CHAPTER 2

HAEMATOLOGICAL ALTERATION IN F1 AND F2 GENERATION CHICKS SUBJECTED TO SINGLE IN-OVO TREATMENT OF CHLORPYRIFOS AND CYPERMETHRIN COMBINATION

INTRODUCTION

Pest control measures are used world over to protect largely the agro-ecosystem (Bhattacharya and Bhattacharya, 2006). In mid 1960s with agricultural modernization in developing countries, the global pesticide industry witnessed a prodigious growth (Binswanger *et al*., 1998). Sporadic use of pesticides has now become prevalent and has led to an extensive contamination of the environment. The research analysis shows that annual consumption of pesticides is about 24% in United States, 45% in Europe, and 31% in the rest of the world (Foo and Hameed, 2010). However, because of injudicious use of pesticides in India, 51% of food commodities are contaminated with pesticide residues (Gupta, 2004). The predominant classes of pesticides that are in use include organophosphates, carbamates and pyrethroids (Al Masri *et al.,* 2009).

Chlorpyrifos is one of the most widely used organophosphorous insecticide and belongs to class II of World Health Organization classification of pesticides (Cox, 1997). Chlorpyrifos inhibits acetylcholine decomposition and hence increases the acetylcholine level in the synaptic cleft and stimulates specific receptors (Nostrandt *et al*., 1997). Chlorpyrifos is moderately toxic and a prolonged exposure has been reported to cause anemia although the mechanism has not been elucidated (Ambali *et. al.,* 2010b).

Cypermethrin is an active pyrethroid which is known to stimulate nerves by causing pronounced repetitive activity. It has been reported that synapses are more sensitive to the pyrethroids than nerve fibres (Latuszynska *et al*., 2001). Mechanistically, pyrethroid binds to and delay sodium channel closure, so that a prolonged sodium tail current persists after the membrane repolarization (Perger and Szadkowshi, 1994). Nevertheless, this neuro-impairment might result in other physiological manifestations, which can be gauged through haemogram analysis.

In the present study, a commercially available combination insecticide (chlorpyrifos + cypermethrin) (Ci) was used to analyze the haematological changes in the first filial (F1) and second filial (F2) generations of domestic fowls. The rationale behind the selection of the present test article is that, chlorpyrifos and cypermethrin are amongst the most frequently used insecticides. Further, due to the potentiation of the effects of cypermethrin by organophosphorus compound, has resulted in the promotion of combination insecticide on a commercial scale. Therefore it was thought pertinent to analyze the toxic effect of Ci rather than individual pesticide.

It is well known that haematological values indicate the state of health and has been used as a diagnostic tool by the clinicians for effective treatment. Hence, the objective of present study was to evaluate the two generation developmental toxicity potential of the chlorpyrifos and cypermethrin combination in domestic fowl through complete blood cell count of both F1 and F2 generation.

MATERIAL AND METHODS

Test chemical

Commercially available insecticide Nurelle D 505 EC (chlorpyrifos 50% EC + cypermethrin 5% EC, Dow AgroSciences, India) was used in the study.

Egg procurement

The fertilized Rhode Island-Red (RIR) eggs were collected from Poultry Science Division of Anand Agricultural University, Anand, and refrigerated at 4^{o}C until used. Eggs were swabbed with povidone-iodine to avoid infection. The investigation covered four groups of fifty fertilized RIR eggs each; three for experimental and one control. Each egg was marked to receive the respective dosage treatment of the Ci for experimental groups and corn oil for control group. The study was conducted on the F1 (Parents) and F2 (Chicks) generations as the animal model.

Insecticide injection

Combination insecticide was diluted in corn oil and three sub-lethal doses namely low, mid and high were made as described in our earlier studies (Uggini *et al*., 2012). A single dose of 0.01, 0.05 and 0.1 µg/egg of Ci diluted in corn oil was administered to the airspaces of fertilized eggs from low, mid and high group respectively on day zero of incubation. The eggs from the control received corn oil in the similar manner. The injected portion of each egg was

sealed by molten paraffin wax. The whole experiment was done in a sterile environment under a laminar air flow. The dose volume of Ci and corn oil was maintained at 25µl/egg.

Incubation

A cleaned, disinfected and fumigated automatic egg incubator and hatcher were used. Incubator regulates the factors such as temperature of 37.5 ± 0.5^{o}C, 75-80% relative humidity and periodic turning of the eggs. Immediately after treatment, both the treated and control eggs were kept in the incubator with proper marking for 18 days. On 18th day the egg candling was done and the viable eggs were transferred to hatcher till the day of hatch. On day 21 after hatching, the hatchling groups (F1 generation) from the treated and control eggs were tagged with wing bands with respect to their dosage treatment and were housed in separate pens with immediate supply of water. After three hours starter mash feed was provided to the chicks. To promote the survival of the chick, assisted hatching was involved in case of those chicks who failed to progress normally at hatching stage due to deformities or weakness caused by Ci.

The F1 generation chicks when reached 25 weeks old were randomized within the respective treatment groups and the similar weighed ones were selected for breeding. Twenty hens and two roosters from each of the four groups were selected and pen mated for a week. The eggs collected from the F1 generation were marked as per the treatment groups of F1 parental generation and incubated. On hatch out the F2 generation chicks were wing banded with respect to their parent's group and reared on standard diet for 4 weeks. The haematological analyses were performed on 25 week old parents and 4 week old second generation chicks.

Haematological Estimations:

Blood samples were drawn from brachial vein of 25 week old parent and 4 week old F2 chick using EDTA rinsed 2ml disposable syringes and collected into EDTA rinsed or heparinized vials. After collection, the samples were refrigerated and processed within 6 hr.

Hematology analyzer, Mindray BC2300 (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) was used to evaluate haematological parameters including total erythrocyte (RBC) count, haemoglobin (Hb) concentration, packed cell volume (PCV), total leukocyte (WBC) count and differential leukocyte counts. Complementary indices of the erythrocytic component, including mean corpuscular haemoglobin concentration - MCHC, mean corpuscular haemoglobin - MCH, and mean corpuscular volume - MCV, were also computed.

Effect of treatment on differential white blood cell counts was enumerated in blood smears prepared and stained panoptically with Microkrom Microscopy Stains (Giemsa Stain) immediately after sampling.

Raw data were processed and analyzed by one-way ANOVA followed by Dunnet's multiple comparison test. Results were expressed as mean ± standard deviation. A 'p' value less than or equal to 0.05 was considered as statistically significant. Statistical analysis was performed using GraphPad Prism 5. The protocol mentioned herein was approved by the IAEC of the department in full compliance with the CPCSEA norms.

RESULTS

The purpose of the present study was to evaluate the possibility of haematologic abnormalities, if any, in the F2 generation induced by Ci treatment to the eggs that gave rise to F1 generation. The haematological parameters in F1 and F2 generations of chicks were analyzed after administering a single dose of 0.01, 0.05 and 0.10 µg/egg of Ci to the air cell of eggs and apparent variations in the haematological parameters with that of a control group were recorded.

F1 Generation

At 0.01 µg/egg and 0.05 µg/egg of Ci, the F1 generation did not show any significant changes in haemoglobin content when compared to the concurrent controls whereas treatment with 0.1µg/ egg of Ci induced severe anaemia ($p \leq 0.05$) in the first generation of chicks.

