CHAPTER 3

HAEMATOLOGICAL AND BIOCHEMICAL CHANGES IN INSECTICIDE INTOXICATED DAY OLD HATCHLINGS AND EARLY EMBRYOS

INTRODUCTION

Several of the toxic chemicals have become an integral part of the ecosystem and pesticides are one among them. With the introduction of many pesticide regulation acts and the environmental protection agencies, there has been an increased awareness regarding the pros and cons of the pesticide usage. Although efforts are being made to make the newer class pesticide chemistries more selective to insects and less persistent than many of the older insecticides, they still are hazardous when used inappropriately. The risk gets amplified when developing organisms get prone to these exposures. In the developing embryo the various organ systems and their functionality are yet incomplete and may fall an easy prey to xenobiotics, at far lower levels which do not show noticeable maternal toxicity (**Slotkin, 2004**). The adverse outcomes of these exposures may manifest as congenital birth defects or be latent and get expressed in the later part of the life.

Evaluation of the toxic effects induced by pesticide exposure is done by several methods. The haematological and biochemical evaluations are often used to take a preliminary picture of the toxicosis. These estimations have been widely used in the diagnosis of a variety of diseases or pathologies induced by industrial compounds, drugs, dyes, heavy metals and pesticides by several researchers (**Morgan and Stockdale, 1980; Ali *et al.*, 1988; Ali and Shakoori, 1990; Mansour and Mossa, 2005**). An evaluation of the haematological parameters can be related to the prevailing physiological condition including the immune status of the organism, while the monitoring of various enzyme activities (like ALT, AST, ALP, etc) in the serum would reveal a normalcy or injury to the detoxifying organ i.e. the liver.

OBJECTIVE

The results of Chapter 1 have given a clear depiction of the various teratogenic effects the insecticides would impose on to the developing embryos. However, many a times the

manifestations are not visible as morphological anomalies. Rather, the competence of a surviving organism may be altered by various changes in the physiological functioning. The blood picture and the enzyme activities often give important preliminary clues as to which internal tissue function has suffered a setback and to what extent. Therefore, the haematological and biochemical parameters were evaluated in this part of the study.

MATERIALS AND METHODS

The embryos were dosed with 0.01, 0.05 and 0.1µg/egg of Ci and the vehicle control was dosed with corn oil (VC1) on zero day of incubation; while the Sp was dosed as 0.15, 0.75 and 1.50mg/egg and vehicle control was dosed with 0.4% methylcellulose (VC2). The dose volume was 50µl/egg. The haemato-biochemical analyses were performed on day old hatchlings. Also, a few biochemical tests were conducted on the early embryos (8 day old). A detailed description of the following methods was given elsewhere under the title materials and methods.

Haemato-Biochemical Analysis

Blood samples were drawn from day old hatchlings by cardiac puncture using EDTA rinsed 2 ml disposable syringes and collected into EDTA rinsed or heparinised vials. After collection, the samples were used immediately or refrigerated and processed within 6hr.

Haematological Estimations

For both **total red blood cell count** (TRBC) and **total white blood cell count** (TWBC), the whole blood was diluted using Natt and Herrick's solution (**Natt and Herrick, 1952**) in blood-cell dilution pipettes. The TWBC and TRBC were done with the help of a Neubauer haemocytometer, as per **Campbell, 1995**. The **differential white cell count** was determined by fixing and staining air-dried blood smears with Wright stain. The **haemoglobin** (Hb) content was determined using the Drabkin's technique (**Drabkin, 1945; Henry, 1974**). **Packed cell volumes** (PCV) were determined by centrifugation of whole blood in microhaematocrit tubes at 3,000*g* for 5 min. **Mean corpuscular volume** (MCV), **mean corpuscular haemoglobin** (MCH) and **mean corpuscular haemoglobin concentration** (MCHC) were calculated as per **Campbell, 1995.**

Biochemical parameters

Glucose was estimated by the GOD/POD method (**Trinder, 1969**; **Henry *et al.*, 1974**).

Serum albumin (Rodkey, 1965; Doumas *et al.*, 1971) was estimated by the BCG method.

Serum globulin was obtained by subtracting the value of serum albumin from the total serum protein.

Serum protein was estimated using the **Bradford's assay (Bradford, 1976).**

Alkaline phosphatase (ALP) activity was estimated by the method of **Kind and King, (1954).**

Alanine aminotransferase (ALT) or serum glutamate pyruvate transaminase (SGPT) activity was estimated by the **IFCC Method (1974).**

Aspartate aminotransferase (AST) or serum glutamate oxaloacetate transaminase (SGOT) activity was determined by **IFCC Method (1986).**

Blood Urea Nitrogen (BUN) was estimated by the enzymatic UV- kinetic initial rate method **(Gutmann and Bergmeyer, 1974; Sampson and Baird, 1979).**

Acetylcholinesterase (AChE) and **butyrylcholinesterase** (BChE) activities were estimated in the serum, liver and brain homogenates as per **Ellman *et al.* (1961) and Gorun *et al.* (1978).**

Estimations on 8th day post incubation

The control and pesticide dosed embryos were collected on day eight of incubation and homogenates of embryonic brain were prepared. Acetylcholinesterase activity, NA^{+}- K^{+} ATPase activity, DNA and RNA content were estimated.

Acetylcholinesterase activity in the embryonic brain was estimated by microplate method **(Ellman *et al.*, 1961; Lesser *et al.*, 2000; Steevens and Benson, 1999).**

Protein Estimation of the embryonic homogenate was determined by microanalysis modification of **Bradford's method (1976)** using Coomassie blue stain.

Nucleic acid content was quantified as per **Labarca and Paigen (1980)** in the embryonic homogenates.

Embryonic homogenates were analyzed for Na^+- K^+ **ATPase** activity by the method of **Post and Sen, 1967**.

RESULTS

Haematological estimations

At 0.01 µg/egg of Ci, the hatchlings did not show any changes in terms of TRBC and TWBC in the blood, when compared to the concurrent controls. The blood picture showed a decline ($p \leq 0.05$) in the total number of red blood cells as well as white blood cells in hatchlings dosed 0.05µg/egg of Ci. With a dose of 0.1µg/egg of Ci, there was a decrement in the number of RBC and WBC count which was even more significant ($p \leq 0.01$) than with 0.05µg/egg of Ci (**Table 3.1**) treatment.

The differential counting of the white blood cells showed that, there were no significant changes when treated with 0.01µg/egg of Ci. The hatchlings of the 0.05µg/egg of Ci treatment group showed no change in the heterophil number but the lymphocyte number was significantly lowered ($p \leq 0.05$). On the contrary, the treatment with 0.1µg/egg of Ci brought about a highly significant increase ($p \leq 0.01$) in the heterophil number and a highly significant decrease ($p \leq 0.01$) in lymphocyte number. The eosinophils, basophils and monocytes in all the three groups of Ci treatment did not undergo any significant treatment related changes (**Table 3.1**).

The haemoglobin content in the chicks of 0.01µg/egg of Ci treatment group was in the normal range (relative to the control groups). With a dose of 0.05µg/egg of Ci, the haemoglobin levels were significantly lowered ($p \leq 0.05$) and the treatment with 0.1µg/egg of Ci induced severe anaemia ($p \leq 0.01$) in the hatchlings. The MCV, MCH and MCHC values in the three Ci treated groups showed no alterations, however, the PCV in 0.1µg/egg of Ci treatment group was significantly lower ($p \leq 0.05$) than the chicks of the control group (**Table 3.1**).

