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Immunosuppressive effect of ibuprofen in Japanese quails

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Abstract
This experimental work was conducted to elucidate the immunosuppressive effect of ibuprofen in Japanese Quails. A total number of 100 healthy Japanese quails were reared up to two weeks of age and then divided into four major groups i.e. Control (C) group and the three Treatment groups namely B1, B2 and B3 respectively. Experiment was carried out on 100 birds out of which 25 birds were kept in each group. The birds in control group received normal starter feed without any medicine and the drug was given with feed @ 15, 30 and 45 mg /kg body weight to the birds of B1, B2 and B3 groups for 15 days. The intoxicated birds revealed severe lesions of gout and immune suppression which was evident by the deposition of white chalky material on the surface of visceral organs and lymphoid depletion.

Keywords: Ibuprofen, immunosuppression, lymphoid depletion and gout

1. Introduction
Non-steroidal anti-inflammatory drugs are one of the most widely prescribed medication in the world. Their main benefit derives from their anti-inflammatory and analgesic effect, but continuous use of these agents increases the risk of toxicity in animals and birds. Ibuprofen is a non-steroidal anti-inflammatory drug which was discovered in 1961 by Stewart Adams and was initially marketed under various trade names including Brufen, Advil and Motrin. It is on the World Health Organization's list of essential medicines, which lists the most effective medicines against pain needed in health care system. Non-steroidal anti-inflammatory drugs (NSAIDs) produce their effect by inhibiting cyclooxygenase enzymes which are involved in the formation of prostaglandins. There are at least three variants of cyclooxygenase enzymes namely COX-1, COX-2 and COX-3. Ibuprofen is believed to inhibit both COX-1 and COX-2 in order to produce analgesic, antipyretic and anti-inflammatory effects which were achieved principally through COX-2 inhibition whereas COX-1 inhibition is responsible for antiplatelet effect and is hence also known as anti-platelet drug. Ibuprofen is toxic for cats and inhibits prostaglandins synthesis needed for normal blood circulation to the stomach. Without normal blood flow, the stomach cannot produce a proper protective layer of mucous to protect its tissues from the harsh digestive acid resulting in the ulceration. Ibuprofen poisoning may cause variety of symptoms in both cats and dogs which may include bloody stool, staggering, lethargic seizures, increased thirst, foaming at the mouth, depression and frequent urination [5]. The Birds of Ibuprofen groups also showed dehydration, anorexia, loss of body weight, decrease in feeding, increase in thirst and even death. On necropsy, the birds revealed deposition of white chalky materials on internal organs giving cooked appearance which was very severe in succumbed birds [9]. The birds succumbed in the treatment groups revealed proliferation and desquamation of endothelium lining along with depletion of lymphocytes in lymphoid organs. Microscopic examination further revealed atrophy, oedema, hemorrhages, decrease in size of the lymphoid follicles and necrosis of Caecum [7]. Nitrobluetetra Zolium (NBT) salt reduction test was performed to assess the functional ability of spleenic macrophages of intoxicated birds. The result of this study clearly indicated that intoxication with ibuprofen caused significant decrease in the number of active spleenic macrophages [1].
Keeping the above facts in view, this investigation was designed to study the immunosuppressive effects of ibuprofen in Japanese quails with fallowing immunological Parameters

a) Nitroblue-tetrazolium blood test
b) Pathomorphological changes in the lymphoid organs
c) Relative change in the weight of lymphoid organ

2. Materials and Methods

A total number of 100 one-day old Japanese quails were procured from the local market of Indore situated in the vicinity of the faculty of veterinary sciences. All the birds were vaccinated against Marek's disease prior to the procurement. The birds were housed in the deep litter system and dried paddy straw was used as bedding material for the birds. The birds were reared up to 2 weeks of age and then divided into 2 major groups i.e. control (C) group and the treatment (T) group. The Treatment group (T) was further divided into 3 sub-groups namely B1, B2 and B3 respectively. Experiment was carried out on 100 birds out of which 25 birds were kept in each group. All the birds irrespectively of their groups were maintained on the same type of chick starter mash. The birds were fed in the first week @ of 10g /bird but from second week onwards ad libitum feeding practice was adopted. All the birds were vaccinated with F1 (Lasota) vaccination against Ranikhet disease on the 6th day of age. The control birds received normal starter feed without any medicine and the Ibuprofen was given with feed @ 15, 30 and 45 mg /kg body weight to the birds of B1, B2 and B3 groups for 15 days. Each bird was critically inspected every day for the appearance of clinical symptoms of toxicity at every stage of the experiment. The mortality rate in each group was also recorded every day until the end of the experiment which lasted for 30 days.

