



## RESEARCH ARTICLE

### Immuno-Toxicological Effects of Allethrin (Active Ingredient in Mosquito Repellent Coils) in BALB/c Mice Following Oral Administration

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#### ARTICLE HISTORY (18-415)

Received: November 03, 2018

Revised: December 24, 2018

Accepted: January 01, 2019

Published online: March 07, 2019

#### Key words:

Allethrin

Immuno-toxicology

Mice

Mosquito coils

Pathology

#### ABSTRACT

The use of synthetic pyrethroids as an insecticide in households has surpassed the other pesticides like organophosphates because of its likely to be safer for humans. This study was designed and executed to find out the toxic effects of allethrin (a synthetic pyrethroid) when they are overused or have an accidental oral exposure in humans or animals. For this purpose, 60 BALB/c mice were randomly divided into four equal groups. Group A served as a control group. Groups B, C and D were given allethrin @ 0.5, 1 and 1.5 g/kg BW, respectively orally on the 14<sup>th</sup> day of the experiment. Animals were monitored twice daily for clinical signs and mortalities. Randomly selected five mice from each group were euthanized humanly at 21<sup>st</sup> and 42<sup>nd</sup> day of the experiment to collect blood/serum for determination of immunological parameters. Clinical signs such as hair coat roughness, alopecia, conjunctival hyperemia, salivation, frequent defecation and nervous signs like tremors and convulsions were observed. The highest mortality (53.3%) was recorded in group D (1.5 g/kg BW) followed by group C (1 g/kg BW) and group B (0.5 g/kg BW) with 46.6% and 26.7% mortalities, respectively. The absolute weight of the spleen and thymus was significantly ( $P \leq 0.05$ ) decreased in all treated groups at 21<sup>st</sup> and 42<sup>nd</sup> day of the experiment. Total antibody titer against SRBCs at the 14<sup>th</sup> and 28<sup>th</sup> day of experiment decreased significantly ( $P \leq 0.05$ ) in treated groups. At 29<sup>th</sup> day, the lymphoproliferative response to tetanus toxoid at 24 and 48 h of time was significantly decreased in all the treatment groups as compared to control group. It is concluded that exposure to the allethrin can lead to immunosuppression by inducing pathological alterations in immune organs.

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**To Cite This Article:** Gul ST, Khan A, Saleemi MK, Ahmad M, Zahoor A, Naseem MN and Hussain R, 2019. Immuno-toxicological effects of allethrin (Active ingredient in mosquito repellent coils) in BALB/c mice following oral administration. Pak Vet J, 39(2): 256-260. <http://dx.doi.org/10.29261/pakvetj/2019.036>

#### INTRODUCTION

In Pakistan, during last two decades many outbreaks occurred and among those Malaria is an important problem. Malaria accounts for 16% of the disease load across the country and hence stated the second most important and devastating disease. Moonsoon and summer season are considered to be the predisposing factors, however, diseases is prevalent throughout the year near the western borders and coastal regions of the country (Khan *et al.*, 2011). As this disease is vector borne like mosquitoes, so the use of repellants has increased tremendously (Riaz, 2011).

A lot of repellent products either natural or synthetic are available in the market. Among these United State produced allethrin (a pyrethroid), which is considered to be the firstly synthesized commercial product in 1949. Allethrin is being extensively used in different forms like aerosol sprays, coils, mats and liquid lotions (Avicor *et al.*, 2017). Rational use of products containing allethrin was thought to be safe, but with every day, increasing marketing and need of such products, there is also an increase in the effective residual level of insecticides those are acting as major environmental pollutants of food and water. It is potentially considered to be a neurotoxic and results in nerve excitation through alterations in the

membrane permeability for Na<sup>+</sup> and K<sup>+</sup> ions (Baltazar *et al.*, 2014; Chedik *et al.*, 2017; Gui *et al.*, 2018).

