

EVALUATION OF IMMUNOTOXIC POTENTIAL OF FIPRONIL IN WISTAR RATS WITH SPECIAL REFERENCE TO CELLULAR IMMUNE RESPONSE

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Abstract: The immune system of mammals is highly complex, and various cells of this system interact with one another to produce the desired effects. Lymphocytes and macrophages are cellular units of the immune system. Fipronil, is an insecticide that was discovered and developed by Rhone-Poulenc in year 1985-1987 and was approved for marketing in year 1993. Although it is effective in veterinary field, there are concerns about its environmental and human health effects. In view of this, it was imperative to explore the immunotoxic effects of fipronil. The results indicated adverse effect of cellular immunity in rats exposed with fipronil. This indicated the depression of cellular immunity due to fipronil exposure. Considering these results, the present study warns immunotoxicity in terms of cell mediated immunity, as one of the risks associated with chronic exposure to fipronil.

Keywords: Cellular immunity, Fipronil, immunotoxicity.

INTRODUCTION

The immune system of mammals is highly complex, and various cells of this system interact with one another to produce the desired effects. Lymphocytes and macrophages are cellular units of the immune system. The two major forms of the lymphocytes, T cells and B cells, differentiate in the thymus and foetal liver respectively. The T cells are involved in cell-mediated immune responses, such as delayed hypersensitivity reaction and immune surveillance against foreign or altered cells.¹The immune system is evolved to protect the host from potentially pathogenic agents including microorganisms (virus and bacteria), parasites and fungi, to eliminate neoplastic cells. The structural and functional alterations of the immune system may lead to immunosuppression, which may modify the host defence mechanisms against infection, cancer and may induce of abnormal immune responses resulting in allergy and autoimmunity.²A number of biological or chemical agents have the

capacity to alter the functionality of the immune system in humans and animals. The immune system functions in resistance to infectious agents, homeostasis of leucocyte maturation, immunoglobulin production and immune surveillance against arising neoplastic cells. Leucocytes play vital role in these functions. They arise from pluripotent stem cells within the bone marrow, where they undergo highly controlled proliferation and differentiation before giving rise to functionally mature cells. The functionally mature cells are divided into granulocytes, lymphocytes and macrophages. Lymphocytes can be subdivided into thymus-derived (T-lymphocytes) and bursa-equivalent (B lymphocytes) depending on the primary lymphoid tissue where maturation occurs. The interaction of environmental chemicals or drugs with lymphoid tissue may alter the delicate balance of the immune system and result in immunosuppression, uncontrolled proliferation (leukaemia and lymphoma), alterations of the host defense mechanisms against pathogens and neoplasia, allergy or autoimmunity.³

Fipronil, is an insecticide that was discovered and developed by Rhone-Poulenc in year 1985-1987 and was approved for marketing in year 1993. Although it is effective in veterinary field, there are concerns about its environmental and human health effects. Fipronil is a member of the phenyl pyrazole class of pesticides. Fipronil became the leading imported product in the area of veterinary and agriculture field. Fipronil is used in a wide variety of pesticide products, including granular products for grass, gel baits, spot-on pet care products, liquid termite control products, and products for agriculture. There are more than 50 registered products that contain fipronil all over the world.

People can be exposed to chemicals in four ways: contact with skin, contact with eyes, breathing them in, or ingesting them. Direct contact to the skin or eyes may occur while applying fipronil products. Pets may be exposed to fipronil by products that are applied to their skin for fleas and tick treatments. It may also be possible to swallow fipronil if the hands are not washed following skin exposure. Exposure to fipronil can be limited by reading the pesticide label and following all the directions for use. There are several studies on immunotoxicity of most groups of pesticides viz. organochlorines, organophosphates, carbamates and pyrethroids. However, no reports seem to be available on immunotoxicity of phenylpyrazole class of pesticides in animals. Although a few pharmacological and toxicological studies have been conducted, substantial reports are lacking on immunotoxicity studies of the phenylpyrazole.