Nitroblue-tetrazolium (NBT) blood test is performed for the functional evaluation of phagocytic cells. The chemical NBT is added to the white blood cells and the cells are then observed under a microscope. Normally, the white blood cells turn blue when NBT is added. This means that the cells should be able to protect the bird from infection. In order to perform Nitroblue tetrazolium (NBT) salt test, NBT salt was dissolved in sodium chloride solution and mixed with an equal volume of Phosphate Buffer Solution with pH 7.2. After that glass slides with impression smears of the spleen were kept in petridishes and the NBT salt solution was poured over the smears. Moist cotton was kept in the petridishes in order to maintain humidity. These slides were incubated at 36ºC for 25 minutes and then at room temperature for 15 minutes. After then NBT salt solution was poured off and smears were stained with Wright's stain. The smears were examined for NBT positive macrophages having formazan crystals deposited in their cytoplasm and the percent positivity was calculated from 400 macrophages in each smear [2].

All the surviving experimental birds were scarified at the end of experimental period and each organ was critically examined for macroscopic lesions. The lesions were recorded and compared at the end of the experiment. For histopathological studies, all the dead and killed birds were subjected to detailed necropsy examination. At necropsy table, pieces of different lymphoid organs like spleen, bursa, thymus, and caecal tonsils were collected and fixed in 10% buffered formalin for histopathological examination. Later, paraffin embedded tissue sections were cut at 5 µm thickness, thereafter, stained with Haematoxylin and Eosin (H & E) stain and examined under microscope [4]. A significant reduction in the relative weight of lymphoid organs indicates that there was a depletion of lymphoid tissue from the lymphoid organs which produces a negative impact on the immune status of the intoxicated birds. For the relative weight of lymphoid organs like spleen, thymus, caecal tonsils and bursa of Fabricius were excised from birds of each group at the end of the experimental period. All these organs were lightly blotted and weighed and the relative weights were calculated in order to evaluate the immunological status of the intoxicated birds.

3. Immunological Observations

3.1 NBT Salt Test: The mean values of B1, B2 and B3 groups were $28.62^{b} \pm 2.05$, $27.54^{b} \pm 0.27$ and $25.89^{c} \pm 3.08$ respectively. The mean value of the Control group was $32.65^{a} \pm 0.24$. There was significant change observed in different treatment groups as shown in the table no.1

3.2 Response on Cell Mediated Immunity (CMI)

The mean values of positive macrophages (%) in the birds of different treatments as compared to control are given in the table. There is significant change observed in different groups in percentage of active macrophages on 30th day past treatment as shown in the table no.1

Table 1: Effect of NBT salt test in different groups of birds with their mean

Name of Groups	Mean values of NBT salt test in different intoxicated groups
Control	$32.65^{a} \pm 0.24$
B1	$28.62^{b} \pm 2.05$
B2	$27.54^{b} \pm 0.27$
B3	$25.89^{c} \pm 3.08$
Significant difference within the groups	

4. Clinical Signs

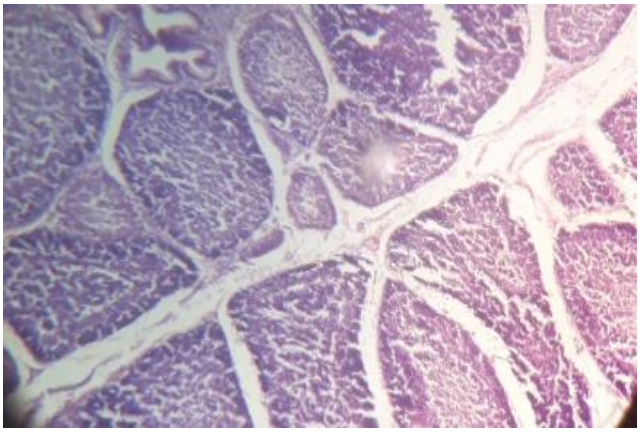
The intoxicated birds were dull and depressed followed by anorexia. After five days of post intoxication, the birds layed down and lacrimation was observed. The birds intoxicated with ibuprofen revealed sunken head with both closed eyes. There was hypersalivation with decreased respiratory rate, pupil was dilated and birds showed a staggering gait. Also, the intoxicated birds vomited suddenly and passed undigested food in faeces. The marked clinical signs observed in ibuprofen intoxicated birds included huddling, dullness, appeared listless, depressed and showed laboured breathing. The birds were reluctant to move and stood with ruffled feathers and cyanosed combs. Some of the affected birds also revealed lameness, enlarged hock joints and sternal recumbency. After 10 days of post intoxication, birds exhibited increased thirst, droopy wings and diarrhoea. The mucus membranes of the affected birds were pale and emaciated. The clinical signs observed in the third week of intoxication included tibial dyschondroplasia, lameness, abnormal bending of the tibial bones, enlarged hock joints, sternal recumbency, convulsion, lacrimation from eyes, rough feather coat, profuse salivation, diarrhoea, restlessness and ataxia. The keel bone appeared sharp due to emaciation. Other major clinical signs observed in intoxicated birds were yellowish diarrhoea, emaciation and polydipsia. The intoxicated birds showed significant increase in temperature beginning from the 1st day till end of the study period. At the end of the experimental period, the intoxicated birds which survived for few days revealed retarded growth, watery droppings, muscle weakness, staggering gait and loss of response to stimulus prior to death. The intoxicated birds stood motionless for a long period of time with puffed feathers. The birds showed repeated sneezing and fluid came from the mouth which appeared to be clogged.

