointestinal tract and brain were found to increase in all five dosage groups i.e. once, twice, thrice and four times daily. The average score of control group was less than 5 whereas the average scores of once, twice, thrice and four times daily groups were 9, 14, 19 and 23 out of 40, respectively. Therefore, after 30 days of inhalation of CM every 6 hourly per day (Group 5), the animals in this group falls in the category of stage III i.e. moderate histopathological injury (Table 2).

4. DISCUSSION
Not much work has been conducted to reveal the toxic effects of CM inhaled through respiratory route. It is considered as a pesticide of choice in tropical countries for indoor spraying for larvicide control purposes. Garcia et al.\textsuperscript{10} in their experimental study on rodents used 1% CM in soy oil and 2% in diesel solution in order to replicate the respiratory signs and symptoms that occurred in Manaus in 2001, in controlled conditions. It was concluded that inflammation is associated with increased respiratory resistance in animals exposed to CM and diesel solution. The data proposed that CM may be associated with the inflammation of organs as observed in experimental animals. This is also in accordance with the present study where the respiratory route of CM exposure has shown a pronounced effect on lungs with marked interstitial inflammation of the alveoli, hemorrhage and congestion. Haratym et al.\textsuperscript{11} evaluated a murine model of pyrethroid poisoning and suggested that all pyrethroids resulted in an increase in the number of white blood cells in peripheral blood irrespective of the dose and sex of the animals. This is in consistent with the present study as an increased red blood index with increased total leukocyte count in a dose dependent manner has been observed. The data of this study has been found partially in accordance with the literature that points at CM toxicity being responsible for alterations in the respiratory system. No comprehensive study has been conducted so far to the best of our knowledge to identify the effects of CM toxicity through inhalation route. Bergmeyer, in 1974, was the first to report the effects after repeated daily oral administration of α-CM on a number of hepatic enzyme systems including cytochrome P450, cytochrome b5, antioxidant status, blood biochemistry and histology of some tissues in rats\textsuperscript{12}.

The vehicle also affects the LD50 of an agent as it influences absorption. Manna et al.\textsuperscript{13} studied the oral administration of CM and compared it with other vehicles for proper absorption. Their findings concluded that the histological investigation depict variable pathologies identified in organs under consideration, such as α-CM associated edema and emphysema was noted in the lungs while liver examination revealed congestion and hemorrhages with disruption of sinusoids. In stomach there was focal desquamation and necrosis of the gastric epithelium but kidneys showed congestion with accumulation of red blood cells. The seminiferous tubules of the testicular tissue revealed edema and vacuolation between tubules as well as congestion and hemorrhages were apparent in meningeal vessels of the cerebellum\textsuperscript{13}. Comparable features were observed in the present study where CM exhibited similar microscopic changes of marked hemorrhage, disruption of blood vessels and inflammation of the interstitium in liver, kidneys and lungs.

According to Giray et al.\textsuperscript{14} after the administration of CM, distinctive motor injury patterns were identified which showed central nervous system injury. A decreased level of free oxygen radicals and increased transamine activity was also found responsible for CM associated liver injury. The other patho-physiological changes were hyperglycemia due to increased catecholamine content in the presence of glycogenolysis. Depression in the erythropoiesis causes an alteration in the RBC count, pack cell volume (PCV) and hemoglobin concentration, whereas an increase in neutrophils number correspond to the inflammation in visceral organs. This is one of the possible reasons that the organs including liver, kidneys, lungs and brain
showed inflammatory responses. The damage to liver cells causes the spillage of hepatic enzymes in blood stream that leads to hepatic damage. The most likely pathogenesis is through production of free oxygen radical (O²⁻) after the induction of CM that undergoes metabolism in the liver via hydrolytic ester cleavage and oxidative pathways by the cytochrome P450 microsomal enzyme system. This in turn decreased the P450 contents in liver and causes an oxidative stress produced depletion of activity of a potent anti-oxidant leading to hepatic degeneration and necrosis. The present antioxidant status and biochemical changes are in correlation with the histopathological changes of tissues as reported by Giray et al.

The investigation executed by Attar in 2010, indicated that an oral administration of another pyrethroid family pesticide, malathion, to rats caused significant alterations in hematological and biochemical parameters. The serum hepatic enzymes and the levels of creatinine, urea, and uric acid were largely increased but the values of total protein and total albumin were statistically decreased. These results are in agreement with different previous researches who studied the adverse effects of pyrethroid class of pesticides including CM and malathion that were found responsible for causing severe physiological and biochemical disturbances in experimental animals including buffalo, goats, mice, cockerels. It was concluded that administration of malathion for one month resulted in the distortion and damage of liver structure along with distortion of hepatocytes, focal necrosis, sinusoidal enlargement and apparent formation of vacuole in hepatocytes. Scattered infiltration of lymphocytic, neutrophils and plasma cell infiltrations, dilation, and congestion of blood vessels with hemorrhage were also identified in liver of rats exposed to malathion.

Areas of renal cortex harboring glomeruli and tubules showed more remarkable changes in treated animals as compared to control group. Therefore, these areas were selected for histological examination with the light microscope. The histologically normal renal corpuscle which consists of a bunch of capillaries is known as the glomerulus. It is surrounded by Bowman’s capsule. Between the visceral and parietal layer there is a space known as Bowman’s space. The histological features in the renal tissue revealed swelling of the glomerulous appearances, increasing of bowmen’s spaces and glomeruli while tubules exhibited remarkable degeneration. Pari et al. studied the ultra structure of the proximal tubular cells and epithelial changes including vacuole formation, damaged external membrane, and a few swollen mitochondria. The similar histological changes were noted in the current study where the renal parenchyma exhibited degeneration and shrinking of glomerulus, cloudy swelling of the renal tubules and scattered inflammation of the interstitial tissue associated with hemorrhage and congestion.

5. CONCLUSION
The study concluded that CM administration through inhalation route may cause irreversible damage to lungs, kidneys and liver in terms of moderate inflammatory infiltration and tissue damage.

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