Total red blood cell concentration values were recorded for the birds that received low dose (0.01µg/egg) of Ci, and were found comparable to that of controls. The analysis of blood showed a decline in the total number of red blood cells at 0.05 µg/egg and 0.10 µg/egg of Ci as compared to the control group (Figure 2.1-A). The PCV value also showed significant reduction in the group that received 0.1µg/egg. However, the MCV values showed an apparent hike in F1generation (Figure 2.2-A) while MCH values were found decreased in all the three treatment groups compared to control group. Though MCHC value showed a similar declining trend, it was found statistically non significant. Quite similar to the trend showed by erythrocytes, the WBC count too was found reduced in birds that received Ci (Figure 2.3-A). Nevertheless, the differential white blood cell count showed an increase in the number of

polymorphs only in the birds from high dose group (Figure 2.4-A), whereas the lymphocyte numbers in this group of birds were found significantly lowered with respect to that of controls (Figure 2.5-A). The eosinophil showed an increase in mean number in birds from the F1 groups that received Ci (Figure 2.6-A), whereas monocyte number declined in all the three first generation groups of chicks (Figure 2.7-A). The platelet count however, recorded significant reduction in count in birds that received high dose of Ci (Figure 2.8-A). Basophil did not show any significant change in its number at any of the selected doses. (Table 2.1).

F2 Generation

The haemoglobin content has not shown any significant variation in any group of the F2 generation chicks that descended from a parent who received Ci during development. However, the total RBC count was lowered in the groups whose parents received 0.05 and 0.10 µg/egg of Ci (Figure 2.1-B). The other parameters like PCV, MCV, MCH (Figure 2.2-B), MCHC count have not shown any significant alterations in any of the F2 generation chicks. However, birds from the F2 generation of the high dose group showed an increase in the mean WBC count (Figure 2.3-B). The differential count of the white blood cells showed that the level of polymorphs was significantly increased in all the Ci treated groups compared to the control (Figure 2.4-B). Nonetheless, the lymphocyte (Figure 2.5-B), eosinophil (Figure 2.6-B), monocytes (Figure 2.7-B) and platelet counts were found significantly decreased in all the three F2 generation chicks descended from Ci treated parents (Figure 2.8-B). However, basophil did not show any significant treatment related changes in any of the treated animals with respect to the control group animals (Table 2.2).

Table 2.1: Complete blood count of F1 generation RIR domestic fowl subjected to various doses of combination insecticide during their embryonic development.

Parameters	Control (Corn Oil)	Low (0.01µg/egg)	Medium (0.05µg/egg)	High (0.1µg/egg)
Haemoglobin (gm/dl)	10.13 ± 0.79 [a]	9.47 ± 0.91 [a]	9.03 ± 0.85 [a]	5.93 ± 0.57[b]
RBC Count (10^6/cumm)	3.77 ± 0.24[a]	3.70 ± 0.35[a]	3.09 ± 0.31[b]	2.84 ± 0.28[b]
P.C.V (%)	30.10 ± 2.71[a]	29.72 ± 2.84[a]	27.02 ± 2.70[a]	18.65 ± 1.79[b]
M.C.V (fl)	80.72 ± 7.89[a]	85.96 ± 8.42[a]	88.57 ± 8.74[a]	97.68 ± 9.62[b]
M.C.H (pg)	27.14 ± 2.43[a]	25.71 ± 2.57[b]	23.14 ± 2.38[c]	20.27 ± 1.98[c]
M.C.H.C (g/dl)	33.68 ± 2.33[a]	33.30 ± 2.58[a]	32.86 ± 3.10[a]	31.94 ± 2.99[a]
WBC Count (/cumm)	13450 ± 505.77[a]	11883 ± 527.65[b]	10700 ± 532.82[b]	10400 ± 530.09[b]
Polymorphs (%)	57.50 ± 3.51[a]	64.67 ± 4.33 [a]	66.33 ± 5.97 [a]	67.33 ± 6.73[b]
Lymphocytes (%)	38.00 ± 2.12 [a]	29.50 ± 2.27[b]	28.33 ± 2.80[c]	25.33 ± 2.12[d]
Eosinophils (%)	2.17 ± 0.19[a]	3.17 ± 0.29[b]	3.33 ± 0.32[b]	3.83 ± 0.39[c]
Monocytes (%)	2.33 ± 0.17[a]	2.00 ± 0.21[b]	1.50 ± 0.14[c]	1.33 ± 0.11[c]
Basophils (%)	1.02 ± 0.33[a]	2.05 ± 0.70 [a]	1.50 ± 0.72 [a]	2.04 ± 0.65 [a]
Platelet Count (K/cumm)	2.33 ± 0.14[a]	2.26 ± 0.19[a]	2.14 ± 0.20[a]	1.79 ± 0.16[b]

Values are expressed in mean ± SD; Values with same superscript are not statistically significant for each parameter.

RBC-Red blood corpuscles, WBC-White blood corpuscles, P.C.V.-Packed cell volume, M.C.V.-Mean corpuscular volume, M.C.H.-Mean corpuscular haemoglobin, M.C.H.C-Mean corpuscular haemoglobin concentration.

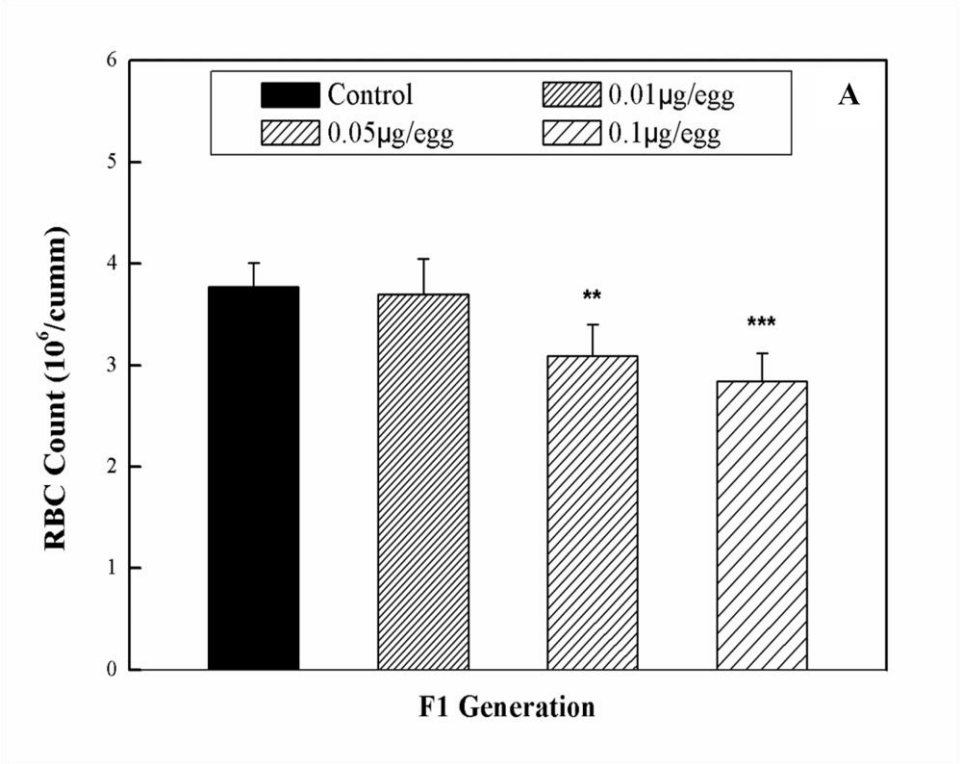
Table 2.2: Complete blood count of F2 generation chicks descended from parents who received low, medium or high doses of Ci during their embryonic development.