5. Pathomorphological changes in the lymphoid organs

5.1 Bursa: The succumbed birds of Ibuprofen groups showed a severe degree of chalky white material deposited on the

surface of the bursa. There was a severe atrophy of bursa in association with hemorrhages and necrotic changes observed at the end of the experimental period. At day 10 after the post intoxication of drug, Bursa revealed moderate vascular congestion, oedema and was filled with fibrinous exudate as seen in the Photograph no1.Histopathological examination of bursa of ibuprofen intoxicated birds revealed degenerative changes and marked depletion of lymphocytes in the lymphoid follicles of bursa as seen in the Photograph no-2.Therefore, the decrease in the number of circulating lymphocytes is mainly due to the effect of drugs on lymphoblasts and thereby decreasing overall population of cells of lymphocyte series.

Photograph 1: Fibrinous exudate in Bursa

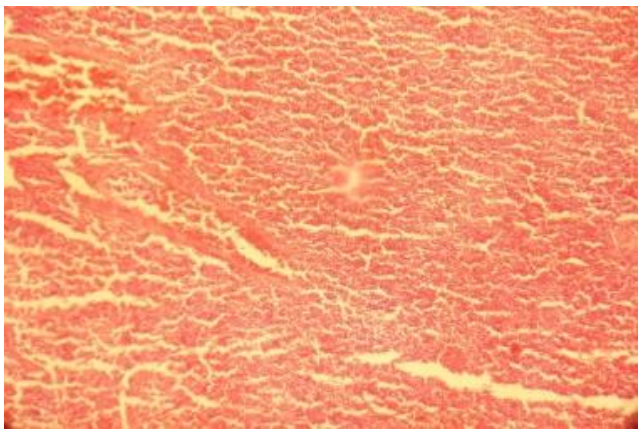


Photograph 2: Lymphoid depletion in Bursa

Bursa also revealed necrosis and hemorrhages in bursal follicles which were more predominant in birds died at the end of experimental period.

5.2 Spleen: The succumbed birds of Ibuprofen groups shows the moderate degree of chalky white materials deposited on the serosal surface of the spleen. Microscopically, the birds died during the experiment reveals proliferation and desquamation of endothelial lining in central artery of the white pulp**.** Severe congestion and hemorrhages were consistently observed at day 10 after post intoxication of ibuprofen drug as seen in Photograph-3**.** Moderate rarefaction of periarteriolar lymphoid sheath and mild histeocytosis was evident at the end of study period. Spleen also revealed severe degenerative changes in lymphoid cells along with the necrosis. In ibuprofen intoxicated cases central arteries showed fibromuscular dysplasia characterized by massive proliferation of endothelial cells. There was moderate depletion of lymphoid tissue in white pulp of the spleen as shown in the Photograph-4.