In view of this, it was imperative to explore the immunotoxic effects of fipronil. These investigations will help in understanding the specific immunological alterations caused by

fipronil. The present study was therefore undertaken with the objectives of generating data on immunotoxic potential (if any) of fipronil by assessing parameters related to cellular immune responses and histopathology of lymphoid organs.

MATERIAL AND METHODS

Experimental animals:

The study was conducted in healthy Wistar rats weighing 200-250g. Rats (54) were procured from institutional animal house and maintained in the Department of Pharmacology and Toxicology, Bombay Veterinary College, Parel, Mumbai under standard laboratory conditions. All the animals were approved by the IAEC under CPCSEA of the institution.

Experimental Design:

Study was carried out in 54 rats. Considering the LD₅₀ (97 mg/kg) 4 and finally three dose levels of exposure were selected as 5 mg/kg, 7.5 mg/kg and 10 mg/kg. Rats were divided into seven groups, out of which group III, IV and V used for phagocytosis study which were exposed to *Staphylococcus aureus* culture and phagocytic index was calculated. Group III, IV and V comprised of six rats in each that received 5 mg/kg, 7.5 mg/kg and 10 mg/kg fipronil respectively via oral gavage for consecutive 28 days. Group I and II served as control for group III, IV and V whereas group VI and VII were subjected to delayed hypersensitivity by contact sensitivity to DNCB method described by Hari Babu.⁵ and rats in group VI received highest dose of fipronil (10 mg/kg) for consecutive 28 days and group VII act as DTH control (Table 1).

Table 1: Design of experiment

Group	Number of animals	Treatment	Dose	Sensitization	Challenge	Parameters studied
I	6	Distilled Water	1 ml/ 100g BW	-	-	Phagocytic activity
II	6	Tween80 0.01% (v/v)	1 ml/ 100g BW	-	-	
III	6	Fipronil	5 mg/ kg BW	-	-	
IV	6	Fipronil	7.5 mg/ kg BW	-	-	
V	6	Fipronil	10 mg/ kg BW	-	-	
VI	12	Fipronil	10 mg /kg BW	2% DNCB at 0.05ml	2% DNCB at 0.05ml	DTH
VII	12	Tween80 0.01% (v/v)	2 ml/ 100g BW	2% DNCB at 0.05ml	Acetone 0.05 ml	

Chemicals:

Standard Fipronil was obtained from Cipla Ltd Mumbai, whereas Nutrient broth, Hank Balanced Salt Solution (HBSS) and Culture of *Staphylococcus aureus* procured from Himedia laboratories Pvt. Ltd. Mumbai.

Phagocytic Index:

Phagocytic index was determined by using method suggested by Hari Babu⁵

In vivo Phagocytosis:

Rats were injected intraperitoneally with 10% peptone followed by 0.5 ml of *Staphylococcus aureus* culture grown overnight in nutrient broth. After 18 hours, rats were injected 0.5 ml of HBSS intraperitoneally. After injecting 0.5 ml HBSS, all the rats were sacrificed by anaesthetizing with chloroform. Peritoneal fluid was collected and smears were prepared on clean grease-free glass slides. The smears were allowed to air dry and were subsequently stained with Leishman's stain. The phagocytes were observed microscopically for the presence of intracellular cocci under the oil immersion objective. A total of 100 polymorphonuclear neutrophils were counted and the numbers of PMN containing phagocytosed bacteria as well as those containing no bacteria were recorded. Percentage of phagocytosis was calculated by following formula.⁶

$$\% \text{ Phagocytosis} = \frac{\text{No. of PMN that have engulfed bacteria}}{\text{No. of PMN counted}} \times 100$$

Phagocytic index was calculated by considering average number of bacteria per phagocyte.⁷

Delayed Type of Hypersensitivity (DTH): Contact Sensitivity to DNCB:**Procedure-**