Parameters	Control (Corn Oil)	Low (0.01µg/egg)	Medium (0.05µg/egg)	High (0.1µg/egg)
Haemoglobin (gm/dl)	10.19 ± 0.85^{a}	10.03 ± 0.92^{a}	9.80 ± 0.91^{a}	9.42 ± 0.90^{a}
RBC Count (10^6/cumm)	4.16 ± 0.31^{a}	3.97 ± 0.33^{a}	2.74 ± 0.27^{b}	2.32 ± 0.25^{b}
P.C.V (%)	30.51 ± 3.03^{a}	29.92 ± 2.94^{a}	26.77 ± 2.69^{a}	26.32 ± 2.55^{a}
M.C.V (fl)	78.83 ± 7.56^{a}	79.50 ± 7.82^{a}	85.99 ± 8.49^{a}	87.84 ± 8.73^{a}
M.C.H (pg)	26.34 ± 2.33^{a}	24.95 ± 2.41^{a}	24.26 ± 2.42^{a}	23.20 ± 2.34^{a}
M.C.H.C (g/dl)	33.38 ± 2.98^{a}	33.15 ± 3.24^{a}	32.59 ± 3.19^{a}	32.22 ± 3.16^{a}
WBC Count (/cumm)	14057 ± 578.32^{a}	14116 ± 620.30^{a}	14533 ± 655.67^{a}	15150 ± 724.47^{b}
Polymorphs (%)	27.14 ± 2.13^{a}	56.50 ± 3.78^{b}	59.67 ± 4.66^{b}	61.83 ± 5.77^{b}
Lymphocytes (%)	68.00 ± 3.21^{a}	39.33 ± 2.39^{a}	34.50 ± 3.22^{a}	34.33 ± 3.01^{a}
Eosinophils (%)	2.57 ± 0.17^{a}	2.17 ± 0.21^{b}	1.83 ± 0.18^{c}	1.67 ± 0.16^{c}
Monocytes (%)	2.29 ± 0.19^{a}	2.00 ± 0.39^{b}	1.50 ± 0.14^{b}	1.33 ± 0.11^{b}
Basophils (%)	1.71 ± 0.42^{a}	1.94 ± 0.62^{a}	1.34 ± 0.55^{a}	2.12 ± 0.64^{a}
Platelet Count (K/cumm)	3.16 ± 0.23^{a}	2.55 ± 0.21^{b}	2.24 ± 0.22^{b}	2.11 ± 0.20^{c}

Values are expressed in mean ± SD; Values with same superscript are not statistically significant for each parameter.

RBC-Red blood corpuscles, WBC-White blood corpuscles, P.C.V.-Packed cell volume, M.C.V.-Mean corpuscular volume, M.C.H.-Mean corpuscular haemoglobin, M.C.H.C-Mean corpuscular haemoglobin concentration.

Figure 2.1: RBC count in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci



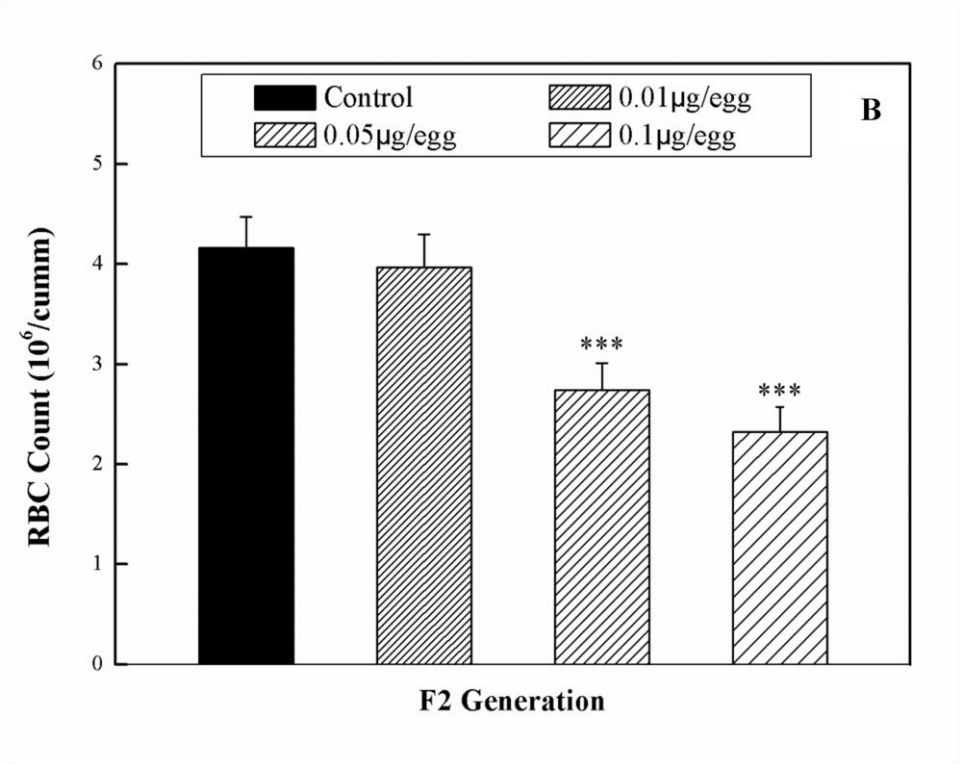
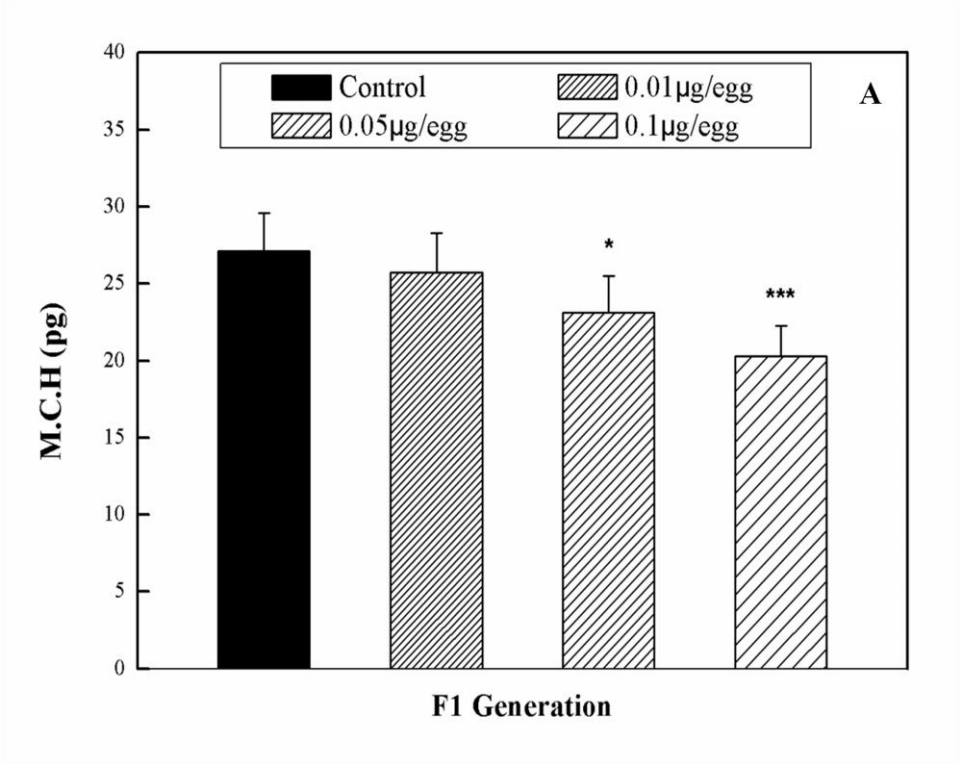