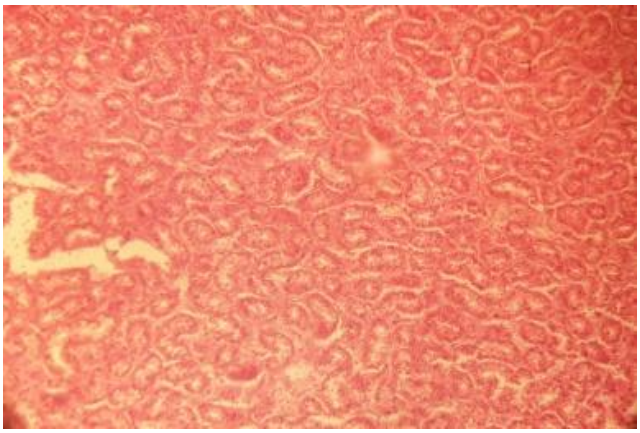
Photograph 3: Severely congested Spleen



Photograph 4: Edema and lymphoid depletion in white pulp of Spleen

5.3 Thymus: Thymus in all intoxicated birds appeared to be reduced in size with deposition of white chalky material on the surface. No gross changes were evident up to day 5 after the intoxication of drug. Thereafter varying degrees of congestion, hemorrhage and marked atrophy were consistently observed up to the end of experimental period as seen in the Photograph no-5. Microscopically, the birds died during the experiment revealed complete degeneration of thymic lobules and interstitial fibrous tissue proliferation. Further analyses of the thymus of the affected birds revealed irregularly shaped lymphocytes. Histopathological examination of thymus of ibuprofen intoxicated birds also revealed hypoplasia and decrease in size of the follicles. Further there was a significant cellular loss at the cortex of thymus and numbers of thymocytes in the cortex and medulla which indicates that the drug interferes with the development of thymus and T cell maturation. Histopathological examination also revealed necrosis and marked depletion of lymphocytes in the lymphoid follicles of thymus as seen in the Photograph no-6. Further analysis of thymus of ibuprofen intoxicated birds revealed dilation and fibrosis in the cortex. Thymic cells of treatment birds revealed lost of their cytoplasmic material and their cytoplasm was hyaline in nature. Nucleus abnormalities like pyknotic nucleus and chromatin clumping were also evident at the end of the experimental period.

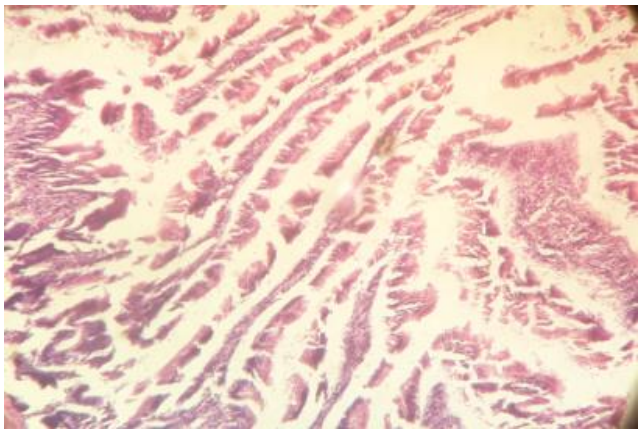
Photograph 5: Hemorrhagic Thymus



Photograph 6: Necrosis and lymphoid depletion in thymus

5.4 Ceacal tonsils: In birds, ceacal tonsils are considered the largest lymphoid aggregates of avian gut-associated lymphoid tissue. Grossly ceacal tonsils appeared to be reduced in size and necrotic in nature. There was a significant decrease in the height and length of villus as compared to control. Although villi were shorter and more flattened in ibuprofen intoxicated birds. Follicles were wider in all treated groups in comparison with control. Histopathological examination also revealed degeneration and decrease in the number of follicles in all treated groups. At day 10 after the post intoxication of birds with drug, Caecal tonsils mostly revealed marked necrosis, denudation and degenerative changes which were evident throughout the study period as seen in the Photograph no-7.Birds sacrified at day 30, revealed hypertrophy of caecal wall and mild infiltration of mononuclear cells. Microscopically, the birds died during the experiment revealed vascular congestion, edema and necrosis**.** Birds died or sacrified after day 30, showed cellular infiltration in the lamina propria, distortion of villi, and thickening of mucosa. At the end of the experimental period, the most severe lesions were observed in the cecal tonsils which consisted mainly of necrosis, ulcer in the cecal tonsils, accumulation of macrophages and lymphocyte depletion as shown in the Photograph no-8. In the intestines, the severe necrosis of the lymphoid-dependent areas was associated with extensive ulceration of the epithelium and accumulation of necrotic material within the intestinal lumen.

