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Original Article

Cypermethrin-induced Repeated-dose Immuno-toxicity in *Oryctolagus Cuniculus*: An Investigation into Pathology of Spleen and Blood

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ABSTRACT

Background: *Cypermethrin is a pyrethroid insecticide widely used in agriculture and medicines. Its toxicity effects on spleen and blood in male and female rabbits is studied in this research.* ***Methods:*** *Age- and weight-matched does/females (n=24) and bucks/males (n=24) of Japanese White rabbits were subjected to intraperitoneal cypermethrin administration @50, 100 and 150 mg.kg^{-1} b.wt. in groups B, C and D, respectively. The experimental rabbit does and bucks were tested for hematology alterations after each of 5 CY-treatments, at days 1, 8, 15, 22 and 29. One rabbit doe and one rabbit buck were sacrificed fortnightly (days 15, 29, 43, 57 and 71) to obtain spleen for histological studies.* ***Results:*** *CY-treated rabbit bucks developed anemia, leukopenia, neutropenia, monocytosis, eosinophilia and basocytopenia mostly in dose and time dependent manner. In contrast, rabbit does show transient but significant leukocytosis, neutropenia, lymphopenia, monocytosis, eosinophilia and basophilia only in high dose group. Moreover, spleen histology revealed congestion, depletion of white pulp with increased red pulp and hemosiderin deposition in CY treated rabbit bucks, but not rabbit does.* ***Conclusions:*** *This study concludes that immuno-toxicity by cypermethrin insecticide is not similar in male and female subjects.*

Key words: *Malpighian corpuscles, pesticide poisoning, lymphocyte production, macrophages, erythrocytes*

INTRODUCTION

It is believed that the women, as a group, has longer lifespan than men. There are increasing evidences that action of various treatment regimens is different in the two sexes and same is the case with toxicity. The major pollutants of industrial revolutions are pesticides, a prominent culprit to decreasing longevity. The pesticide poisoning causes about 220,000 human deaths yearly, mainly in third world; many countries had devised various policies to reduce their use.[1] But pesticide consumption is even feared to be increasing due to growing of pests and drop in pest enemy population.[2] Among various categories of pesticides, insecticides comprise a higher proportion of total pesticide usage.[3] Numerous insecticides are used to control insects and acarian (ticks, mites), making insecticides ubiquitous environmental contaminants. Among their various classes, pyrethroid insecticides are considered safe, but due to frequent use become most important to clinical toxicologists and eco-toxicologists.[1] Cypermethrin (CY) is highly active artificially prepared pyrethroid, commonly used in household, medicines and agriculture related industries.[4] Although, a bulk of research work is available about CY-toxicity, the focus on the male/female dissimilarity and resemblance is negligible. Our previous publications have described

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lowered testosterone levels along-with decreased testicular and epididymal sperm counts and anemia in male rabbits, but not females after repeated-dose CY-treatment.[1,5] Both of these studies implied that CY-poisoning in male subjects is typical relative to females.

Frequent use and misuse of pyrethroids (including CY) especially in developing and poor countries culminates in several health hazards to non-target species. Although many studies have investigated the toxic effects of CY, spleen toxicity has rarely been addressed. Moreover, despite relevance of pathology and patho-physiology of spleen to the blood, no study addressing CY toxicity related the spleen histopathology with hemato-pathology. Immuno-histologic studies of spleens of animals poisoned with pyrethroids implied that pyrethroids contribute to disturbances of the process of lymphocytes (Ls) production in the spleen.[6,7] The spleen is the major site of immune responses to blood born antigens. It also stores large number of platelets and granulocytes (and in a virtual sense red cells), forming part of flight or fight physiological reaction. Moreover, slow blood flow through spleen and intact specialized microcirculation of the spleen enables the macrophages to engulf and remove aged erythrocytes.[8] Recently, some researchers have related engulfing of aged erythrocytes mainly with liver, instead of spleen.[9] Current awareness about tissue macrophages is inadequate and it is difficult to relate them precisely with abnormality and clinical symptoms.[10] Among contents of red cells (after breakdown) iron, folate etc. are recycled, most amino acids are returned to the pool, un-conjugated bilirubin circulates as such, while most other catabolites are excreted.[8]

It is due to such important relation of blood with spleen, that chronic congestion, infiltrations, myeloproliferative disorders and work hypertrophy are among the commonest causes of splenic pathology. Knowing the CY-induced alterations in erythrocyte and leukocyte indices along-with importance of blood and spleen relation, this study was planned to investigate whether CY affects hematology and spleen histology in the two sexes of New Zealand White rabbit (*Oryctolagus cuniculus*).