The delayed type of hypersensitivity (DTH) test was carried out on rats from Group VI and VII. The procedure employed was as follows. A test area of about 3 cm diameter on the skin of the neck region was chosen. Hairs were clipped off close to the skin. Shaved area was marked. The diameter of the area of exposure was kept same for both sensitization and challenge. 0.05 ml of 2% DNCB in acetone was applied slowly drop by drop on to the marked area to the animals from group VI for DTH sensitization on day 14th of experiment. The solution was made to evaporate quickly by gently blowing. The solution was prevented to run down from the neck region. The thickness of the skin before challenge (0 hours) was measured by using Vernier calipers. The same procedure was repeated with challenge dose on day 28th of experiment and skin thickness was measured at 24, 48 and 72 hours along with clinical observations. Group VII was maintained as DTH control in which, 0.05 ml of 2%

DNCB in acetone was applied slowly drop by drop on to the marked area for sensitization on day 14th of experiment. On day 28th of experiment, 0.05 ml of acetone as a challenge dose was applied slowly drop by drop on to the marked area. The solution was made to evaporate quickly by gently blowing and thereby preventing the solution to run down the neck region.

Statistical analysis:

All the data was compiled together & subjected to ANOVA to find out significant differences among the groups at 1 & 5 % level. However % phagocytic activity and phagocytic indices were analyzed by applying Completely Randomized Design (CRD) and Skin thickness (mm) in DTH reaction by was analysed by double mean 't' test as per Snedecor and Cochran⁸

RESULTS AND DISCUSSION

Phagocytic activity and phagocytic index:

Both macrophages and neutrophils play critical roles in the defense body system by eliminating microorganisms from the infected tissues. From the phagocytic index it was observed that control peritoneal macrophages phagocytosed the *S. aureus* at higher rate which reduced upon exposure to fipronil. Fipronil exposed rats are likely to be more prone to infection since the ability to clear the infectious microorganisms would decrease as evident from the phagocytic activity parameter.

The clearance of bacteria can be measured in two ways. Phagocytic activity takes into account the percentage of phagocytes involved in engulfing bacteria whereas phagocytic index takes into account number of bacteria engulfed by the phagocytes. *In vivo* method followed in rats in the present study involved use of non-pathogenic *Staphylococcus aureus* organism from operator's safety point of view. Peritoneal fluid was conveniently obtained which is a rich source of phagocytes. This method has been followed by quite a few researchers.⁹⁻¹²

In the present study, phagocytic activity (%) and phagocytic indices were calculated at the end of experiment immediately after sacrificing the rats. Phagocytic activity (%) and phagocytic indices are presented in Table 2 and Fig 1 and 2 respectively. Macrophage engulfed bacteria shown in Plate 1.

Table 2: Mean (\pm S.E.) % phagocytic activity and Phagocytic index from different groups of rats

Groups	Group I	Group II	Group III	Group IV	Group V
PA	70.67 \pm 1.145 ^a	69.67 \pm 1.145 ^a	51.17 \pm 2.10 ^b	40.33 \pm 2.06 ^c	31.5 \pm 1.67 ^d
PI	4.83 \pm 0.477 ^a	5.16 \pm 0.654 ^a	3.67 \pm 0.67 ^{ab}	3.17 \pm 0.60 ^{bc}	2.17 \pm 0.48 ^c

Value (n=6) in the same row bearing common superscript do not differ significantly. * Significant at $P \leq 0.05$. PA- % phagocytic activity and PI- Phagocytic index

Phagocytic activity (%) significantly differed among treated groups whereas, phagocytic index differed among group II, IV and V but not between group III & IV and group IV & V. There was no significant difference in the two control groups for both the parameters. Phagocytic activity (%) in group V, receiving fipronil was lowest while that of group I and II i.e. untreated control groups, it was highest.

Reduced average phagocytic activity and phagocytic index in fipronil exposed groups in dose dependent manner in the present study is indicative of adverse effect of fipronil on cellular immunity. This can indirectly permit to infer that population chronically exposed to high fipronil levels is prone to catch bacterial infections. Reduced phagocytic activity and phagocytic index is also reported in rats and mice exposed to different chemicals chronically.⁹⁻¹¹

Similar finding were also reported by Gatne and associates in 2006 who, studied immunotoxicity of imidacloprid in rats at doses of 16, 48 and 160 mg/kg for 28 days. The difference between phagocytic indices was highly significant ($P \leq 0.01$) at all doses as compared to control (1% w/v gum acacia).¹³