Analysing the results of Spinosad treatment (**Table 3.2**), the 0.15mg/egg of Spinosad treatment did not evoke any significant changes in any of the haematological estimations

performed in the present study. The dose of 0.75mg/egg of Spinosad lead to an increase ($p \leq 0.05$) in the number of WBC while rest of the haematological counts i.e. TRBC, DC, Hb, PCV, MCV, MCH and MCHC showed no biologically significant variation. The 1.5mg/egg of Spinosad treatment however lead to a decrease ($p \leq 0.05$) in TRBC and TWBC. Also, the lymphocytes were found to be in low numbers ($p \leq 0.05$) compared to the control group. While Hb, PCV, MCH and MCHC values showed no change, the MCV values in 1.5mg of Spinosad treatment showed a significant increase ($p \leq 0.05$).

Biochemical estimations

The embryos treated with 0.01µg/egg of Ci after hatching showed no significant changes compared to the control group after analysing the results of the biochemical estimations in the present study, i.e. serum glucose, albumin, globulin, total protein, ALP, ALT, AST, BUN and BChE activity (**Table 3.3**). However, the dose of 0.01µg/egg of Ci led to a decrease ($p \leq 0.05$) in brain AChE activity while the values were within the normal range in serum and liver. The dose of 0.05µg/egg of Ci showed an increase ($p \leq 0.05$) in serum glucose, ALP and ALT. The AChE and BChE activities in the serum, liver and brain showed a significant decrement ($p \leq 0.05$), and of these, the most significant decrease was sighted in the AChE in brain ($p \leq 0.01$). The 0.1µg/egg of Ci treatment to embryos made the hatchlings show a significant alterations in all the biochemical estimations made. There was an increase in glucose content ($p \leq 0.01$) and a decrease in albumin ($p \leq 0.05$), globulin ($p \leq 0.01$) and total protein ($p \leq 0.01$). The enzyme activities of ALP ($p \leq 0.01$), ALT ($p \leq 0.01$), AST ($p \leq 0.05$) (**Figure 3.1**) and BUN ($p \leq 0.05$) were also present in a range higher than the control chicks. The AChE as well as BChE activities were all lowered in serum, liver and brain (**Figure 3.2a and 3.2b**). Amongst the three tissues tested, the decrease in AChE activity in brain was highly significant ($p \leq 0.001$) followed by liver ($p \leq 0.01$) and then serum ($p \leq 0.05$). The decline of BChE was highly significant ($p \leq 0.01$) in serum and liver and followed by the brain ($p \leq 0.05$).

The estimated biochemical parameters in the 0.15mg/egg Sp treated group showed no biologically significant variation from control group. The 0.75mg treatment showed an increase in ALP ($p \leq 0.05$) and a decrease ($p \leq 0.05$) in AChE activity in serum, liver and brain; while the BChE activity was found to be lowered ($p \leq 0.05$) only in serum and liver, but not in brain. With the dose of 1.50mg/egg of Spinosad to embryos, the day old chicks showed elevated levels of glucose ($p \leq 0.05$) and a decline in globulin ($p \leq 0.01$) and total protein ($p \leq 0.05$). The enzyme activities too were disturbed at this dose i.e. the ALP ($p \leq 0.01$), ALT

($p \leq 0.05$), AST ($p \leq 0.05$) and BUN ($p \leq 0.05$) activities showed a significant raise; the serum, liver and brain AChE activities were found decreased, with the highest significance in decline shown by the brain ($p \leq 0.01$). The BChE activity also got lowered ($p \leq 0.05$) in serum, liver and brain (**Table 3.4, Figure 3.3, 3.4a and 3.4b**).

Early embryonic Estimations

On day eight, the brain homogenates of all the Ci treated embryos showed a fall in AChE activity in a dose dependent manner i.e. the highest variation shown by the group given the highest concentration of Ci, i.e. 0.1μg/egg ($p \leq 0.001$) and then 0.05 and 0.01 μg/egg (**Table 3.5, Figure 3.5**). The Sp at a concentration of 0.15 and 0.75mg/egg induced no comparable changes in the AChE activity. However, 1.50mg/egg of Spinosad brought about a highly significant decrease ($p \leq 0.01$) in AChE activity in the brain (**Table 3.5, Figure 3.6**).

On estimating the nucleic acid content (**Table 3.5, Figure 3.7 and 3.8**), no significant changes occurred when the DNA and RNA in embryos treated with 0.01μg/egg of Ci; however, at 0.05 and 0.1μg/egg of Ci the embryos had a low content of both DNA as well as RNA ($p \leq 0.05$). The Spinosad treatment at a concentration of 0.15 and 0.75mg/egg brought about no changes in the amount of DNA and RNA (**Figure 3.9 and 3.10**) in the 8 day embryos while with 1.5mg of Spinosad the embryos had a decreased quantity of both DNA and RNA ($p \leq 0.05$).

The Na^+-K^+ ATPase activity (**Table 3.5, Figure 3.11**) in the embryos given 0.01μg/egg of Ci was within a normal range relative to the control group of embryo; while the activity of the same was found elevated in embryos treated with 0.05 ($p \leq 0.05$) and 0.1μg/egg ($p \leq 0.01$) of Ci. The Spinosad at a concentration of 0.15 and 0.75mg did not make the embryos susceptible to changes in Na^+-K^+ ATPase activity (**Figure 3.12**), while a significant increase was found in the same with a dose of 1.5mg/egg ($p \leq 0.01$).

DISCUSSION

Haematological estimations

The haematological parameters in hatchlings of 0.05 and 0.1μg/egg of Ci treated groups varied significantly with that of the control chicks. The PCV denotes the volume occupied by the RBC in the whole blood. The MCV reflects the size of the RBC while the MCH and MCHC reflect the Hb content of the RBC. 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, cytotoxicity and/or internal haemorrhages inflicted by the insecticide. When internal haemorrhages occur, the erythrocytes are absorbed by the lymphatic vessels or are lysed or phagocytised (**Latimer *et al.*, 2003**) and hence lead to a reduced RBC count. Therefore, the reduction in the total red blood cell count, the Hb content and the PCV, while the MCV, MCH and MCHC being normal (in 0.1μg/egg of Ci treatment) here in this study could be due to cytotoxic nature of the insecticide or its effect on the erythropoiesis. Chlorpyrifos as well as cypermethrin toxicities were individually studied earlier in rats (**Barna-Lloyd *et al.*, 1991; Goel *et al.*, 2006; Ambali *et al.*, 2007, 2010**) and chicks (**Qadri *et al.*, 1987**) and were shown to be lowering the values of RBC, Hb and PCV. It was opined that both chlorpyrifos (**Ambali *et al.*, 2007, 2010**) and cypermethrin (**Spiteller, 1996**) induce oxidative stress and thereby pose a cytotoxic effects on the RBC through increased lipoperoxidative changes which finally lead to the erythrocyte membrane fragility. **Michelangeli *et al.*, (1990)** have accounted the hydrophobic nature of the cypermethrin to be responsible for creating disturbances in the membrane structures. 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 effected 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 as the organophosphate induced toxicity in rats. The number of circulating lymphocytes in peripheral blood is an index of functional ability of lymphoid organs and lymphocytes are the main agents of immunogenic response in birds and mammals. Thus, a reduction in the number of total lymphocyte may be an indication of lowering of the immunocompetence of the animals. The dose of 0.01μg/egg of Ci showed no effect on the hatchlings haematological parameters which mean that the chick embryos have shown a tolerance to this dose of Ci.