Photograph 7: Necrotic Caecal tonsils



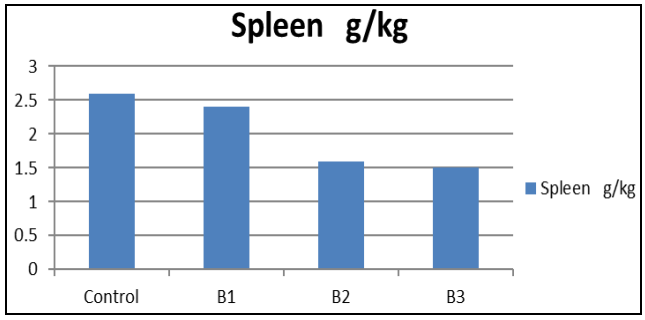
Photograph 8: Necrosis, denudation and lymphoid depletion in Caecal tonsils

6. Relative organ weight ratio

6.1 Spleen: The mean values of ibuprofen treatment groups B1, B2 and B3 were $2.40^{a} \pm 0.23$, $1.60^{b} \pm 0.26$ and $1.50^{b} \pm 0.24$ gm/kg body weight and control group mean was $2.60^{a} \pm 0.14$ gm/kg body weight. The analysis of table no 2 and graph no.2 revealed significant decrease in the weight of the spleen which produces negative effect on the immune status of the birds.

Table 2: Effects of ibuprofen on spleen weight in control and intoxicated birds with their mean

Groups	Spleen g/kg
Control	$2.60^{a} \pm 0.14$
B1	$2.40^{a} \pm 0.23$
B2	$1.60^{b} \pm 0.26$
B3	$1.50^{b} \pm 0.24$
Significant difference within the groups	

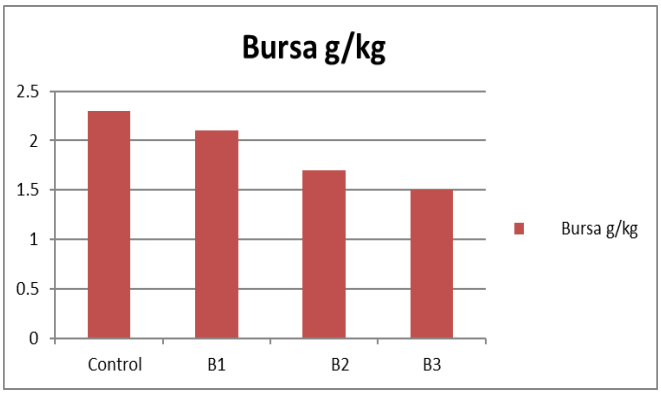


Graph 2: Alteration in spleen weight in control and intoxicated birds

6.2 Bursa: The mean value of Ibuprofen groups B1, B2 & B3 are $2.1^{a}\pm 0.03$, $1.7^{b}\pm 0.17$ and $1.5^{b}\pm 0.05$ gm/kg body weight. The mean of Control group was $2.3^{a}\pm 0.14$gm/kg body weight. The statistical analysis shows the significant decrease in the weight of Bursa in different treatment groups as shown in table no.3 and graph no.3 leading to the negative impact on the immunological status of the birds.

Table 3: Effects of ibuprofen on weight of bursa in control and intoxicated birds with their mean

Groups	Bursa g/kg
Control	$2.3^{a}\pm 0.14$
B1	$2.1^{a}\pm 0.03$
B2	$1.7^{b}\pm 0.17$
B3	$1.5^{b}\pm 0.05$
Significant difference within the groups	

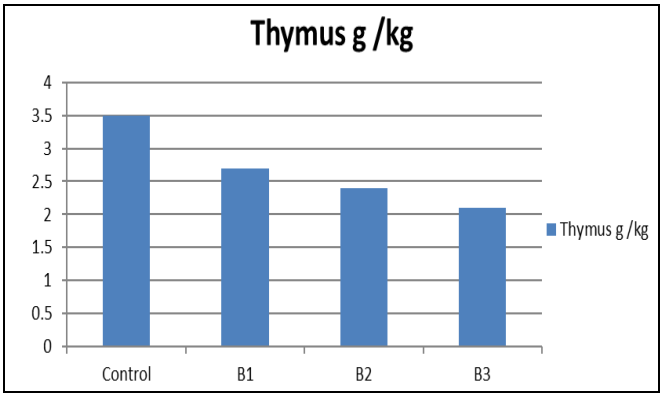


Graph 3: Alteration in bursa weight in control and intoxicated birds

6.3 Thymus: The mean of Ibuprofen intoxicated groups B1, B2 and B3 were $2.70^{b} \pm 0.27$, $2.40^{ab}\pm 0.51$ and $2.10^{a} \pm 0.25$ gm/kg body weight and the mean of control group was $3.50^{a} \pm 0.31$ gm/kg body weight. The statistical analysis reveals significant difference in different treatment groups as shown in table no. 4 and graph no.4