Delayed type of hypersensitivity (DTH) Contact sensitivity to DNCB:

In the present study, group VI (DTH) and VII (DTHC) were sensitized with 2 % DNCB in acetone. In group VI (DTH), challenge dose of DNCB was applied on day 14th whereas group VII (DTHC) was challenged with acetone only. Application of the challenge dose of DNCB caused erythema, edema and vesiculation (Plate 2). There was no significant difference in the skin thickness at 0 hrs and 24 hrs whereas significant decrease in skin thickness in group VI was observed as compared to group VII at 48 and 72 hrs of post challenge (Table 3 and Figure 3). This indicated the depression of cellular immunity upon exposure to fipronil.

The ability of an individual to develop contact sensitivity is a measure of cellular immunity and DNCB is one of the chemicals that has been employed to evaluate the CMI response in

man and animals. Direct application of compounds like DNCB to skin will result in systemic sensitization to various metabolites of the sensitizing compound. DNCB is known as a strong irritant binding to membrane proteins of the Langerhans cells of the skin. The conjugate is a potent neoantigen able to induce local tumour-destruction and raise cellular immune defense. DNCB reacts with skin compounds to form hapten carrier molecules. DNCB is considered as highly reactive substance which could form dinitrophenyl protein complex with various skin components. ⁵DNCB compound enhances the CD8+ cells and decrease CD4+ cells and also stimulates IL-10 proliferation. DNCB functionally impairs the immune function of T- cells. Following interaction with a DNCB, T-cells secrete cytokines. In DTH reaction, the primary lymphocytes response involved appears to be responsible for accumulation of mononuclear cell infiltrate and increased vascular permeability occurs in the vicinity of the stimulus. ¹⁴ The procedure of use of DNCB described was followed by various workers in laboratory animals. ^{5,15,16}

Table 3: Mean (\pm S.E.) effect of fipronil on the cellular immunity in rats (Delayed hypersensitivity to DNCB)

Groups	Skin thickness (mm)			
	Before Challenge	After Challenge		
	0 hrs	24 hrs	48 hrs	72 hrs
DTH (Group VI)	0.965 \pm 0.04	1.04 \pm 0.06	1.21 \pm 0.01	1.00 \pm 0.04
DTHC (Group VII)	1.05 \pm 0.04	1.24 \pm 0.09	1.72 \pm 0.03	1.17 \pm 0.02
T- table	1.397	1.767	17.986	3.407

Value (n=12); T value at $P \leq 0.01 = 2.819$, T value at $P \leq 0.05 = 2.074$.

Delayed type of hypersensitivity in chickens after administration of carbaryl @ 20 ppm in feed for 3 months by using dinitrochlorobenzene (DNCB). Peak reaction was observed at 24 hours followed by subsequent decrease by 48 hours and negligible at 72 hours post challenge with DNCB. Carbaryl lowered the cell mediated immune response as shown by significant reduction in DTH reaction to DNCB. ¹⁶

Immunotoxicity of imidacloprid in rats by injecting SRBCs into hind footpad on day 28 for assessing the DTH response which differed significantly in treated groups as compared to controls. ¹³

In the present study, rats exposed to highest dose of fipronil in group VI showing clinical signs such as piloerection, excessive jumps, and convulsions. Those rats sensitized and challenged with DNCB showed blackish circular erythema on scrotum of 3 rats (Plate 3). However, there neither has been report of such finding in the reviewed literature nor the reason for this discoloration could be explained.

Conclusions

This results indicating adverse effect of cellular immunity in rats exposed with fipronil. This indicated the depression of cellular immunity due to fipronil exposure. Considering these results the present study warns immunotoxicity in terms of cell mediated immunity, as one of the risks associated with chronic exposure to fipronil.