The haematological parameters of the hatchlings were unaffected by the treatment of eggs with 0.15mg of Spinosad. Significantly reduced levels of haemoglobin were observed in the 1.50mg treatment group. This was recognized as macrocytic hypochromic anaemia, since the MCV was significantly higher, while PCV was unaffected. According to **Barger (2003),** increased activity of bone marrow or haemolysis could lead to impaired Hb synthesis, which results in macrocytic hypochromic anaemia. With the dose of 0.75mg/egg, an increase in TWBC occurred, which might be due to an immune response developed by the chicks against

the Spinosad toxicosis. Further, when the dose was increased to 1.5mg of Spinosad per egg, the occurrence of leukocytopenia and lymphocytopenia might have resulted due to deficits in the precursor cell and/or cytotoxic effect of the insecticide. The Spinosad was earlier reported to have induced haemato-toxicological changes in rats (**Yano *et al.*, 2002; Mansour *et al.*, 2007**) and now in the present study also, it showed similar effects in chicks treated at embryonic stage with a dose of 1.5mg/egg.

Biochemical estimations

Abnormal glucose metabolism in laboratory animals when exposed to the pesticides was reported several times (**Dybing and Sognen, 1958; Matin and Siddiqui, 1982; Fletcher *et al.*, 1988**). In the present study also, hyperglycaemia was observed in the 0.05 and 0.1µg/egg of Ci treatment groups. In a review by **Rahimia and Abdollahi (2007)**, it was discussed that the organophosphate induce hyperglycaemias by several mechanisms including physiological stress (**Clement, 1985; Fletcher, 1988**), oxidative stress, inhibition of cholinesterase of the central or peripheral synapses that act in endocrine regulation of glucose metabolism (**Kant *et al.*, 1988; Matin and Siddiqui, 1982**) and by causing disturbances in metabolism of liver (**Joshi and Rajini, 2009**). Cypermethrin was also reported to induce hyperglycaemia in rodents (**Lock and Berry, 1981; Shakoori *et al.*, 1988; Manna *et al.*, 2004**) and chicks (**Qadri *et al.*, 1987**). Also, treatment with 1.50mg/egg of Sp showed an increase in serum glucose. Therefore, embryonic exposure to Spinosad might also lead to physiological disturbances pertaining glucose metabolism. However, in another study by **Stebbins *et al.* (2002)**, a 13-week dietary treatment of 0.12% Spinosad lead to a decrease in the mean glucose values in the CD-1 mice.

Proteins are the most abundant components of the serum. The albumins and globulins are the major groups of serum proteins. The albumins are synthesized by the liver and are responsible regulating the blood volume maintaining the osmotic pressure. The globulins include gamma globulins (antibodies) and a variety of enzymes and carrier or transport proteins. Antibodies are produced by mature B lymphocytes called plasma cells, while most of the other proteins are made in the liver. Damage to the liver or reduction in its synthesis by the plasma cells or decrease in number of plasma cells might have led to the decline in the globulin and consequently, the albumin and total protein were reduced in the Ci (0.1µg/egg) treated chicks.

Serum enzymes like AST, ALT, and ALP represent the functional status of the liver. Chemical-induced cellular alteration varies from simple increase of metabolism to death of cell. The increase or decrease of enzyme activity is related to the intensity of cellular damage. A report by **Manna *et al.*, (2004)** described the increased levels of the serum enzymes ALP, ALT and AST to be attributed to the damage caused to the liver due to the liberation of free radicals via esoteric and oxidative pathways by the cytochrome P450 microsomal enzyme system 14 during the pyrethroids metabolism in the liver. Therefore a marked increase of these enzymes in the present study could be a sign of Ci (at a dose of 0.05 and 0.1µg/egg) induced toxicosis in the hatchlings' liver. The Spinosad however, showed an increase in 1.50mg/egg dosed group, though with a dose of 0.75mg/egg, only ALP was increased, ALT and AST were comparable to the controls. Further, the blood urea nitrogen is a measure of the amount of urea nitrogen in the blood. Urea is cleared from the bloodstream by the kidneys. Therefore a test measuring how much urea nitrogen remains in the blood can be used to test the renal functioning, as it reflects the changes in glomerular filtration. However, there are many factors besides renal disease that can cause BUN alterations, including protein breakdown, hydration status, and liver failure. In the present study only the highest tested doses of both Ci and Sp (0.1µg/egg Ci and 1.5mg/egg Sp) showed a significant increase in the BUN value indicating a possible alteration in the renal function.

The enzyme acetylcholinesterase is abundant in central and peripheral nervous system and is localized in many neuronal perikarya as well as fibres (**Christoph *et al.*, 1992**). Its function at the post synaptic receptors is to hydrolyse and remove the ACh and ultimately initiate an action potentials at precise, exact intervals. The organophosphates are generally very long or even irreversible inhibitors of acetylcholinesterase (**Scharf, 2003**). Inhibition of AChE after an exposure to the organophosphates was extensively reported in various experiments with rodents and aves (**Garcia-Cabero *et al.*, 1998; Abu-Qare *et al.*, 2001; Ashry *et al.*, 2002**). In both aves and mammals, the inhibition of brain cholinesterase levels is a reliable biomarkers for organophosphate exposure (**Lesser *et al.*, 2000**). The pyrethroids act on the voltage gated sodium channels. However, former studies have shown that cypermethrin treatment also results in a decline in the AChE activity in rats and fish (**Reddy *et al.*, 1991; Kale *et al.*, 1999**). **Latuszynska *et al.* (2001)** reported that a Nurelle D 550 (Commercial formulation of chlorpyrifos and cypermethrin) induced an inhibition of acetylcholinesterase in blood and brain of the Wistar rats. The results of the present study are in agreement with the above reports, as 0.05 and 0.1µg/egg of Ci led to decline of AChE activity in serum, liver

and brain. **Ludke *et al.*, 1975** demonstrated that a decline in the brain AChE activity less than 80% of normal indicates exposure to an anti-cholinesterase compound, whereas activities below 50% of normal indicate potentially lethal intoxication. For avians, brain AChE levels are more reliable than blood AChE because concentrations of AChE in blood vary greatly with species and are less reliable for predicting nervous system AChE activities than in mammals (**Mortensen *et al.*, 1998**). Sp treatment also led to a lowered AChE activity, though less intense than in the Ci treatment group. Similar results indicating the potential of Spinosad to decrease the acetylcholinesterase activity was reported by **Mansour *et al.* (2008b)** in rats.

Early embryonic Estimations

The results here indicate that the Ci as well as Sp induces changes in the early AChE activities of the embryos, though the Ci being highly potent inhibitor than Sp. The AChE inhibition at an early stage during development would mean more than the disruption of the classical regulation of neurotransmission. **Koenigsberger *et al.*, (1997, 1998)** demonstrated that the disruption of cholinesterases in the developing axons of rat leads to a faulty axonogenesis. Further evidences were provided to the facts that, developmental exposure to cholinesterase inhibitor like chlorpyrifos, disrupt the fundamental process of brain development such as DNA synthesis (**Dam *et al.*, 1998; Whitney *et al.*, 1995**), expression and function of macromolecular constituents and transcription factors that control cell differentiation (**Johnson *et al.*, 1998; Crumpton *et al.*, 2000; Garcia *et al.*, 2001; Schuh *et al.* 2002**). In the investigation here, the Ci treatment at a concentration of 0.05 and 0.1µg/egg showed significant disturbances in the synthesis of DNA and RNA in the brain, while the Spinosad showed lowered DNA and RNA quantity only after a treatment with 1.5mg/egg. Nonetheless, considering the above discussed non-enzymatic roles of the acetylcholinesterase, it could be presumed that the disruption of the acetylcholinesterase might have further lead the deficits in the nucleic acid content. Another consideration in a developing embryo is rapid increase in the cell populations, for which protein biosynthesis associated with the nucleic acids plays a key role. Hence nucleic acids could be sensitive indices to access the biochemical changes (**Pushpanjali *et al.*, 2005**) in response to the insecticide toxicity. **Pushpanjali *et al.* (2005)** observed a decrease in the DNA and RNA content in the chick embryonic brain after treating them with endosulfan while **Pal and Kushwah (2000)** reported a decrease in DNA and RNA in the brain and liver of chick embryo. Contrastingly, increased hepatic DNA and RNA in chick following penchlorophos

and heptachlor intoxication were noted by **Manciulea and Giurgea (1976)**. The differences in observation may be due to differential action of different types of pesticides in embryonic stages.