Table 4: Effects of ibuprofen on thymus weight in control and intoxicated birds with their mean

Groups	Thymus g /kg
Control	$3.50^{a} \pm 0.31$
B1	$2.70^{b} \pm 0.27$
B2	$2.40^{ab}\pm 0.51$
B3	$2.10^{a} \pm 0.25$
Significant difference within the groups	



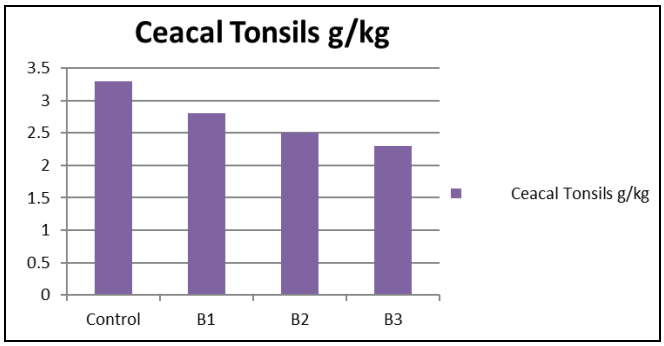
Graph 4: Alteration in thymus weight in control and intoxicated birds

6.4 Caecal Tonsils: The mean of Ibuprofen group B1, B2 & B3 were $2.8^{b} \pm 0.96$, $2.50^{ab}\pm 0.42$ and $2.30^{ab}\pm 0.17$. gm/kg body weight and the control group mean was $3.3^{a}\pm 0.28$ gm/kg body weight

Table 5: Effects of ibuprofen on weight of caecal tonsils in control and intoxicated birds with their mean

Groups	Ceacal Tonsils g/kg
Control	$3.3^{a}\pm 0.28$
B1	$2.8^{b} \pm 0.96$
B2	$2.50^{ab}\pm 0.42$
B3	$2.30^{ab}\pm 0.17$
Significant difference within the groups	

The statistical analysis reveals significant increase in different treatment groups of ibuprofen as shown in table no.5 and graph no.5



Graph 5: Alteration in ceacal tonsils weight in control and intxicated birds

7. Discussion

In this study, Nitroblue-tetrazolium (NBT) blood test was performed for the evaluation of functional activity of phagocytic cells. The result revealed a significant decrease in the percentage of active macrophages in the intoxicated birds. The results were comparably similar with the findings observed in monocytes of chicken during aflatoxicosis [1]. The weight of the lymphoid organs helps to assess the immune status of the birds. This study revealed significant reduction in the relative weight of lymphoid organs in the birds of treatment group. These observations were in accordance with the results of immunosuppression observed in the chicken produced by Marek's disease virus [8]. The succumbed birds of Ibuprofen treatment groups showed the moderate degree of chalky white materials deposited on the serosal surface of the spleen. Microscopically, the birds died during the experiment revealed proliferation and desquamation of endothelial lining in the central artery of the white pulp and moderate depletion of lymphoid tissue in a white pulp. Similar results were earlier documented in gout in poultry [7]. Hemorrhages and atrophy was more prominent in bursa of Fabricius in B3 toxicity group which was easily visualized on gross examination. Microscopically, Bursa revealed vascular congestion, hemorrhages, interstitial oedema and depletion of lymphoid cells. These results are similar to the findings of pesticides toxicity on lymphoid organs in sheep [3]. Thymus in all intoxicated birds appeared to be reduced in size with deposition of white chalky material on the surface. Microscopically, hemorrhages with severe depletion of lymphoid cells were consistently observed in all intoxicated birds. The birds died during the experiment revealed complete degeneration of thymic lobules and hypoplasia. The results

were comparably similar with the findings of carbendazim toxicity in rats [10]. The caecum of the ibuprofen intoxicated birds revealed deposition of white chalky material on gross examination and decrease in the number of follicles and necrosis which were earlier documented in the gout in poultry [6].

8. Conclusion

It was concluded that ibuprofen was markedly toxic to birds, resulting in severe clinical toxicosis and changes in the lymphoid organs were suggestive of immunomodulatory effects of ibuprofen in birds.

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