Higher exposure of allethrin is one of the causes of dermal and respiratory allergies. Although, the pyrethroids severe toxicity case in human and animals are uncommon in developed countries, yet such effects appear commonly in developing countries (Madhubabu and Yenugu, 2017). Use of allethrin and other pyrethroids in closed and poorly ventilated areas is expected to be a major cause of severe toxicity cases in humans through inhalation and it is the main and quicker route of exposure (Kasumba *et al.*, 2016). The common signs observed after exposure may be headache, dizziness, palpitations, chest tightness and less commonly fatigue and blurred vision (Garba *et al.*, 2007). There is also seen a substantial absorption and accumulation in epidermis of these toxic compounds. It is slow but one of the common occupational hazard to the persons working with sprays and chemical manufacturing. Ingestion is very rare and accidental, but have very lethal results, including sore throat, nausea, vomiting and abdominal pain, mouth ulceration, increased secretions and/or dysphagia, coma and convulsions those are life-threatening (Kasumba *et al.*, 2016). In Asian region including Pakistan, India, Japan, China, Korea, Thailand and Malaysia mosquito coils are being used extensively as repellent (Zhang *et al.*, 2011). According to an estimation the annual consumption of mosquito repellent coils are approximately 29 billion pieces worldwide. Annually, more than 25-77 million poisoning cases are reported (Zhang *et al.*, 2011). The present study has been conducted in terms of human health concerns those occur due to overuse of the mosquito repellent insecticides and however, it is connected to immunosuppression in patients recovering after exposure. The objectives of this experimental trial were the evaluation of the possible side effects and immunological toxicities of household insecticides.

## MATERIALS AND METHODS

**Chemicals:** Allethrin (C<sub>19</sub>H<sub>26</sub>O<sub>3</sub>) in the form of mosquito repellent oil was purchased from the local market which contains allethrin 2.6% w/v. Formalin (CH<sub>2</sub>O) 10% was purchased from Scharlau (Spain). Di-sodium hydrogen phosphate (HNa<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O), Sodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O), Xylene (C<sub>8</sub>H<sub>10</sub>), Histological wax (C<sub>n</sub>H<sub>2n+2</sub>) and Ethyl alcohol (C<sub>2</sub>H<sub>5</sub>OH) were purchased from Merck (Darmstadt, Germany). Tetanus toxoid adsorbed (R<sub>x</sub> Imatet, Amson Vaccines & Pharma (Pvt) Ltd Karachi) was purchased.

**Experimental design:** Basically, allethrin is a pyrethrin. Singh *et al.* (2012) used orally in their study as mosquito repellent/killer, thereby, allethrin has been selected for oral use in the present study. A total of 60 BALB/c mice were procured from local market. These were acclimatized for 14 days providing basal feed and water *ad libitum*. Temperature was maintained at 22-25°C and lighting schedule of 12L:12D. The mice were randomly divided into four equal groups, i.e. A, B, C and D where A served as a control. At 14<sup>th</sup> day of experiment, allethrin was administered orally to the other groups B, C and D @ 0.5, 1 and 1.5 g/kg BW, respectively. For the clinical signs, animals were monitored twice daily, and scoring was based

on the severity of clinical symptoms. Mortality if any, was recorded on daily basis. At 21<sup>st</sup> and 42<sup>nd</sup> day of experiment, five animals from each group were selected randomly and euthanized humanely. Serum samples were collected according to standard procedures. Immune organs like thymus and spleen were collected and absolute and relative organ weights were calculated.

### Physiological parameters

**Clinical signs, feed intake, body weight and mortality:** Animals were monitored twice daily to observe clinical signs and scored subjectively from 0 to 3. A cumulative picture of clinical signs was developed for description. Feed intake for each group was measured daily as grams/mice/day and then was calculated on weekly basis. The average body weight of individual mice in each group was determined on weekly basis. Number of mice died during the experiment in each group was recorded. Mortality percentage was calculated.

**Immunological parameters:** Humoral immune response was assessed through antibody response to sheep RBCs following procedure as per description by Pérez Berezo *et al.* (2011). Briefly, after 7 days of the allethrin dose administration (21<sup>st</sup> day of the experiment), 0.5ml of 3% washed SRBCs (sheep red blood cells) were inoculated to three mice from each group including control. A booster dose was inoculated 14 days post to the primary injection. Blood samples were taken at 7 and 14 days post to the primary treatment and kept undisturbed until the serum was separated and then it was stored at -20°C. To analyze the antibody titer against sheep RBCs serum was thawed at 56°C for 30 minutes and antibody titer was calculated by observing the button formation.