Na^+-K^+ ATPase is an integral membrane bound hydrolytic enzyme concerned with immediate release of energy. Recent studies focus on the role of Na^+-K^+ ATPase in signal transduction (**Liu *et al.*, 2000; Xie, 2001**). The increase in the activity of Na^+-K^+ ATPase in the Ci and Sp dosed embryos could be due to solubilisation of the membrane protein as a result of structural and functional damage to the plasma membrane. The organophosphates as well as pyrethroids are known inducers of lipid peroxidation, which might have led to loss of membrane integrity and release of the Na^+ K^+ ATPase (**Datta *et al.*, 1994; Bagchi *et al.*, 1995; Rauchova *et al.*, 1995; Kale *et al.*, 1999**).

SUMMARY

Analysing the results of the haematological and biochemical parameters in the early embryos and in the hatchling, it can be derived that the Ci treatment at a concentration 0.05 and 0.1µg/egg lowered the DNA and RNA content, thereby indicating a hampered protein synthesis. This might also imply an imbalance in the making up and functioning of certain growth factors and signalling protein. Even minute change in these early molecules might lead to large functional changes. For example, evidences support that AChE has extra-synaptic and non-cholinergic roles during embryonic development (**Bigbee *et al.*, 1999**). The *in vitro* as well as *in vivo* studies have shown that AChE takes up a morphogenic role, and its expression is correlated with the neurite outgrowth (**Cochard and Coltey, 1983; Bigbee and DeVries, 1987; Dupree and Bigbee, 1994**). The embryonic exposure to the insecticide in this study has been carried out at an early stage before the organogenesis, therefore the haematological and the biochemical alterations might as well mean a disruption of terminal differentiation and/or debilitation of the functional capabilities of the organ systems other than the direct effects i.e. cytotoxicty or lipid peroxidation. The Sp also might show similar inflictions since there were alteration in the tested parameters, however the changes were prominent only at the highest of the dose tested. Nonetheless, comparing the doses and effects of the two insecticides, Ci is more hazardous than Sp in terms of inflicting the toxicity even at very low concentrations.

TABLE 3.1 Haematological estimations in Ci treated chicks

Attribute	Treatment (Ci µg/egg)			
	VC1	0.01	0.05	0.10
TRBC (10^6/mm^3)	2.44 ± 0.03[@]	2.42 ± 0.07	1.79 ± 0.18↓*	1.74 ± 0.21↓**
TWBC (10^3/mm^3)	16.42 ± 0.37	16.64 ± 0.43	14.65 ± 0.60↓*	13.71 ±0.44↓**
DC				
Heterophils	26.16 ± 0.60	26.55 ± 0.76	28.66 ± 0.80	30.66 ± 1.08↑**
Eosinophils	02.33 ± 0.42	03.50 ± 0.42	03.16 ± 0.40	03.16 ± 0.47
Basophils	03.83 ± 0.30	04.16 ± 0.30	04.00 ± 0.57	04.00 ± 0.36
Monocytes	06.50 ± 0.50	05.83 ± 0.60	06.00 ± 0.25	05.33 ± 0.49
Lymphocytes	62.33 ± 1.02	59.83 ± 1.66	58.16 ± 0.87↓*	56.66 ± 0.84↓**
Hb (gm/dl)	12.01±0.7	11.29±0.44	9.92±0.23↓*	9.17±0.34↓**
PCV (%)	30.93 ± 0.46	30.02 ± 1.37	29.67 ± 1.64	25.83 ± 1.35↓*
MCV (µ^3)	126.8 ± 3.37	125.3 ± 9.09	174.2 ± 19.03	168.7 ± 25.79
MCH (pg)	49.31 ± 3.04	46.99 ± 3.00	59.52 ± 8.17	57.36 ± 8.34
MCHC (%)	38.82 ± 2.23	37.76 ± 1.19	33.92 ± 1.89	34.45 ± 1.54

[@] Values expressed as mean ± SEM; VC1: Corn oil: ↑Significant increase; ↓ Significant decrease; *p ≤0.05; **p ≤0.01; TRBC-Total Red Blood Corpuscles, TWBC-,Total White Blood Corpuscles, DC-Differential Count, Hb- Haemoglobin, PCV- Packed Cell Volume, MCV- Mean Corpuscular Volume, MCH- Mean Corpuscular Haemoglobin, MCHC- Mean Corpuscular Haemoglobin Concentration.

TABLE 3.2 Haematological estimations in Sp treated chicks

Attribute	Treatment (Sp mg/egg)			
	VC2	0.15	0.75	1.50
TRBC (10^6/mm^3)	2.475 ± 0.05@	2.54 ± 0.05	2.43 ± 0.08	1.95 ±0.21↓*
TWBC (10^3/mm^3)	16.42 ± 0.39	16.11 ± 0.42	18.11 ± 0.32↑*	14.35 ± 0.65↓*
DC				
Heterophils	26.3 ± 1.14	26.00 ± 0.81	24.83 ± 1.30	29.66 ± 0.84
Eosinophils	2.83 ± 0.60	3.33 ± 0.42	3.66 ± 0.42	3.50 ± 0.42
Basophils	3.50 ± 0.42	3.6 6 ± 0.42	3.16 ± 0.40	3.83 ± 0.40
Monocytes	5.83 ± 0.54	5.16 ± 0.54	5.50 ± 0.42	5.16 ± 0.47
Lymphocytes	61.50±0.88	61.83 ±1.13	63.16 ± 0.60	57.83 ± 1.01↓*
Hb (gm/dl)	12.14±0.44	12.15± 0.37	11.65±0.65	10.65±0.32
PCV (%)	30.65 ± 0.36	31.00 ± 1.55	30.18 ± 0.51	30.00 ± 0.65
MCV (μ^3)	124.1 ± 2.82	122.0 ± 2.31	125.0 ± 5.18	163 ± 18.66↑*
MCH (pg)	49.23 ± 2.32	47.83 ± 1.46	48.27 ± 3.39	58.44 ± 7.54
MCHC (%)	39.65 ± 1.65	39.32 ± 1.69	38.62 ± 2.20	35.58 ± 1.27

@ Values expressed as mean ± SEM; VC2: Methyl Cellulose; Sp: Spinosad; ↑Significant increase; ↓Significant decrease; *p ≤0.05

TRBC-Total Red Blood Corpuscles, TWBC-,Total White Blood Corpuscles, DC- Differential Count, Hb- Haemoglobin, PCV- Packed Cell Volume, MCV- Mean Corpuscular Volume, MCH- Mean Corpuscular Haemoglobin, MCHC- Mean Corpuscular Haemoglobin Concentration.