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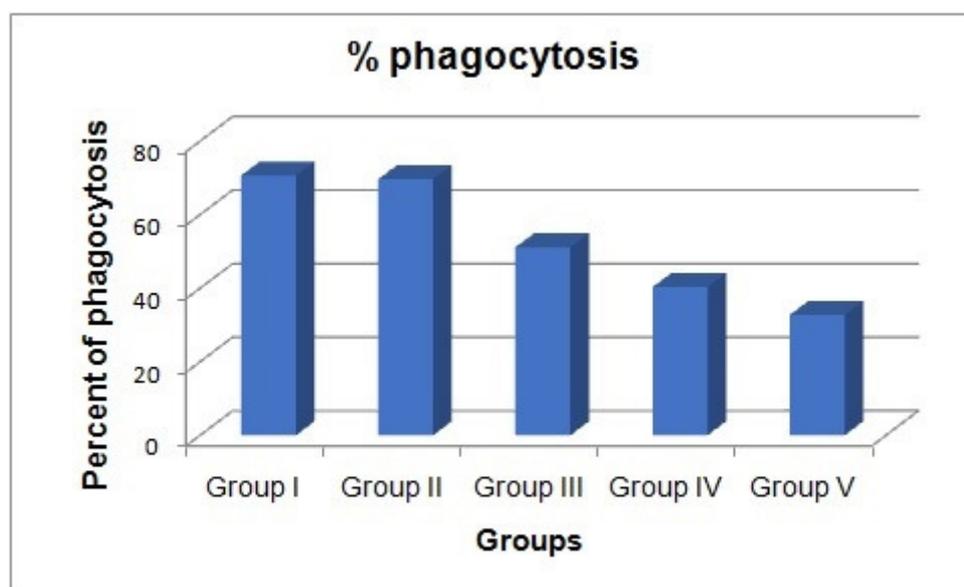


Figure 1: Average percentage of phagocytic activity from different groups of rats.