Cell mediated immunity was evaluated by producing lymphoproliferative response to tetanus toxoid in mice as per following the procedure described by Paul *et al.* (2009). Three mice from each group were randomly selected and 0.2ml of tetanus toxoid was injected in their foot pad of right limb. Physiological normal saline (0.2ml) was injected in the left foot pad as a self-control. With the help of screw gauge, skin thickness was measured of the right and left foot before and after 24, 48 and 72 hours of the injection. Difference in thickness of right from left foot pad and before and after tetanus toxoid injection was considered as the cutaneous basophilic hypersensitivity response.

**Data analysis:** The data collected from this research trial was subjected to statistical analysis through analytical software named "Statistix". One-way analysis of Variance (ANOVA) was applied and group means were compared by least significant difference at significance level P≤0.05.

## RESULTS

### Physical parameters

**Clinical signs:** All the mice in control group (A) were alert with no behavioral abnormalities and responded to the external stimuli very well throughout this experiment. However, mice in all other groups showed the symptoms of cholinergic toxicity of variable intensity in dose dependent manner. More severe (+++++) clinical signs were exhibited by group D followed by group C (+++) and B (++) . The

clinical signs those were observed are hypersalivation, frequent urination, rough hair coat, conjunctival hyperemia, alopecia and some nervous signs like tremors and convulsions were also prominent in the treatment groups. The intensity of these symptoms was highest in the week just after toxin exposure. However, in concurrent weeks it was gradually diminished (Table 1).

**Table 1:** Clinical signs exhibited by mice administered orally different doses of Allethrin

Clinical signs	Groups			
	A (Control)	B (Allethrin @ 0.5 g/kg BW)	C (Allethrin @ 1.0 g/kg BW)	D (Allethrin @ 1.5 g/kg BW)
Hair coat roughness	0	5.75	7.00	8.75
Alopecia	0	5.00	5.75	7.75
Conjunctival hyperemia	0	1.50	2.25	3.75
Salivation	0	2.50	3.25	5.25
Frequent defecation	0	5.00	6.50	8.50
Nervous signs	0	3.75	5.0	6.50
Total	0	23.50	29.75	40.50

Animals were monitored twice daily for clinical signs and scored subjectively from 0 to 3. A cumulative picture of clinical signs (presented in this table) was developed for description.

**Mortality:** Mortality percentage was calculated in the first week after allethrin exposure. The highest percentage (53.3%) was recorded in group D, followed by in groups C (46.6%) and B (26.7%). No mortality was recorded in control group.

**Feed intake:** Similar feed was offered to all the groups throughout this experiment. A significant ( $P \leq 0.05$ ) decrease in feed intake was recorded in a dose dependent manner.

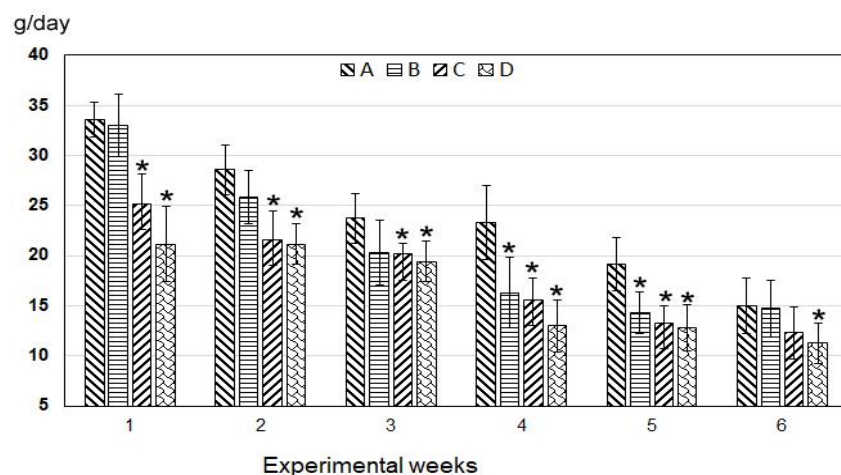
During the experiment, mice in groups C and D consumed significantly ( $P < 0.05$ ) less feed as compared to control mice (Group A) and Group B (Fig. 1). Feed intake was reduced maximally in group D as compared to groups B and C. Group B had a higher feed intake as compared to other two treatment groups (Fig. 1).