METHODS

Study Settings and Design:

Blood specimens with ethylene di-amine tetra acetic acid (EDTA) from all subjects under investigation were obtained prior to start of experiment (d 0) and after each of the 5 experimental treatments (d 1, 8. 15, 22 and 29). All specimens were obtained from jugular vein after sterilizing the area. Blood smears from each animal were made in duplicate from fresh blood, air dried, fixed with methanol and stained for differential leukocyte counts (DLCs). To obtain spleen for these studies, experimental subjects were slaughtered according to specified schedule. One rabbit buck and a rabbit doe from each group were sacrificed fortnightly (i.e., d15, d29, d43, d57 and d71).

Selection and description of Experimental Subjects

Twenty-four does (females) and 24 bucks (males) of Japanese White rabbits were procured, tagged and kept under similar management conditions during 5-day (d) acclimatization and during the trials (71d). All the experimental subjects were apparently healthy, mature and age- and weight-matched; the room temperature was maintained at 20–25°C throughout the study. Drinking water was available *ad libitum* to all the experimental subjects. The green fodder *Trifolium alexandrinum* was offered in the morning and evening.

Intervention

CY-92% used in the study was gifted by M/S Pak-China Chemicals, Lahore-Pakistan. Due to being water insoluble, CY was dissolved in Jequirity seed oil for IP injection to the animals under study. In order to study the effects of intraperitoneal (IP) administration of CY on hematology and histology of spleen, both rabbit does and rabbit bucks were assigned to four groups of six rabbits each. Dose to be injected was adjusted according to body weight (b.wt.). The experimental subjects in groups B, C and D received low (50mg.kg^{-1} b.wt.), medium (100 mg.kg^{-1} b.wt.) and high (150 mg.kg^{-1} b.wt.) CY-doses, respectively. Group A served as control. Each animal in the control group received equivalent volume of Jequirity seed oil (mustard oil). The rabbit does and bucks in all groups received respective CY treatments at weekly interval.

Parameters of the Study

The hematological studies included erythrogram and leukogram (including percentages of neutrophils (Ns), monocytes (Ms), lymphocytes (Ls), eosinophils (Es) and basophils (Bs)). The spleen was obtained for routine histological analysis on each d of sacrifice (d15, d29, d43, d57 and d71). Tissue specimens were collected and preserved in 10% buffered formalin. Fixed tissues were processed by the routine method of dehydration and paraffin embedding. Sections of 4-5 µm breadth were prepared to stain (hematoxylin and eosin dyes).[11] The parameters investigated in this study regarding spleen histopathology included: 1) congestion 2) hemosidrin deposition 3) Ls depletion in Malpighian corpuscle/depleted white pulp.

Ethical issues

The Ethical Committee at University of Agriculture Faisalabad-Pakistan approved in advance and observed all the issues related to ethics during whole the research work.

Statistical analysis

Randomized Complete Block Design was used and means were compared by applying analysis of variance (ANOVA) using Minitab statistical software package on personal computer. A semi-quantitative evaluation on the basis of arbitrary scores (---- to ++++) was performed regarding the histopathological lesions in spleen.

RESULTS

(a) Erythrogram

The anemia was observed in rabbit bucks but not in rabbit does. The detail of the observations and discussions are presented in one of our previous article.[1]

(b) Leukogram

Significantly lower TLC was observed in group D (150mg.kg^{-1} b.wt) at d15 and d29, while in group C (100mg.kg^{-1} b.wt) at d15 and in group B at d29 in rabbit bucks (Table 1). Absolute Ns and Ls counts were significantly lowered at d15 in all CY treated groups of rabbit bucks (Table 1). Moreover, absolute Ns counts were also lower at d29 in groups B and D of rabbit bucks. Absolute Ms counts were significantly higher and lower in group D at d1 and d29, respectively of rabbit bucks (Table 1).

The present study in female rabbits showed transient but significant rise in TLC and Ns on d15 in group B, but lowered Ns and Ls on d22 in group D (Table 2). The absolute counts of Ms, Es and Bs in group B and those of Ms and Es in group D were also found to be significantly higher on d15 (Table 2).