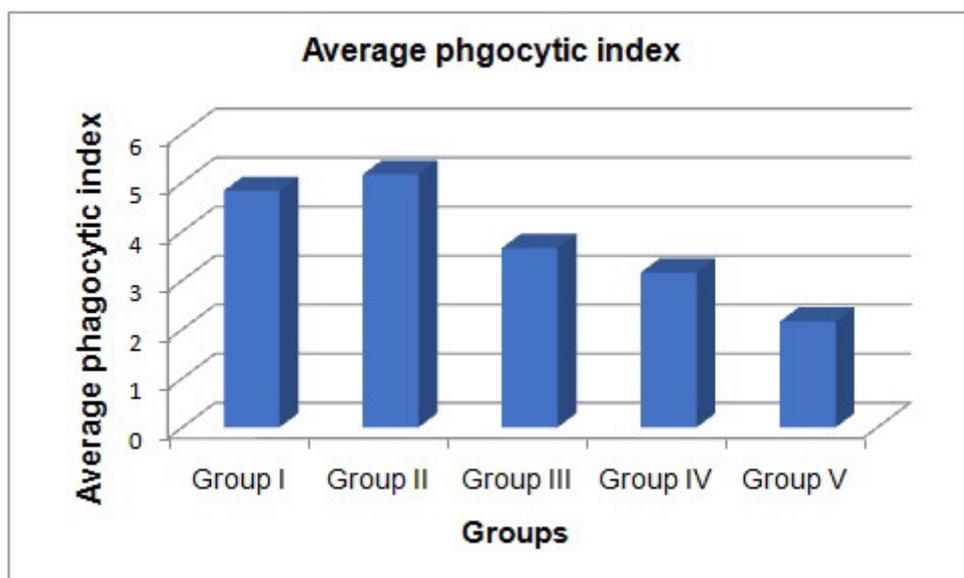


Figure 2: Average of phagocytic indices from different groups of rats

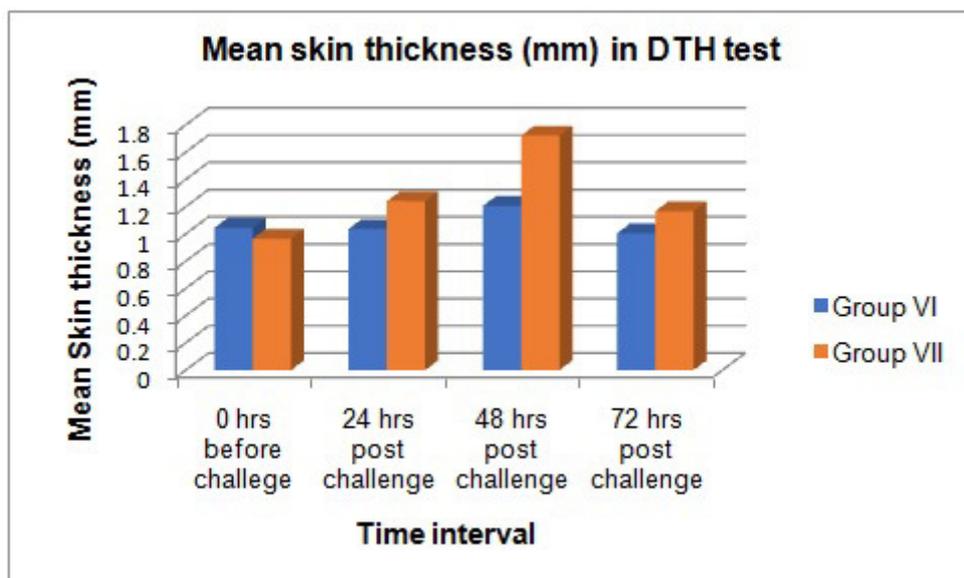


Figure 3: Mean (\pm S.E.) skin thickness (mm) in delayed type of hypersensitivity to DNCB method in rats

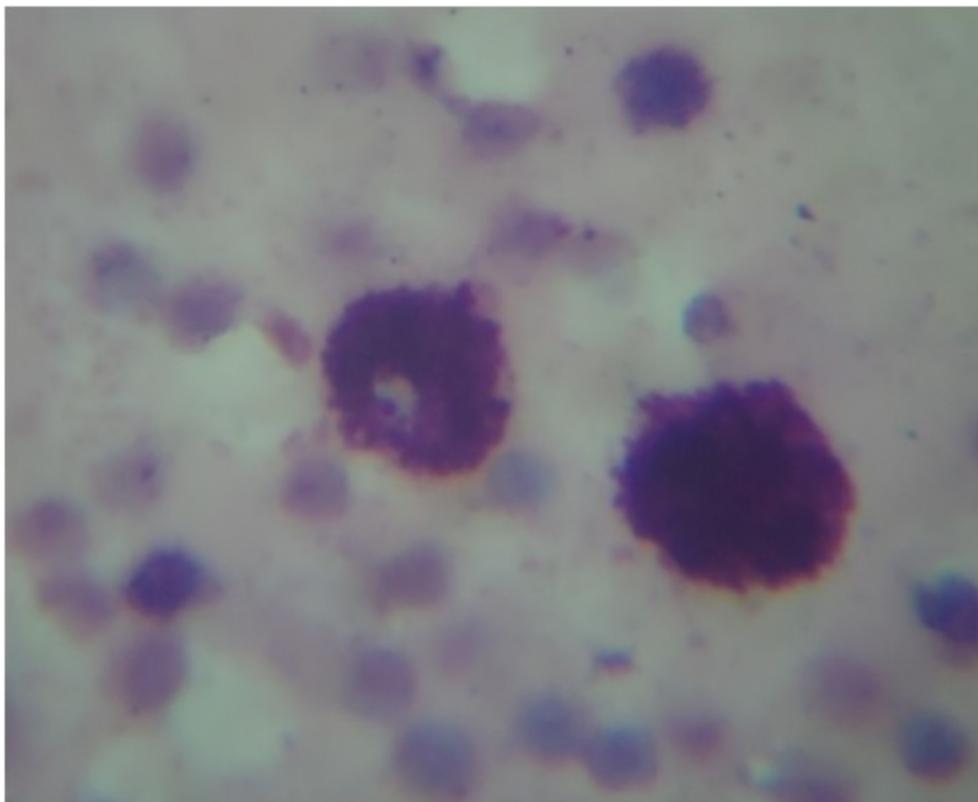


PLATE 1: Phagocytosed macrophages (Leishman's stain $\times 1000$)



PLATE 2: Blackish circular erythema on scrotum of rat.