**Body weight:** Body weight did not differ significantly in treatment groups and control group at 1, 4, 5 and 6 experimental weeks. However, at 2<sup>nd</sup> and 3<sup>rd</sup> experimental week, mice in group D showed significantly increased and decreased body weight as compared to the control (Fig. 2).

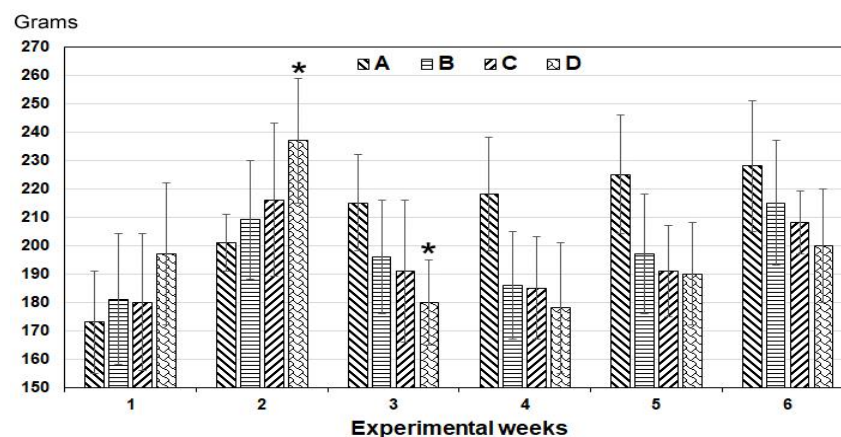
**Absolute organ weights:** At 21<sup>st</sup> and 42<sup>nd</sup> day of experiment, a significant ( $P \leq 0.05$ ) decrease in absolute weights of the spleen and thymus was recorded in all the allethrin treated groups as compared to the control groups. Spleen absolute weights in group B was also significantly higher than that of groups C and D, however, there was a non-significant difference in absolute weights of the thymus in treatment groups (Table 2).

### Immunological parameters

**Humoral immune response to SRBCs:** Total antibody titers decreased significantly ( $P \leq 0.05$ ) against SRBCs in allethrin treated groups including C and D, as compared to group B and control at day 14<sup>th</sup> (after 7 days of primary injection). In contrast to these, at 28<sup>th</sup> day of experiment all the allethrin treated groups (B, C and D) have significantly decreased antibody titers as compared to the control (Fig. 3).



**Fig. 1:** Feed intake of mice administered allethrin. Allethrin was administered orally @ 0.5, 1.0 and 1.5 g/kg BW to groups B, C and D, respectively. Group A served as control. Bars (mean $\pm$ SD) bearing asterisk differ significantly ( $P \leq 0.05$ ) on the same experimental week than control.



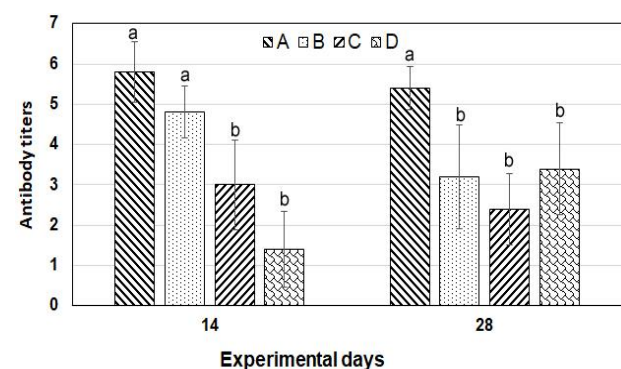
**Fig. 2:** Body weight of mice administered allethrin. Allethrin was administered orally @ 0.5, 1.0 and 1.5 g/kg BW to groups B, C and D, respectively. Group A served as control. Bars (mean $\pm$ SD) bearing asterisk differ significantly ( $P \leq 0.05$ ) on the same experimental week than control.