(c) Spleen Histopathology:

Two rabbits (one male and the other female) from each group were euthanized fortnightly (d15, d29, d43, d57 and d71). Tissue samples of spleen were preserved and processed to prepare histological slides and lesions in each group were scored on the basis of severity (---- to ++++) at various days of experiment (Table 3). From this a cumulative lesion score was derived for the overall intensity of lesions in a particular group (Table 4). Control group (A) and the treated females did not show any lesion. Congestion, depletion of white pulp/Malpighian corpuscle (Figure 1) with increased red pulp and hemosiderin deposition were observed mostly in dose and time related fashion in CY treated male rabbits (Tables 3, 4). Overall picture of spleen seemed to contain increased red pulp. The red pulp, however, contained scattered Ls in it, which made it difficult to be differentiated from the white pulp.

DISCUSSION

Blood investigation is among the best and well-organized scientific tests. Blood test devices on smartphones and tablet computers have become cost-effective and rapid ways of analysis through recent advancements in the information technology.[12] The time-tested knowledge mostly obtained through traditional and manual applications can provide base to generate well-organized and precious knowledge by utilizing modern applications. Linking hematology alterations with microscopic lesions in spleen, gender-linked difference has been addressed in this article. In this study, IP administration of CY (a pyrethroid) led to anemia, leukopenia, neutropenia, monocytosis, eosinophilia and basocytopenia in rabbit bucks. In contrast, the rabbit does show transient but significant leukocytosis, neutropenia, lymphopenia, monocytosis, eosinophilia and basophilia. In normal animals, manufacture of erythrocytes is in balance with their elimination. But, in certain diseases and toxicity, there is brutal disturbance to erythrocyte life span. Such situation produces anemia and iron toxicity in animals.[9] The anemia development in male subjects with CY-treatment might have resulted from immunosuppression.

In a number of studies on mammals, leukopenia has been documented after CY or other pyrethroid treatment.[13,14] Contrarily, other workers have documented leukocytosis with pyrethroids exposure.[7,15,16] Pyrethroid exposure in fish, mammalian, avian and other species has been reported to disturb DLCs, which include neutrophilia, lymphocytosis along with monocytosis or lymphopenia.[17,18] Increased Ns represents inflammation in visceral organs.[19] Several research studies, however, described non-significant modifications in TLC and DLC on treatment with various dose amounts of pyrethroids.[20] The increased TLC in animals may result from the mobilization of the immunological system and/or a shift in the leukocytic pool from the spleen to peripheral blood. Basic module of innate immunity is neutrophils' relocation into inflamed tissues; blood neutrophils undergo polarized movement from first to last endothelial cells (ECs) coating the venular lumen in a luminal-to-abluminal route. During this transendothelial migration, enzymes convert lysophosphatidylcholine (LPC) to lysophosphatidic acid. Neuropathy target esterase (NTE) hydrolyzes LPC *in vitro*. NTE is a secondary target of some insecticides; insecticides damage the leukocyte functioning by attacking NTE and favoring cytotoxic accumulation of LPC to rupture ECs.[21]

Pyrethroids may disturb the processes of production of effector cells of the immunological system because of changes in neuro-hormone activity. Pyrethroids cause shifts in the pool of spleen and peripheral blood Ls, which is manifested by lymphocytosis in the blood, and the decrease in the subpopulation of T-Ls in the spleen.[22] Pyrethroids toxicity leads to disturbed development of lymphatic follicle and decreased number of Ls in the cortex and subcortical region of the spleen, suggesting that pyrethroids contribute to disturbances of the process of Ls production in the spleen.[6,7] Spleen of CY treated male rabbits exhibited hemosiderin deposition, congestion, and depletion of splenic follicles (white pulp) also known as Malpighian corpuscle (Figure 1) in the present study. The lesions were in concurrence with white pulp depletion reported in goats and diminished proliferation of splenocytes and Ls in mice and rats treated with pyrethroids.[23,24,25] The red pulp of spleen had been reported to be atrophied and filled with Ls, along-with marked fibrosis and depletion of Ls in Malpighian corpuscles in the pyrethroid treated animals.[23,26,27]