Figure 2.2: M.C.H. in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci



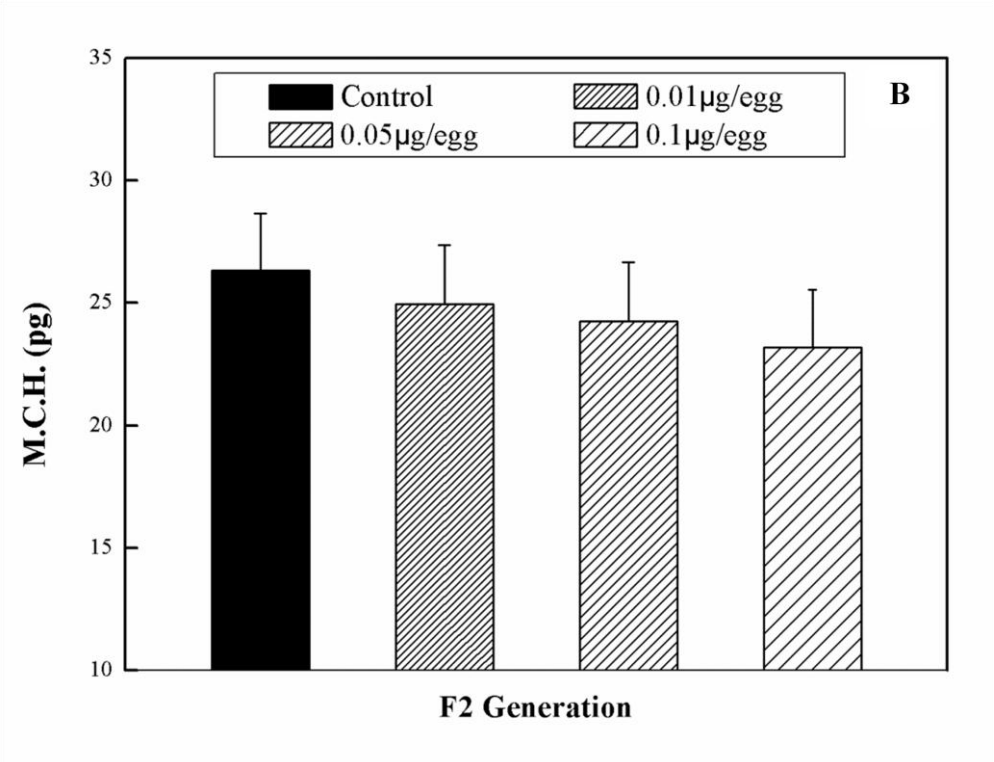
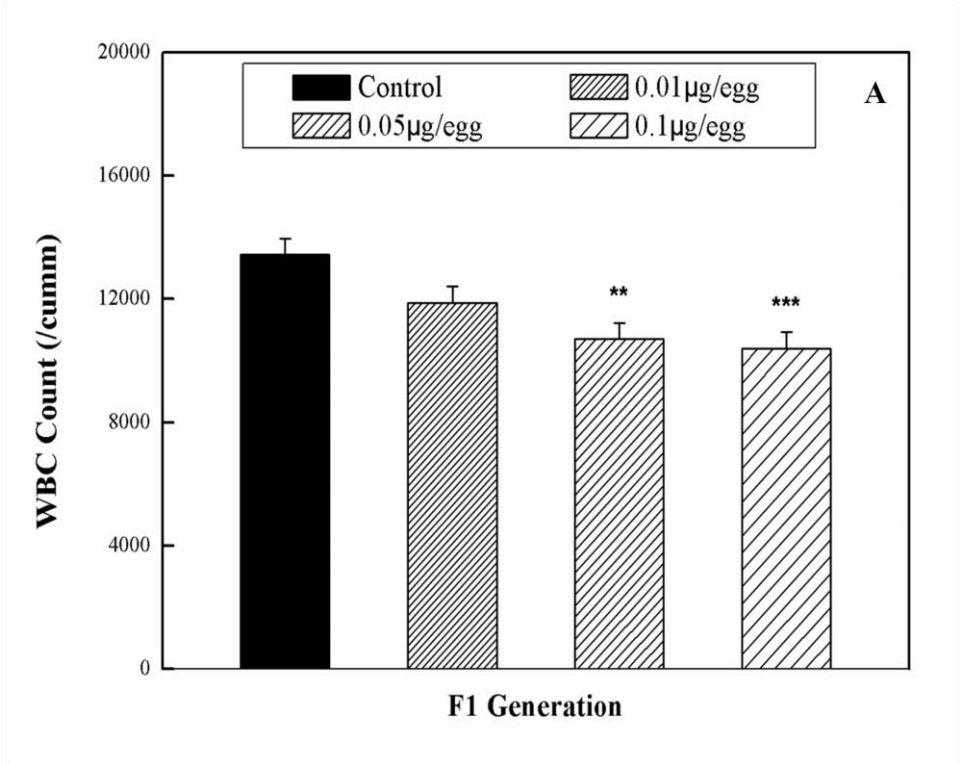