TABLE 3.3 Biochemical parameters in vehicle control and combination insecticide treated groups

Attribute	Treatment (Ci µg/egg)			
	VC1	0.01	0.05	0.10
Glucose (mg/dl)	171.8 ± 4.86[@]	184 ± 11.74	202.7 ± 8.70↑*	213.6 ± 7.08↑**
Albumin (gm/dl)	1.54 ± 0.12	1.51 ± 0.25	1.31 ± 0.17	0.85 ± 0.13↓*
Globulin (gm/dl)	1.98 ± 0.14	2.12 ± 0.18	1.68 ±0.22	1.06 ± 0.15↓**
Protein (gm/dl)	3.53 ± 0.24	3.62 ± 0.31	2.98 ± 0.39	1.92 ± 0.24↓**
ALP (IU/L)	821.3 ± 69.0	1061.1 ± 77.5	1082.6 ± 51.7↑*	1198.78 ±85.2↑**
ALT (IU/L)	2.90 ± 0.56	3.95 ± 0.72	6.54 ± 1.27↑*	8.06 ± 0.73↑**
AST (IU/L)	112.12 ± 8.48	113.41 ± 13.7	139.89 ± 16.2	162.01 ± 12.2↑*
BUN (mg/dl)	0.21 ± 0.03	0.26 ± 0.05	0.33 ± 0.03	0.39 ± 0.05↑*
AChE				
Serum	1.32 ± 0.16	1.10 ± 0.38	0.71 ± 0.14↓*	0.54 ± 0.15↓*
Liver	3.07 ± 0.26	2.39 ± 0.27	1.84 ± 0.31↓*	1.53 ± 0.34↓**
Brain	4.53 ± 0.25	3.11 ± 0.38↓*	2.42 ± 0.33 ↓**	1.83 ± 0.46↓***
BChE				
Serum	4.58 ± 0.32	3.18 ± 0.58	2.67 ± 0.51↓*	2.17 ± 0.42↓**
Liver	3.59 ± 0.29	3.07 ± 0.24	2.41 ± 0.37↓*	2.01 ± 0.25↓**
Brain	0.95 ± 0.09	0.78 ± 0.58	0.61 ± 0.09↓*	0.54 ± 0.10↓*

[@] Values expressed as mean ± SEM; VC1: Corn oil; Ci; ↑Significant increase; ↓Significant decrease; *p value ≤0.05; **p value ≤0.01

ALP- Alkaline Phosphatase, ALT- Alanine aminotransferase, AST- Aspartate aminotransferase, BUN- Blood Urea Nitrogen, AChE- Acetylcholinesterase (µM ATChI hydrolyzed /min/mg protein), BChE- butyrylcholinesterase (µM BTChI hydrolyzed /min/mg protein)

TABLE 3.4 Biochemical parameters in VC2 and Spinosad (Sp) treated groups

Attribute	Treatment (mg/egg)			
	VC2	0.15	0.75	1.50
Glucose (gm/dl)	178.4 ± 5.54[@]	176.9 ± 8.74	192.02 ± 9.10	209.13 ± 4.17↑*
Albumin (g/dl)	1.44 ± 0.13	1.42 ± 0.13	1.37 ± 0.11	1.13 ± 0.22
Globulin (g/dl)	2.16 ± 0.24	2.00 ± 0.21	1.92 ± 0.02	1.02 ± 0.14↓**
Protein (g/dl)	3.60 ± 0.32	3.43 ± 0.31	3.30 ± 0.31	2.15 ± 0.36↓*
ALP (IU/L)	843.16 ± 76.7	870.74 ± 76.2	1099.9 ± 53.6↑*	1176.6 ± 53.8↑**
ALT (IU/L)	2.65 ± 0.41	3.76 ± 0.35	4.10 ± 0.55	4.96 ± 0.86↑*
AST (IU/L)	104.5 ± 8.09	118.4 ± 13.8	119.8 ± 10.6	147.0 ± 13.7↑*
BUN (mg/dl)	0.22 ± 0.15	0.20 ± 0.01	0.25 ± 0.03	0.34 ± 0.05↑*
AChE				
Serum	1.44 ± 0.14	0.95 ± 0.15	0.78 ± 0.21↓*	0.70 ± 0.33↓*
Liver	2.93 ± 0.22	2.94 ± 0.22	1.89 ± 0.35↓*	1.80 ± 0.30↓*
Brain	4.06 ± 0.24	3.79 ± 0.25	2.78 ± 0.37↓*	2.39 ± 0.33↓**
BCHE				
Serum	4.58 ± 0.32	3.18 ± 0.58	2.34 ± 0.48↓*	2.17 ± 0.43↓*
Liver	3.30 ± 0.23	3.58 ± 0.17	2.23 ± 0.30↓*	2.16 ± 0.29↓*
Brain	1.10 ± 0.12	0.88 ± 0.13	0.78 ± 0.10	0.58 ± 0.07↓*

[@] Values expressed as mean ± SEM; VC2: Methyl Cellulose; ↑Significant increase; ↓Significant decrease; *p value ≤0.05; **p value ≤0.01

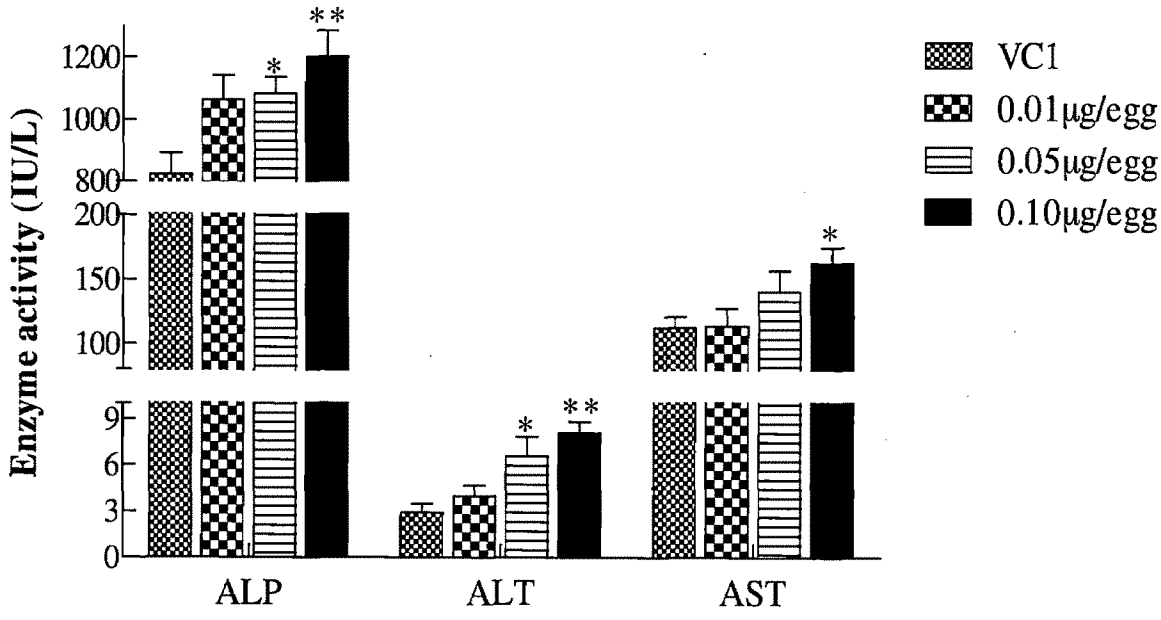
ALP- Alkaline Phosphatase, ALT- Alanine Aminotransferase, AST- Aspartate Aminotransferase, BUN- Blood Urea Nitrogen, AChE- Acetylcholinesterase (μM ATChI hydrolyzed /min/mg protein), BChE- butyrylcholinesterase (μM BTChI hydrolyzed /min/mg protein)