CONCLUSION

This study described difference of results regarding hematology and spleen histology in male and female subjects of *Oryctolagus cuniculus* with CY-treatment. In males, the CY insecticide resulted in splenic white pulp depletion, leukopenia, neutropenia, monocytosis and eosinophilia along-with anemia. It is interesting that we could not find such association in *Oryctolagus cuniculus* females. We hypothesized that interaction of spleen macrophages and erythrocyte may be involved in the

Table 1: Total leukocyte count and absolute counts of neutrophils, lymphocytes, monocytes, eosinophils and basophils in male rabbits injected with different CY-doses

Experimental days		0	1	8	15	22	29
Total leukocyte counts ($nx10^9.L^{-1}$)							
Groups (CY-Doses: $mg.kg^{-1}$ b.wt)	**A (0)**	10.29±1.79	9.71±2.67	13.43±4.13	13.30±2.19	9.50±1.88	10.16±2.36
	B (50)	8.23±0.94	9.19±1.93	8.56±1.66	9.29±1.27*	8.7±1.06	7.88±0.50*
	C (100)	10.51±3.36	13.31±2.60	10.69±1.98	8.74±0.54*	7.94±1.25	10.35±0.37
	D (150)	8.29±0.90	10.03±0.90	9.49±3.52	8.40±0.64*	8.47±0.66	7.79±1.12*
Neutrophils ($nx10^9.L^{-1}$)							
Groups (CY-Doses: $mg.kg^{-1}$ b.wt)	**A (0)**	3.32±0.90	3.29±1.36	4.34±2.13	4.94±0.71	3.48±0.83	3.52±0.46
	B (50)	4.21±1.06	4.05±1.15	3.20±0.71	3.68±0.63*	3.19±0.26	2.59±0.42*
	C (100)	3.70±1.04	5.27±1.86	4.75±1.41	3.73±0.40*	2.89±0.72	3.50±0.37
	D (150)	4.00±0.70	4.11±0.64	3.96±2.07	3.55±0.30*	3.26±0.76	2.78±0.37*
Lymphocytes ($nx10^9.L^{-1}$)							
Groups (CY-Doses: $mg.kg^{-1}$ b.wt)	**A (0)**	3.51±1.37	2.67±2.05	3.39±3.38	6.16±1.07	4.06±0.66	4.74±1.25
	B (50)	3.76±1.03	2.05±1.73	2.76±1.53	3.53±0.70*	3.90±0.80	3.37±0.61
	C (100)	3.68±0.58	3.19±2.71	3.71±0.85	2.98±0.11*	3.14±0.63*	4.42±0.65
	D (150)	3.65±0.49	3.04±0.32	2.65±1.85	2.81±0.45*	3.01±0.39*	2.98±0.67*
Monocytes ($nx10^9.L^{-1}$)							
Groups (CY-Doses: $mg.kg^{-1}$ b.wt)	**A (0)**	0.57±0.45	0.33±0.19	0.55±0.76	0.97±0.33	0.73±0.25	0.93±0.35
	B (50)	0.28±0.09	0.32±0.27	0.41±0.23	0.67±0.35	0.97±0.48	0.55±0.22
	C (100)	0.35±0.20	0.44±0.41	0.85±0.45	0.63±0.13	0.53±0.13	0.62±0.09
	D (150)	0.43±0.42	0.93±0.36*	0.59±0.34	0.74±0.21	0.52±0.10	0.48±0.14*
Eosinophils ($nx10^9.L^{-1}$)							
Groups (CY-Doses: $mg.kg^{-1}$ b.wt)	**A (0)**	0.94±0.46	0.53±0.40	0.55±0.76	0.57±0.24	0.70±0.25	0.73±0.18
	B (50)	0.97±0.31	0.63±0.62	0.71±0.49	0.94±0.56	0.38±0.15	0.37±0.10
	C (100)	1.69±1.93	0.75±0.69	0.91±0.47	0.96±0.21	0.95±0.31	1.17±0.36*
	D (150)	0.92±0.19	1.21±0.23	0.90±0.52	0.95±0.22	1.28±0.32*	1.18±0.37*
Basophils ($nx10^9.L^{-1}$)							
Groups (CY-Doses: $mg.kg^{-1}$ b.wt)	**A (0)**	0.64±0.23	0.29±0.18	0.38±0.48	0.66±0.18	0.53±0.10	0.57±0.10
	B (50)	0.46±0.29	0.43±0.37	0.46±0.27	0.66±0.16	0.40±0.12	0.34±0.08
	C (100)	0.61±0.53	0.35±0.37	0.47±0.12	0.44±0.06*	0.47±0.21	0.65±0.34
	D (150)	0.48±0.09	0.73±0.27	0.52±0.33	0.35±0.08*	0.39±0.18	0.37±0.18

The values (mean±SD) bearing asterisks indicate significant difference in a column of respective count.

pathogenesis of spleen and blood, as anemia was also observed in males and not females. Nevertheless, more studies are essential with major focus on gender linked difference in hematological and splenic histology alterations due to exposure of pyrethroids (cypermethrin, permethrin, deltamethrin, fenvalerate etc.) to non-target species.