Figure 2.3: WBC count in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci



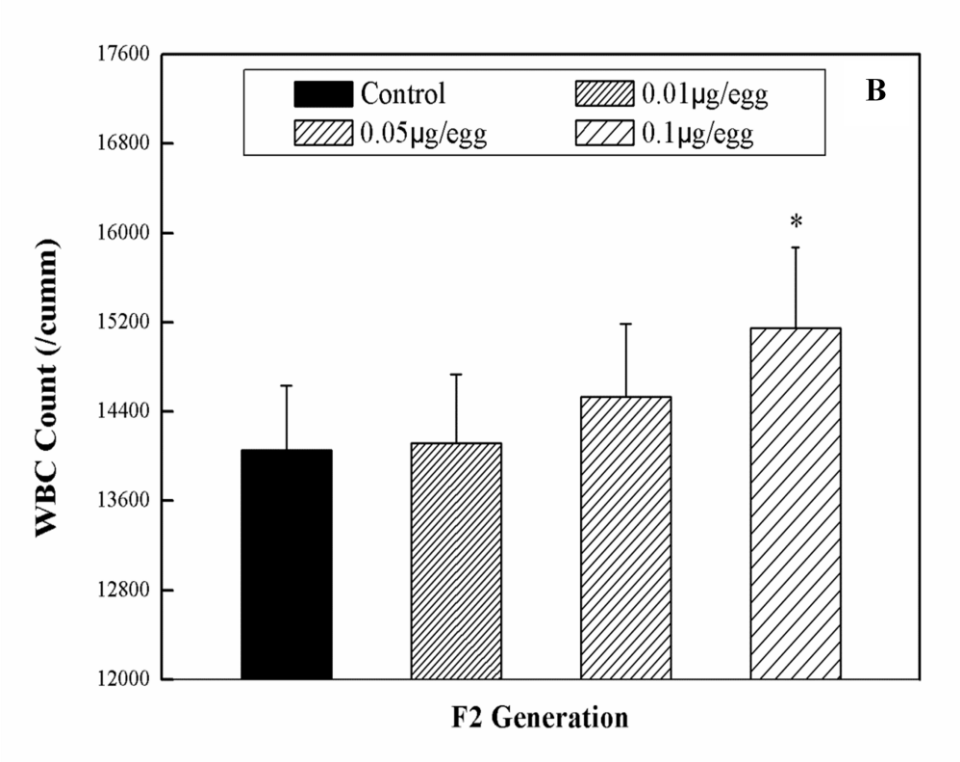
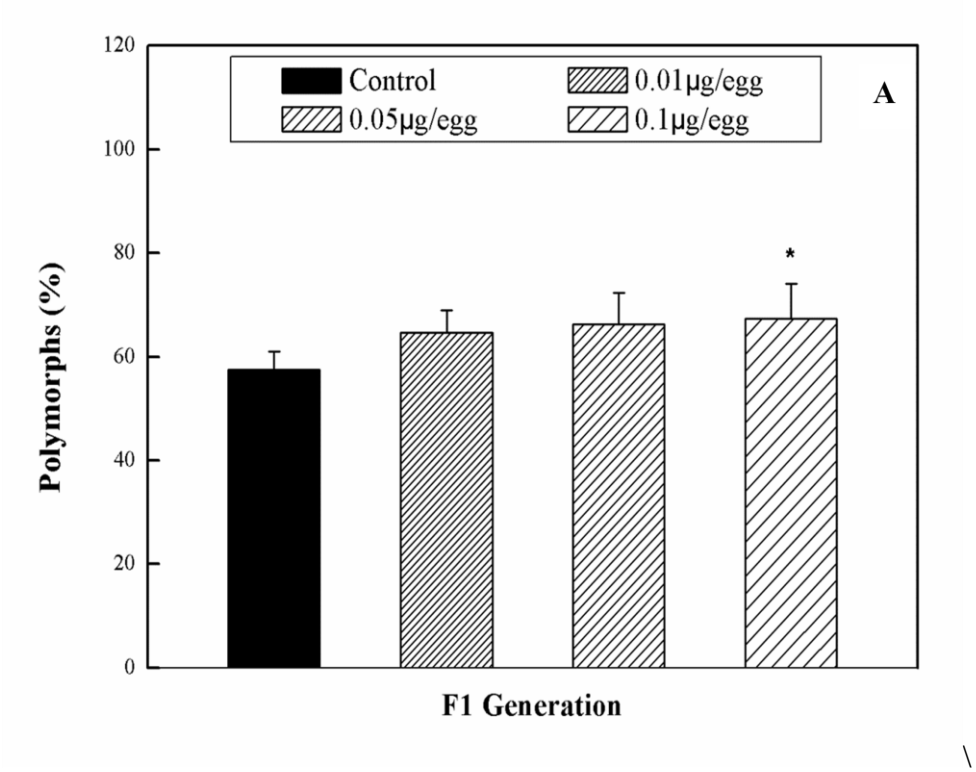


Figure 2.4: Polymorphs in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci



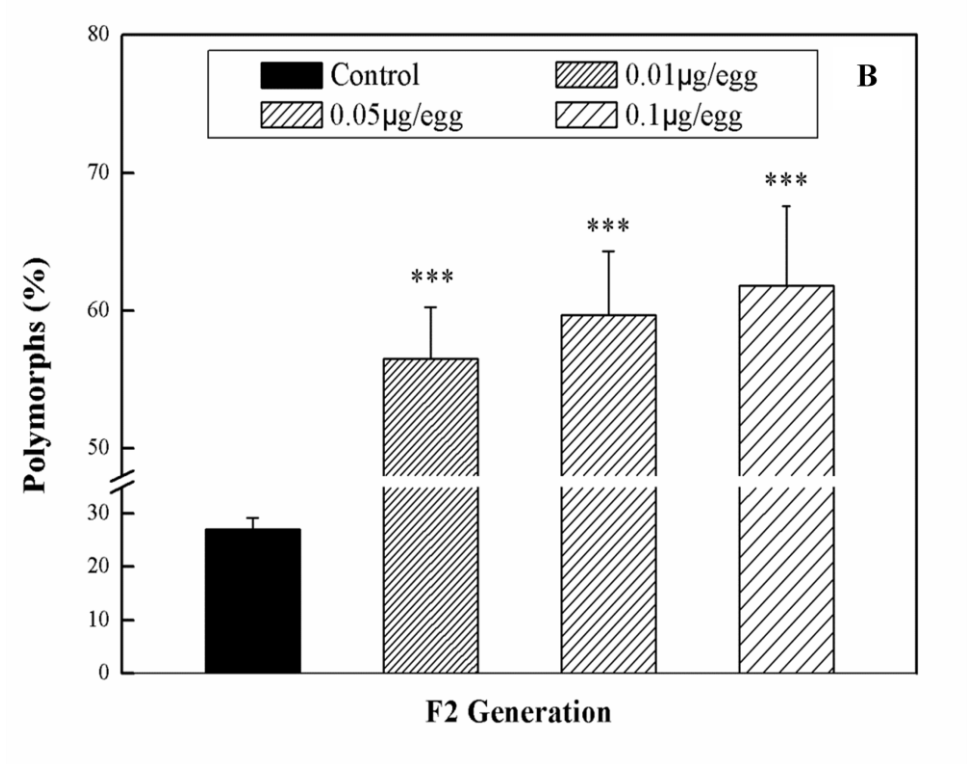


Figure 2.5: Lymphocytes in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci

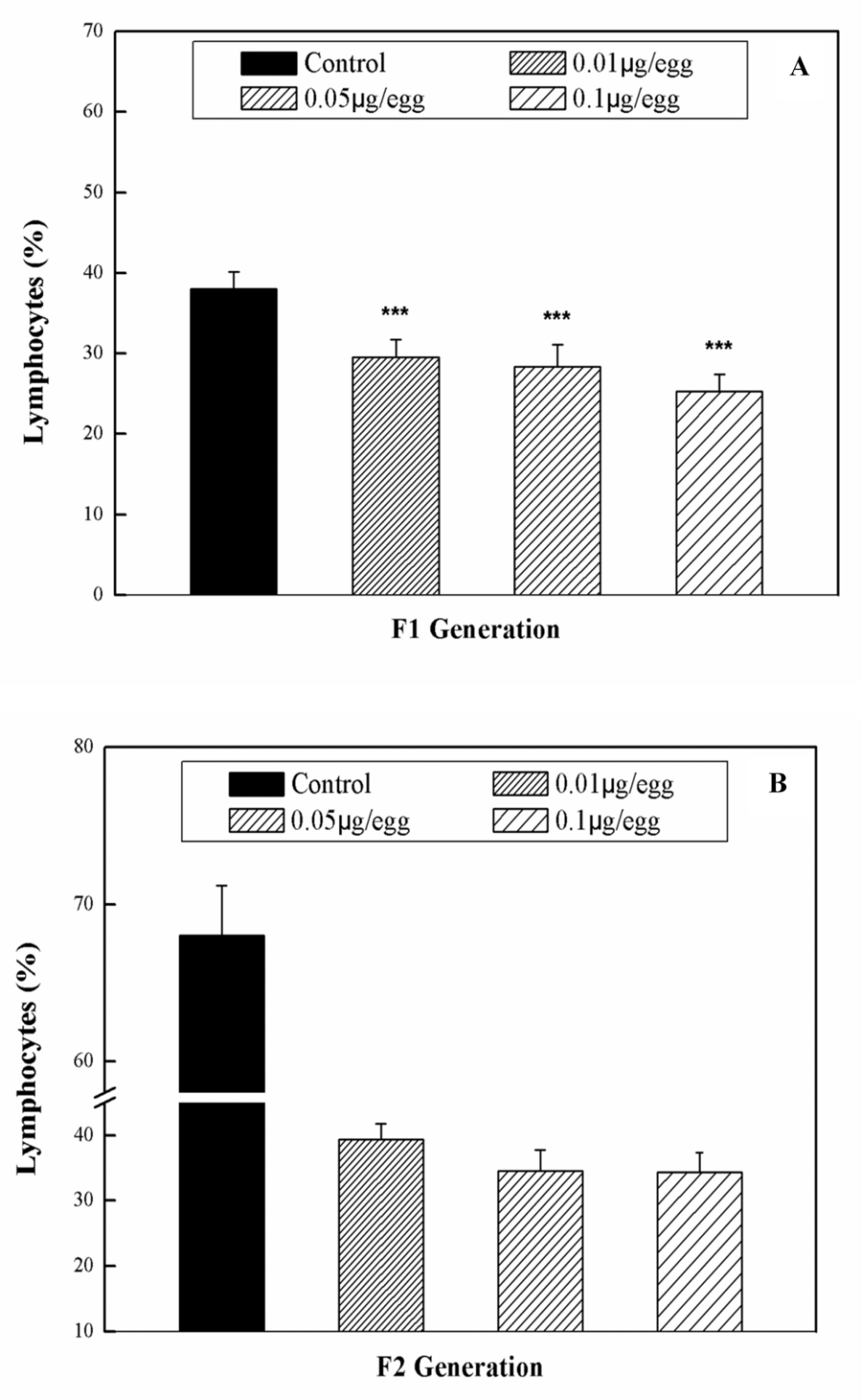
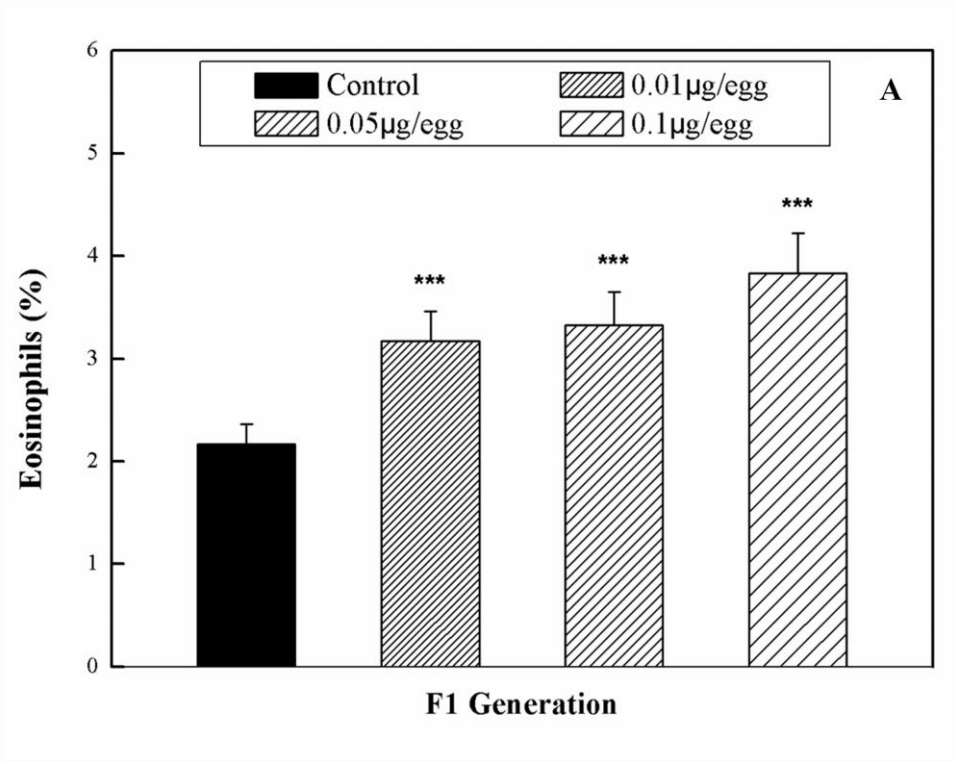


Figure 2.6: Eosinophils in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci



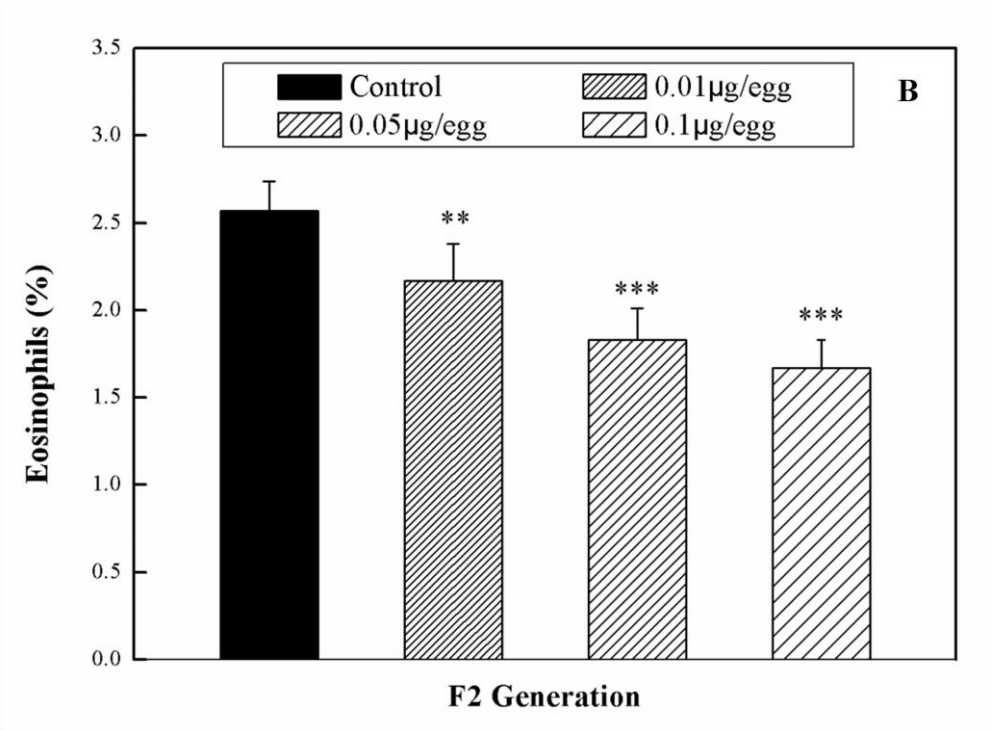
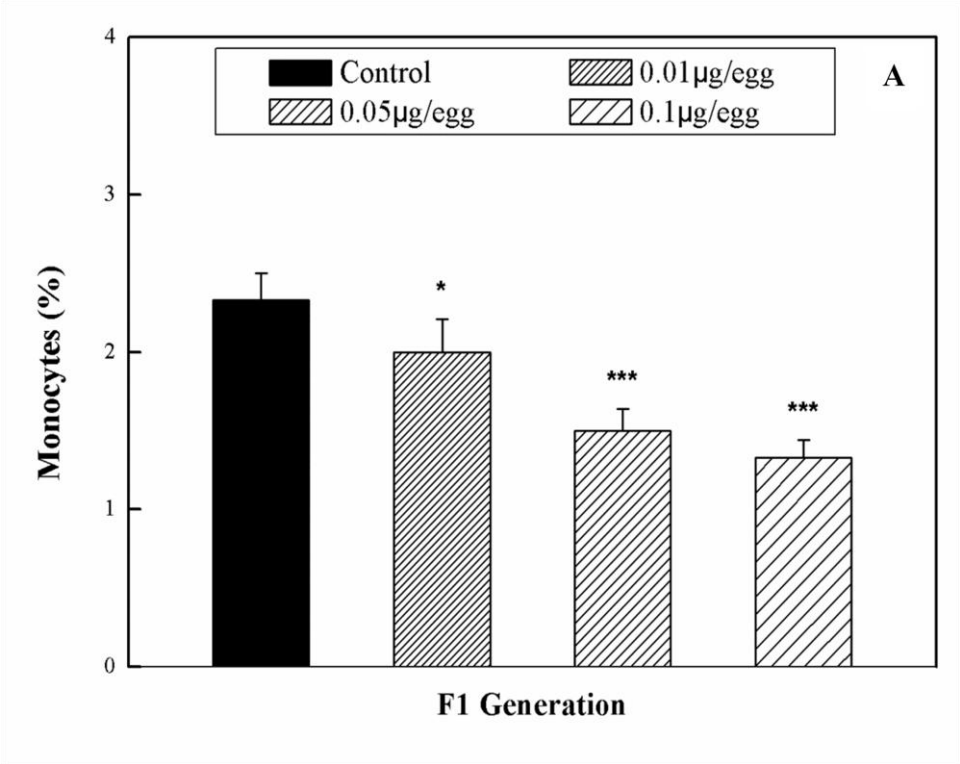