**Table 2:** Absolute organ weight (grams) of mice administered different doses of Allethrin

Experimental days	Groups	Spleen	Thymus
21 <sup>st</sup>	A	0.48±0.12a	0.52±0.19a
	B	0.27±0.04b	0.23±0.09b
	C	0.19±0.06c	0.21±0.05b
	D	0.18±0.05c	0.17±0.08b
42 <sup>nd</sup>	A	0.64±0.12a	0.82±0.21a
	B	0.42±0.03b	0.64±0.05b
	C	0.34±0.02c	0.61±0.07b
	D	0.31±0.04c	0.58±0.04b

Values (Mean±SD) followed by different letters in a column under specific day differ significantly ( $P \leq 0.05$ ). Allethrin was administered orally @ 0.5, 1.0 and 1.5 g/kg BVW to groups B, C and D, respectively. Group A served as control.

**Cell mediated immune response to tetanus toxoid:** At the 19<sup>th</sup> day of the experiment, the response to tetanus toxoid at 24, 48 and 72h of time differed non-significantly in all the treatment groups B to D than that of the control group (Table 3). At 29<sup>th</sup> day, the response to tetanus toxoid at 24 h of time was significantly ( $P \leq 0.05$ ) decreased in all the treatment groups as compared to control group. At 48h of time the lymphoproliferative response to tetanus toxoid was significantly decreased in C and D, while group B differed non-significantly as compared to control group. At 72h of the time the immune response was significantly decreased in group D while the group B and C differed non-significantly than that of the control group (Table 3).



**Fig. 3:** Antibody titers of mice against SRBCs at different days. Allethrin was administered orally Allethrin @ 0.5, 1.0 and 1.5 g/kg BVW to groups B, C and D, respectively. Group A served as control. Bars (mean±SD) bearing different alphabets differ significantly ( $P \leq 0.05$ ) on the same experimental days than control.

## DISCUSSION

Pyrethroids based mosquito repellants are frequently used to prevent the vector borne diseases in humans. They are used as sprays, lotions, coils etc. Particularly new born babies and kids are at risk of toxicity due these vaporizers. Annually, household mosquito repellant products are used in billion units throughout the world that is alarming (Kasumba *et al.*, 2016). Thus, keeping in view such alarming conditions, the current study was planned to find out the immune-toxicological effects of such accidental exposures to the allethrin toxicities in animal model.

The mechanism of action of pyrethroids is different from that of organophosphates in context that they don't interfere acetylcholine-cholinesterase system (Soderlund, 2012). However, their action is comparable to chlorinated hydrocarbons. Type II pyrethroids, such as cypermethrin and allethrin, have an additional  $\alpha$ -cyano group in their

**Table 3:** Lymphoproliferative response (mm) of mice against tetanus toxoid at different days administered orally various doses of Allethrin

Experimental day	Groups	Period (Hours)		
		24	48	72
19 <sup>th</sup>	A	5.26±0.15a	5.01±0.04a	4.69±0.23a
	B	4.72±0.52a	4.65±0.53a	4.59±0.49a
	C	4.41±1.03a	4.42±0.94a	4.52±0.53a
	D	4.35±0.71a	4.34±0.64a	4.47±0.47a
29 <sup>th</sup>	A	5.94±0.41a	5.43±0.41a	5.02±0.22a
	B	5.30±0.12b	5.29±0.15ab	5.03±0.08a
	C	5.15±0.17b	4.88±0.3bc	4.67±0.40a
	D	4.91±0.25b	4.55±0.17c	4.17±0.05b

Footnote remains the same as under Table 2.

structure which enhances their insecticidal activity. Although their mode of action is not completely clear, they are thought to be the potent nervous poisons as they interfere the sodium-flow to the neurons (Forshaw *et al.*, 2000). The main defense method of body against pyrethroids is metabolic system. Since they are composed of esters, metabolic system inactivates them by the ester decomposition. Their toxicity increases if the action of detoxifying esterases is inhibited (Schleier *et al.*, 2008).