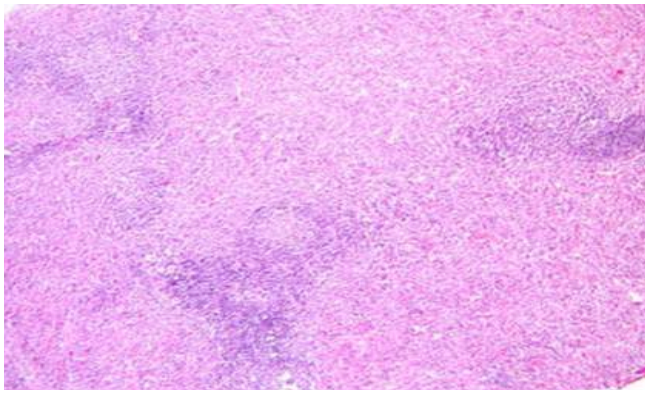

Figure 1: Photomicrograph of spleen of CY (150mg.kg-1 b.wt) treated male rabbit on day 71 showing depletion of white pulp. H and E, Lens 10X.

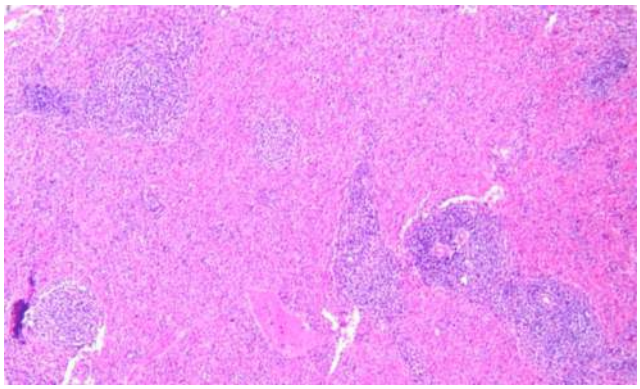

Figure 2: Photomicrograph of spleen of control rabbit with no depletion of white pulp on day 71 of experiment. H and E, Lens 10X

Table 2: Total leukocyte count and absolute counts of neutrophils, lymphocytes, monocytes, eosinophils and basophils in female rabbits injected with different CY-doses