Figure 2.7: Monocytes in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci



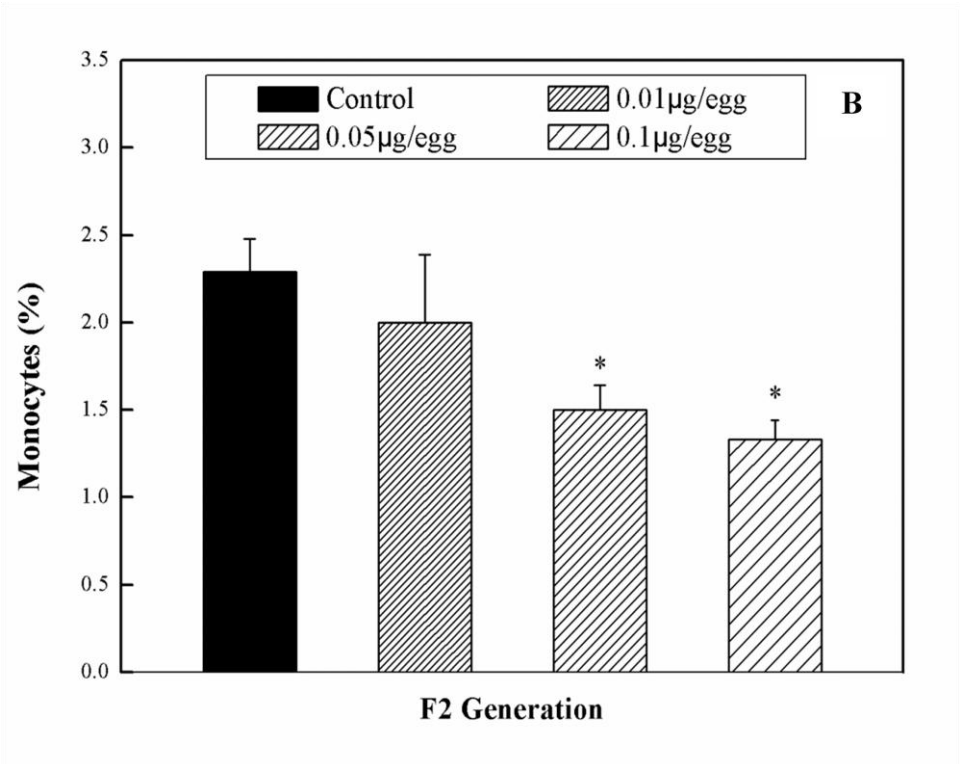
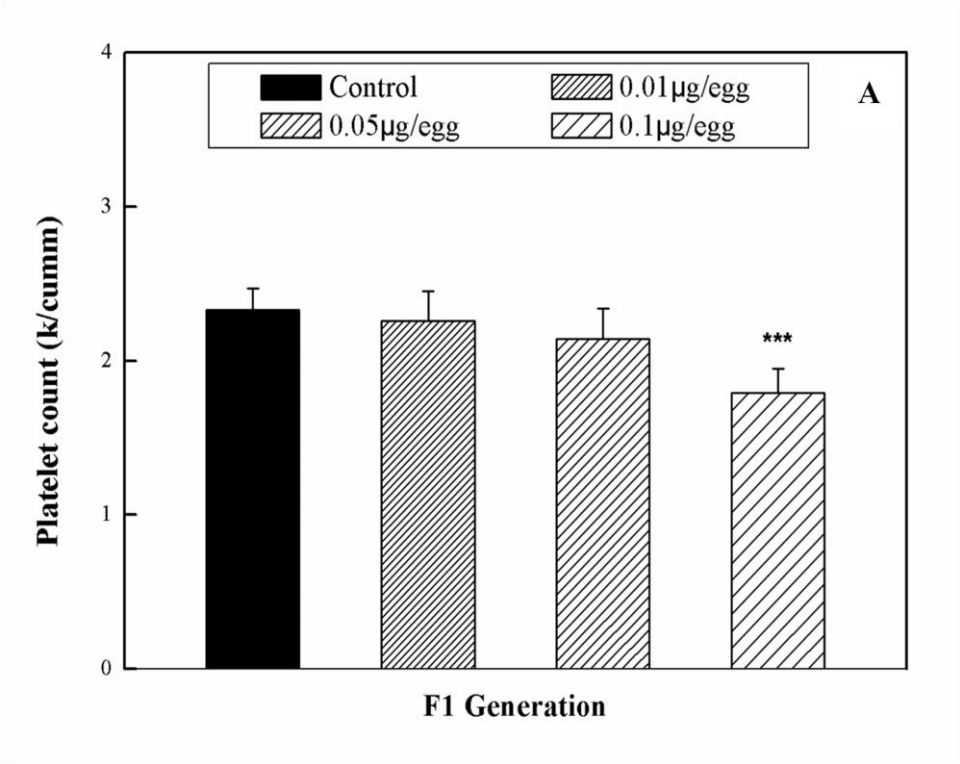
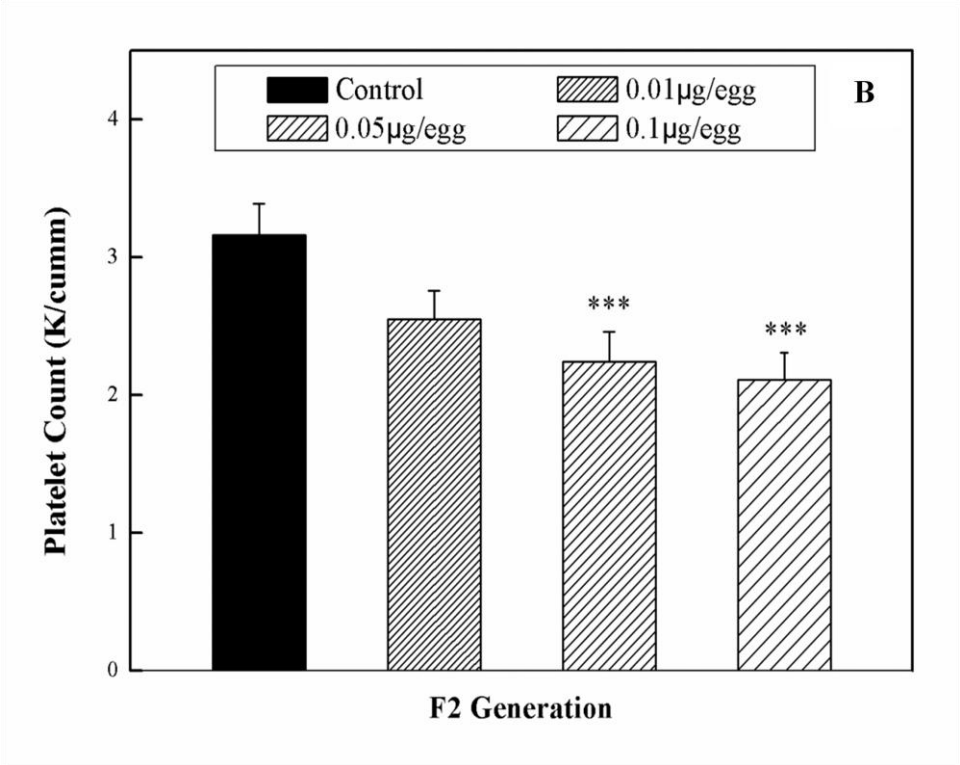