TABLE 3.5 Estimations in the early embryonic stage in vehicle controls and insecticide treated embryos

Treatment	Attribute			
	AChE (nmol/min/ mg protein)	**DNA** (mg/g wet wt. of tissue)	**RNA** (mg/g wet wt. of tissue)	**NA^+ K^+ ATPase** (µmol P_i released /min/mg of protein at 37 °C)
VC1	19.93 ± 1.08[@]	0.45 ± 0.04	0.148 ± 0.01	147.0 ± 10.82
0.01 µg Ci/egg	13.54 ±2.57↓*	0.44 ± 0.05	0.147 ± 0.02	201.3 ±11.20
0.05 µg Ci/egg	11.21 ±1.76 ↓*	0.26 ± 0.04↓*	0.088 ± 0.01↓*	212.3 ± 20.99↑*
0.10 µg Ci/egg	07.75 ± 0.73↓***	0.21 ± 0.05↓**	0.085 ± 0.01↓*	259.3 ± 9.52↑**
VC2	20.34 ± 0.87	0.47 ± 0.02	0.157 ± 0.02	140.0 ± 13.2
0.15 mg Sp/egg	20.99 ± 2.15	0.46 ± 0.05	0.142 ± 0.01	158.3 ± 19.3
0.75 mg Sp/egg	14.96 ± 1.40	0.47 ± 0.05	0.092 ± 0.02	192.7 ± 06.4
1.50 mg Sp/egg	10.86 ± 1.85↓**	0.25 ± 0.04↓*	0.084 ± 0.01↓*	256.7 ± 15.5↑**

[@] Values expressed as mean ± SEM; VC1: Corn oil; VC2: Methyl Cellulose; ↑Significant increase; ↓Significant decrease; *p value ≤0.05; **p value ≤0.01; ***p value ≤0.001.

FIGURE 3.1 Serum enzyme activities in vehicle control (VC1) and combination insecticide (Ci) treated groups



VC1: Corn oil, ALP- Alkaline Phosphatase, ALT- Alanine Aminotransferase, AST- Aspartate Aminotransferase.

*p value ≤ 0.05; **p value ≤ 0.01

FIGURE 3.2a Acetylcholinesterase in vehicle control (VC1) and combination insecticide treated groups

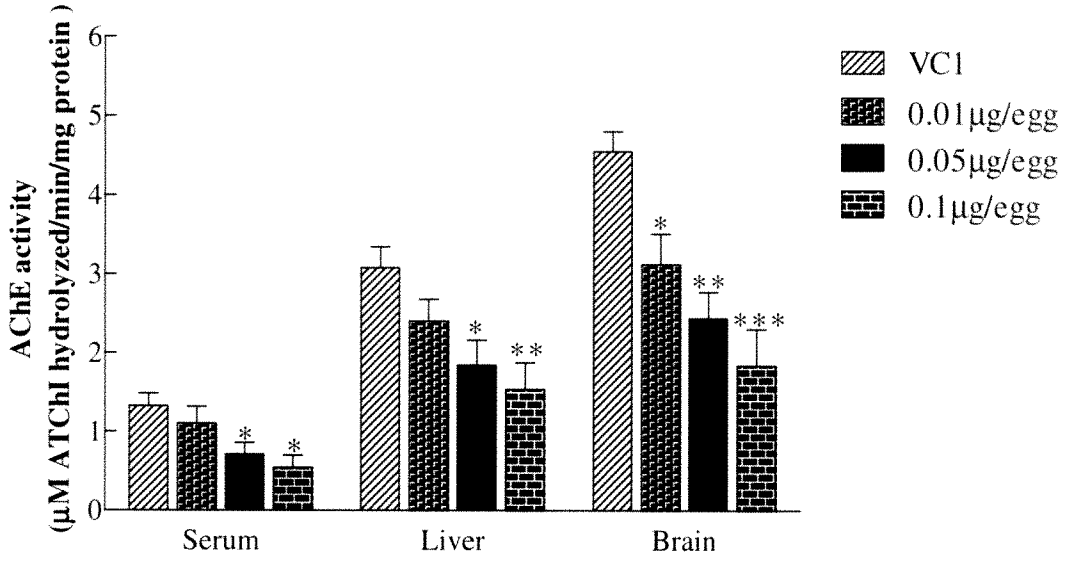
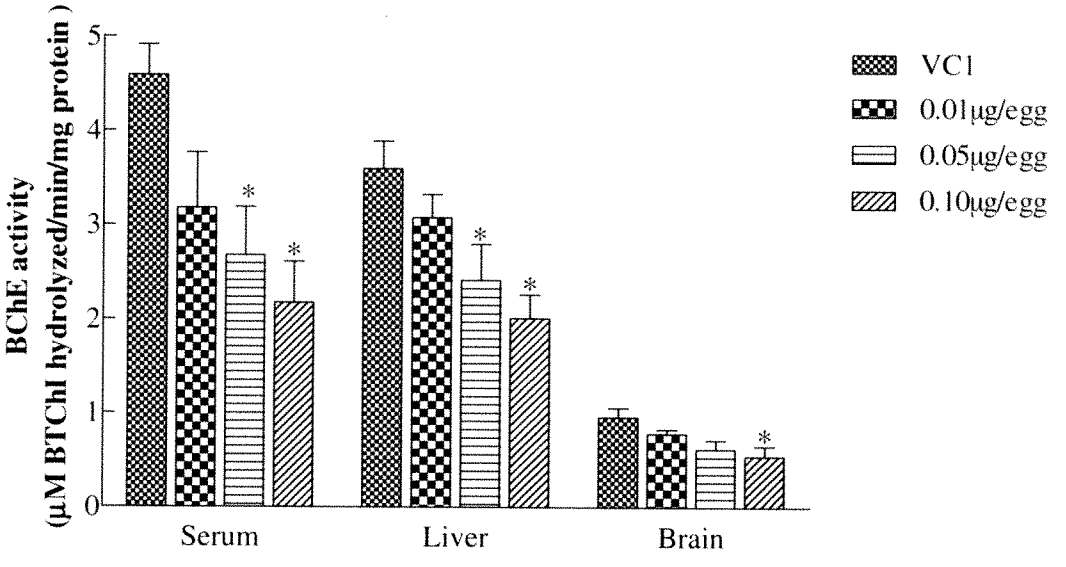