Experimental days		0	1	8	15	22	29
Total leukocyte counts (nx10^9.L^{-1})							
Groups (CY-Doses: mg.kg^{-1} b.wt)	**A (0)**	7.97±1.10	8.53±1.62	8.67±1.96	7.92±2.68	9.43±1.32	7.20±1.79
	B (50)	8.65±1.54	10.12±2.51	8.57±2.10	11.98±1.27*	7.97±1.19	7.73±2.44
	C (100)	7.15±0.49	10.28±2.49	8.53±1.50	8.12±2.20	13.83±5.82	12.23 ±9.95
	D (150)	8.87±1.19	11.30±2.91	7.98±2.07	10.57±0.73	5.93±1.06	5.20±2.60
Neutrophils (nx10^9.L^{-1})							
Groups (CY-Doses: mg.kg^{-1} b.wt)	**A (0)**	2.68±0.32	3.06±0.71	3.68±1.06	3.05±0.94	3.73±0.84	2.18±0.66
	B (50)	2.98±0.59	2.84±0.90	3.52±0.91	4.57±0.95*	2.90±0.63	2.56±0.48
	C (100)	2.29±0.43	3.20±1.80	3.98±0.74	2.79±1.21	4.69±1.44	3.82±2.96
	D (150)	2.37±0.54	5.14±1.93	2.79±0.90	3.61±0.56	2.12±0.26*	1.84±0.88
Lymphocytes (nx10^9.L^{-1})							
Groups (CY-Doses: mg.kg^{-1} b.wt)	**A (0)**	3.48±0.65	3.40±0.94	3.45±0.68	3.58±1.13	4.29±0.68	2.09±0.70
	B (50)	4.09±0.85	4.53±1.44	3.20±1.08	4.74±0.96	3.02±1.22	3.78±1.62
	C (100)	3.51±0.63	4.60±0.96	3.19±0.53	3.67±0.79	5.58±1.66	4.96±3.62
	D (150)	4.41±0.74	3.63±0.61	3.15±0.92	4.06±0.63	2.28±0.31*	1.94±0.78
Monocytes (nx10^9.L^{-1})							
Groups (CY-Doses: mg.kg^{-1} b.wt)	**A (0)**	0.72±0.29	0.82±0.33	0.46±0.11	0.38±0.31	0.67±0.33	0.63±0.23
	B (50)	0.55±0.22	0.81±0.20	0.56±0.20	0.84±0.22*	0.51±0.29	0.43±0.12
	C (100)	0.68±0.57	0.43±0.12	0.42±0.22	0.55±0.20	0.81±0.19	0.57±0.50
	D (150)	1.03±0.69	0.93±0.57	0.50±0.29	0.75±0.23*	0.47±0.23	0.35±0.25
Eosinophils (nx10^9.L^{-1})							
Groups (CY-Doses: mg.kg^{-1} b.wt)	**A (0)**	0.63±0.15	0.75±0.19	0.73±0.37	0.49±0.34	0.27±0.11	0.28±0.11
	B (50)	0.59±0.16	1.37±0.74	0.61±0.45	0.96±0.23*	0.86±0.28	0.70±0.31
	C (100)	0.46±0.20	1.43±1.64	0.61±0.45	0.67±0.52	2.18±2.41	2.05±2.32
	D (150)	0.64±0.19	0.93±0.35	1.02±0.32	1.43±0.38*	0.85±0.26	0.81±0.52
Basophils (nx10^9.L^{-1})							
Groups (CY-Doses: mg.kg^{-1} b.wt)	**A (0)**	0.46±0.15	0.51±0.25	0.35±0.09	0.46±0.18	0.55±0.26	0.35±0.13
	B (50)	0.46±0.17	0.56±0.60	0.46±0.23	0.87±0.28*	0.69±0.38	0.26±0.12
	C (100)	0.22±0.07	0.63±0.46	0.33±0.22	0.44±0.22	0.58±0.20	0.84±0.72
	D (150)	0.41±0.28	0.68±0.25	0.53±0.15	0.72±0.27	0.22±0.08	0.27±0.24

Table 3: The frequency and incidence of histological lesions in the spleen of male rabbits injected with different CY-doses

Lesion	Groups (Dose: mg.kg^{-1} b.wt)	Experimental days 15		29		43		57		71	
		F*	I**	F	I	F	I	F	I	F	I
Congested areas	B (50)	----	0	----	0	----	0	----	0	----	0
	C (100)	----	0	----	0	+--	50	+---	50	+--	50
	D (150)	----	0	+---	50	+---	50	+---	50	++--	50
Depleted white pulps	B (50)	---	0	----	0	+---	50	+---	50	++--	50
	C (100)	+---	50	+---	50	+---	50	++--	50	++--	100
	D (150)	+---	50	++--	50	++--	10	+++-	10	++++	100
Hemo-sidrin deposits	B (50)	----	0	----	0	----	0	+---	50	+---	50
	C (100)	----	0	+---	50	+---	50	+---	50	+--	50
	D (150)	+---	50	+---	50	+---	50	+---	50	++--	50

*F = Frequency; **I = Incidence (%). Control group (A) did not show any lesion.

Table 4: Frequency and incidence of histological lesions in the spleen of male rabbits injected with different CY-doses

Lesion	Groups (CY-Doses: mg.kg^{-1} b.wt) B (50)		C (100)		D (150)	
	F*	I**	F	I	F	I
Congestion	----	0	+---	30	+---	50
Depleted white pulp	+---	30	++--	60	+++-	80
Hemosidrin deposition	+---	20	+---	40	++--	50

*F = Frequency; **I = Incidence (%). Control group (A) did not show any lesion.

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ETHICAL APPROVAL

The *Oryctolagus cuniculus* subjects used in this research were purchased from the local market. The synopsis of this research work was approved by the Ethical Committee at University of Agriculture Faisalabad, Pakistan.

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