In the present study allethrin administered mice exhibited clinical signs of toxicity including dullness, depression, hair coat roughness, alopecia, conjunctival hyperemia, salivation, less feed intake, frequent defecation, and nervous signs like tremors and convulsions, with different stages of severity. No clinical sign was observed in the control group. However, mice in all other groups showed the symptoms of cholinergic toxicity of variable intensity in dose dependent manner. The intensity of signs was highest in week following the exposure to allethrin. However, in concurrent weeks it was gradually diminished. Wolansky and Harrill (2008) almost reported the same results in terms of allethrin and other pyrethroids toxicities. It has been reported in the literature that the target point is CNS for such compounds. But these toxic effects are reversible at sub-lethal doses (Sinha *et al.*, 2004).

In the present study, decrease in feed intake with increasing doses of allethrin was observed. These results were in accordance to the behavioral alternations examined by He *et al.* (2013). Grewal *et al.* (2010) reported the similar trend in feed intake of albino mice after exposure to cypermethrin. Anadón *et al.* (2013) also observed that use of synthetic pyrethroid for prolonged periods have negative effect on feed intake.

In the present experiment the body weight showed significant decrease after exposure to allethrin. The maximum decrease was observed in group D, however, group B showed non-significant decrease as compared to control. Uzunhisarcikli *et al.* (2007) also stated after research in male rats that the allethrin exposure lead to decrease in weight gain.

In the present research mortality was recorded in all experimental groups during first week of the allethrin dosing. Number of mortality was dose dependent and no mortality was observed in control group. In comparison to control group, mortality was significantly higher in group D and C. Mahande *et al.* (2007) reported non-significant mortality in cattle that were grazed on pasture treated with pyrethroid based acaricide. However, Ghosh *et al.* (2009) reported mortality of two brothers who accidentally ingested allethrin.

After 7 days of primary injection, total antibody titer decreased significantly ( $P \leq 0.05$ ) in groups C and D, while of group B differed non-significantly than control group. A booster dose of SRBCs was injected. Total antibody titer against SRBCs, after administration of booster dose, decreased significantly ( $P \leq 0.05$ ) in groups B to D as compared to control group. These results were similar to the findings perceived by Sangha *et al.* (2011). Jin *et al.* (2014) observed that bifenthrin, a synthetic pyrethroid, induces immunotoxicity in male albino mice. Mondal *et al.* (2009) observed immunotoxic effects in female Wistar rats and concluded that pyrethroids produce significant decrease in antibody production.

In the present study, cell mediated immunity was assessed by analysis of lymphoproliferative response to tetanus toxoid in mice. The response to tetanus toxoid differed significantly ( $P \leq 0.05$ ) in all the treatment groups than that of the control group at 19<sup>th</sup> and 29<sup>th</sup> day of experiment. Jankowski *et al.* (2010) observed the similar effects on immune response of broiler chicken after exposure to resmethrin. Holmstrup *et al.* (2010) reported that insecticides are natural stressor to the environment and they significantly alter the immune response of the exposed animal. Eder *et al.* (2008) and Shen *et al.* (2018) reported the immunotoxic effect of pyrethroid insecticides in fish. Mokarizadeh *et al.* (2015) investigated the immunotoxic effects of pesticides specially pyrethroids in human and reported that the immune response to certain mitogens is either decreased, delayed or not shown at all.

**Conclusions:** Allethrin has immuno-pathological properties in terms of immunosuppression and behavioral/physical alterations. Decrease in feed intake and body weight was observed in a dose dependent manner. Similar trends were observed in terms of humoral and cell mediated immune responses. It is concluded that exposure to the allethrin can lead to immunosuppression by inducing pathological alterations in immune organs.

**Authors contribution:** STG, AK, MKS and AZ were actively involved in idea conceiving and project designing and execution. MA and MNN and RH were involved in data analysis, interpretation and write up of the manuscript. All authors approved the manuscript.

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