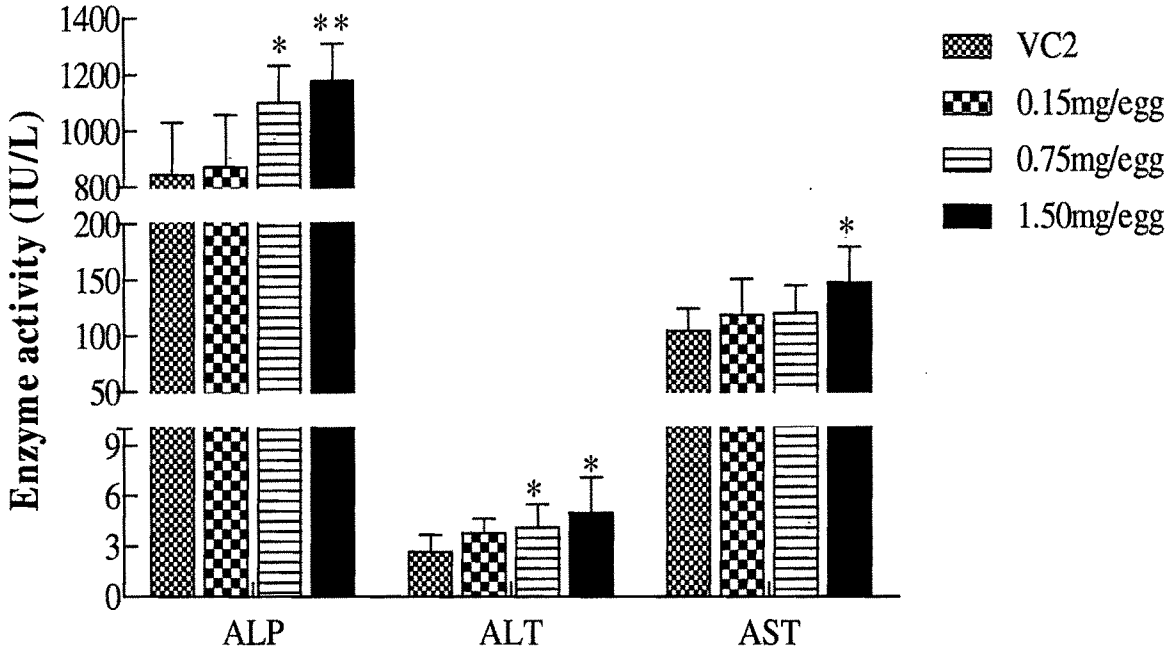
FIGURE 3.2b Butyrylcholinesterase activity in vehicle control (VC1) and combination insecticide treated groups



VC1: Corn oil; VC2: Methyl Cellulose

*p value ≤ 0.05; **p value ≤ 0.01; ***p value ≤ 0.001.

FIGURE 3.3 Serum enzyme activity in vehicle control (VC2) and Spinosad treated groups



VC2: methyl cellulose,-ALP- Alkaline Phosphatase, ALT- Alanine aminotransferase, AST- Aspartate aminotransferase

*p value ≤ 0.05; **p value ≤ 0.01

FIGURE 3.4a Acetylcholinesterase activity in vehicle control (VC2) and Spinosad treated groups

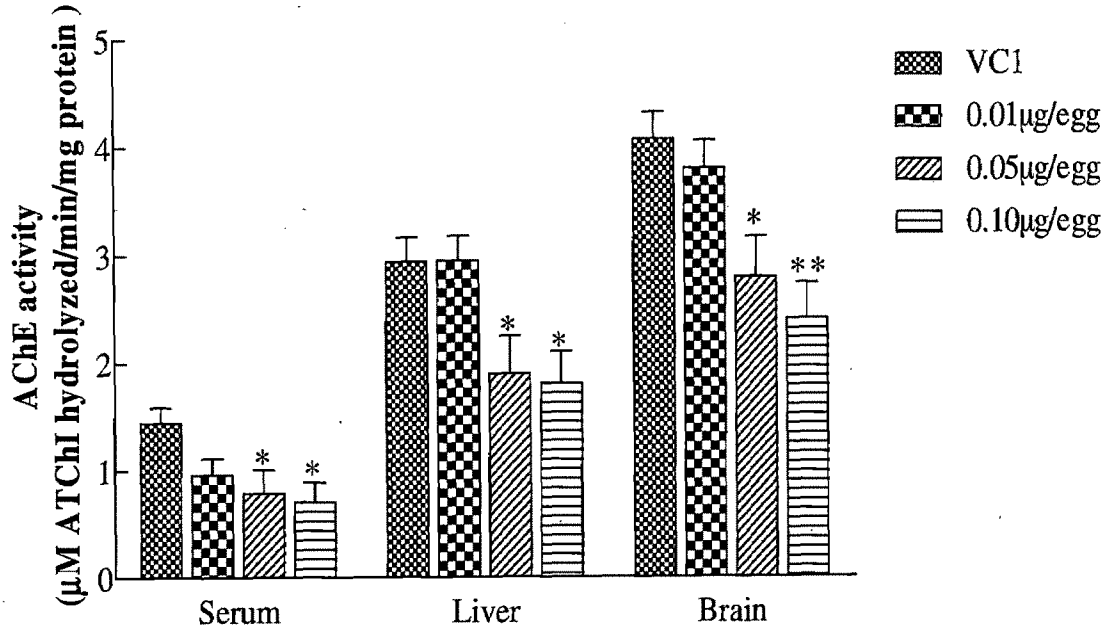
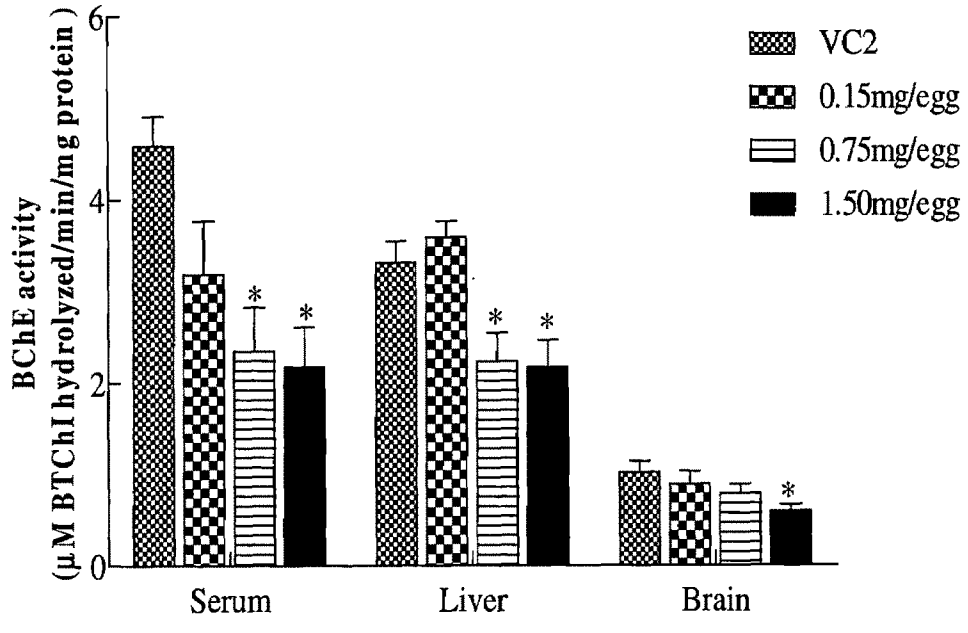


FIGURE 3.4b Butyrylcholinesterase activity in vehicle control (VC2) and Spinosad treated groups



*p value ≤ 0.05; **p value ≤ 0.01

FIGURE 3.5 - 3.8 Biochemical parameters in vehicle control (VC1 or VC2) and combination insecticide (Ci) or Spinosad (Sp) treated embryos on day 8