Figure 2.8: Platelet count in F1 (A) and F2 (B) generation fowls subjected to various doses of Ci





DISCUSSION

A scan through the literature has revealed that the pesticide intoxication often results in altered hemogramme. According to earlier reports, variations in the haematological parameters were observed as a result of cypermethrin toxicity (Morgan *et al*., 1980) and chlorpyrifos toxicity (Barna-Lloyd *et al*., 1991; Goel *et al*., 2006; Ambali, 2009) where they reported a decrease in RBC count, Hb and PCV levels. Tung *et al*., 1975, opined that these changes could be due to the destruction of RBCs with integral disability, which are formed by a hyperactive bone marrow. Moreover, the decrease in RBC counts is reported to be symptomatic of erythrocyte damage or erythrocyte formation (Shakoori *et al.,* 1990). It has been documented that the poisoning by pesticide residues results in anaemia due to interference of haemoglobin biosynthesis and shortening of the lifespan of circulating erythrocytes (Betrosian *et al*., 1995; Jyotsna *et al*., 2003). Furthermore, cypermetrin is known to get accumulated in cell membranes and hence, disturb the membrane integrity which ultimately leads to the lysis of erythrocytes resulting in erythrocytopenia (Michelangeli *et al*., 1990). The low Hb concentration observed in the chicks from the Ci treated group may be attributed to the enhanced Hb destruction or declined Hb formation as opined by several earlier workers (Moss and Hathway, 1964, Vural *et al*., 1986). However, hyperactivity of bone marrow or rupture and destruction of red blood cells might also contribute to the induction of anaemia in case of pesticide poisoning (Barger, 2003).

An increase in MCV and decrease in MCH was observed in all the treated groups of F1 generation compared to their control group indicating macrocytic and hypochromic anemia. Barger, (2003), while explaining the reasons for macrocytic and hypochromic anemia, cited hyper activity of bone marrow or deficiency of some haemopoietic factors as the possible causative factors and a same can be applied to explain the pathology observed in the current study as well. The increase in MCV indicates presence of abnormally large sized erythrocytes (Latimer *et al*., 2004). Increased MCV may also be observed in regenerative anemia due to hemolysis and haemorrhages. However, the F2 generation has not shown any significant changes in Hb, MCV and MCH, though there was a reduction in the total RBC count. Generally, a decline in total red blood cell counts along with a decrement in PCV, while the MCV, MCH and MCHC being normal, indicates that the anaemic condition is due to hampered erythropoiesis. Though direct cytotoxicity and/or internal haemorrhages inflicted by the insecticide might also result in erythrocytopenia, their contribution in the present case

of F2 generation is least likely. There are reports that when internal haemorrhages occur, the erythrocytes are absorbed by the lymphatic vessels or are lysed or phagocytosed (Latimer *et al*., 2004) and hence lead to a reduced RBC count.

Here in this study leucopenia has been recorded in F1 generation the same alteration was previously reported in chicks treated with the pyrethroid compound fenvalerate (Garg *et al*., 2004) and the organophosphates chorpyrifos and methidathion (Ojezele and Abatan, 2009). Further, the decrease in total white blood cell count and lymphocytes in 0.05 and 0.1µg/egg of Ci treated groups may indicate adversely affected lymphoid progenitors. The heterophilia observed in 0.1µg/egg of Ci treated group, might be an immune response to a metabolic or chemical poisoning and /or tissue necrosis. Similar observations were made by Cho *et al*. (1989), who found lymphocytopenia and neutrophilia in rats exposed to subchronic doses of organophosphate. It is well known that the number of circulating lymphocytes in peripheral blood is an index of functional ability of lymphoid organs and the lymphocytes are the main agents of immunogenic response in birds as well as mammals. Thus, a reduction in the number of total lymphocyte may be an indication of compromised immunocompetence of the intoxicated birds. In the F2 generation, though the total WBC counts seemed unaltered, heterophilia and lymphocytopenia were observed, which vividly depicts subdued immunocompetence. Gowri and coworkers (2010) have reported that Ci treatment induces immunotoxicity in chicks of F1 generation. This report consolidates the present notion of reduced immunocompetence even in second generation as evidenced by the lymphocytopenia.

Further in the present study, noteworthy decline in platelets are seen in chicks from all the three treatment groups which can be attributed to the Ci induced compromised redox physiology. Araujo and colleagues (2008) have reported oxidative stress as a possible mediator of thrombocytopenia in malaria patients. This report gives credence to our notion of reactive oxygen species as a possible reason for thrombocytopenia in Ci intoxicated chicks. Finally, since all the selected doses induced toxicity to the chicks in a dose dependent manner it could be postulated that NOAEL dose should be less than 0.01µg/egg.

In conclusion the study reveals the susceptibility of external toxic substances to not only to the adult animals but also to the descendents. The toxicants influenced the health of parent generation when dosed during their in-ovo development to a great extent and also hampered the embryonic development of the next generation. The haematological analysis divulged that

the F2 generation animals had compromised immunity compared to the animals receiving the pesticide dose directly. This finding underlines the need for screening of the known developmental toxicants for more than one generation to garner the much needed evidences behind many mysterious human diseases about which the aetiology is still at large.