FIGURE 3.5 Acetylcholinesterase Ci treated embryos

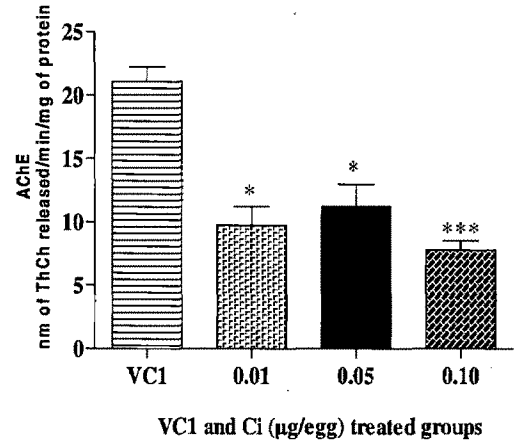


FIGURE 3.6 Acetylcholinesterase in Sp treated embryos

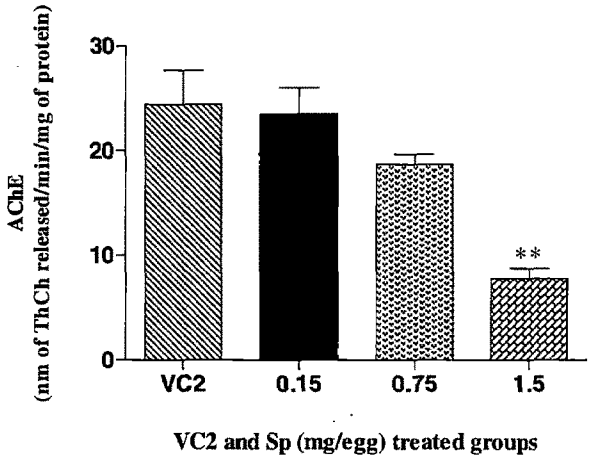


FIGURE 3.7 DNA content in Ci treated embryos

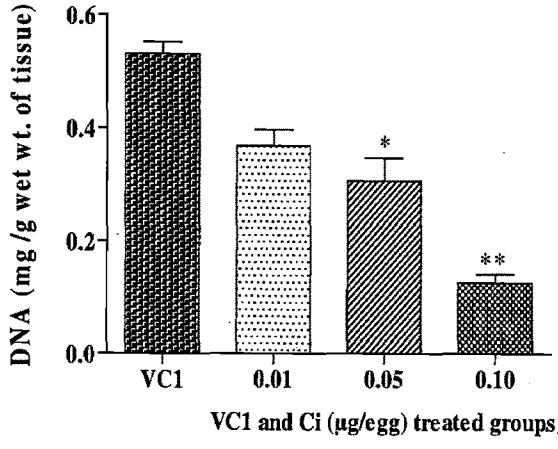
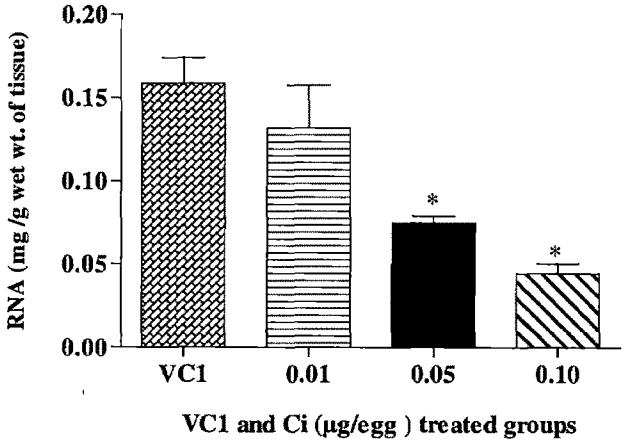


FIGURE 3.8 RNA content in Sp treated embryos



*p value ≤ 0.05; **p value ≤ 0.01; ***p value ≤ 0.001.

FIGURE 3.9 - 3.12 Biochemical parameters in vehicle control (VC1 or VC2) and combination insecticide (Ci) or Spinosad (Sp) treated embryos on day 8

FIGURE 3.9 DNA content in Sp treated embryos

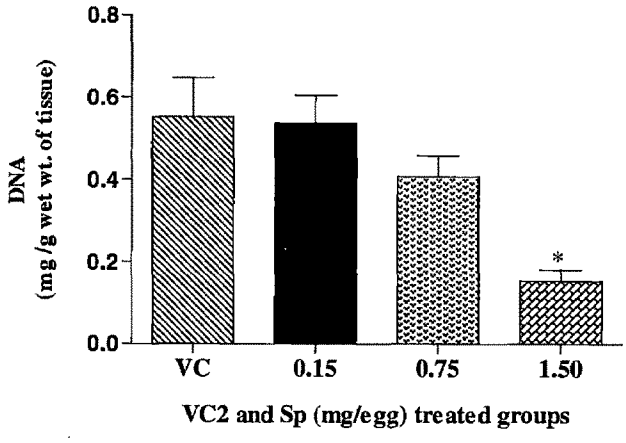


FIGURE 3.10 RNA content in Sp treated embryos

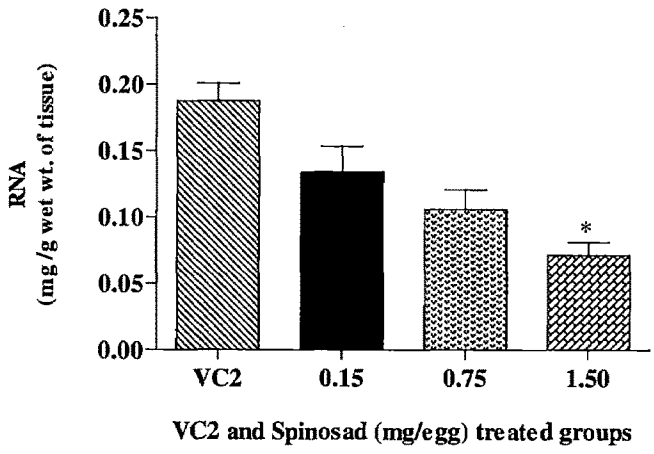


FIGURE 3.11 NA^+ K^+ ATPase in Ci treated embryos

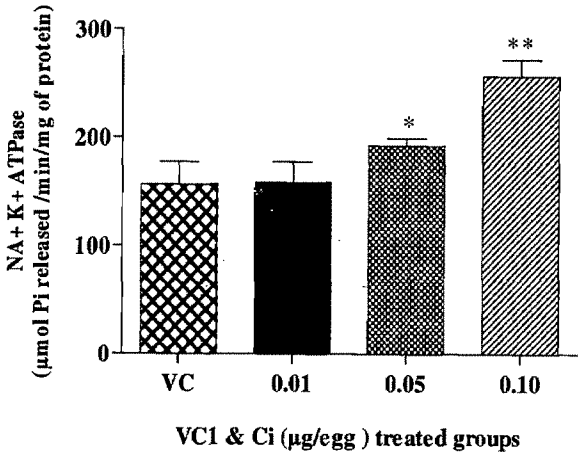
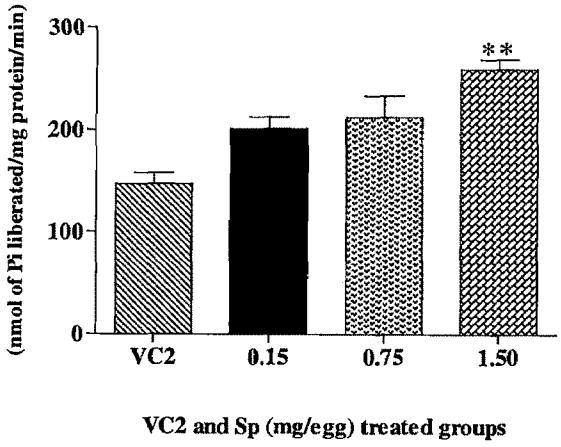


FIGURE 3.12 NA^+ K^+ ATPase in Sp treated embryos



*p value ≤ 0.05; **p value ≤ 0.01