CHAPTER 5

EVALUATION OF GENOTOXIC POTENTIAL USING COMET ASSAY AND MICRONUCLEUS TEST

INTRODUCTION

The biological information contained in all the eukaryotes is stored in its genetic material – the DNA and RNA. Maintaining genome stability is crucial for cell growth and cell survival (**Ragu *et al.*, 2007**) which might otherwise lead to mutation, chromosomal aberration, aging or cancer (**Kim *et al.*, 2000**). In the physiologic state, generation of DNA breaks and subsequent DNA repair is more or less balanced. In other words, there is a "steady-state" as the DNA damage can be repaired by various mechanisms. DNA repair genes have been shown to be expressed in the early stages of developing mammalian embryos (**Lowe *et al.*, 1997, Kim *et al.*, 2000**), and are believed to rely entirely on the oocyte's machinery i.e. the mRNAs and proteins deposited and stored prior to ovulation. Therefore, the survival of the embryo necessitates that the oocyte be adequately equipped with maternal stored products and also that embryonic gene expression commences at the correct time (**Jaroudi and Sengupta, 2007**). However, if the damage induced is greater than the repair capacity, the amount of DNA breaks increases, and it may finally contribute to the development of a disorder.

The category of substances that can induce changes to the structure or number of genes through chemical interactions with the DNA and/or non-DNA targets are said to be genotoxins (**Maurici, *et al.*, 2005**). There are three primary effects that genotoxins can have on organisms by affecting their genetic information. Genotoxins can be carcinogens, or cancer-causing agents, mutagens, or mutation-causing agents, or teratogens, birth defect-causing agents. DNA damage can occur as double-strand breaks or as single–strand breaks. The amount of DNA breaks depends on different factors such as cell type, the age of the cell, and the extracellular environment (**Seo *et al.*, 2000; Simone *et al.*, 2008; Rothkamm *et al.*, 2008**). Single-stranded DNA breaks can result from a variety of factors including UV light (**Choy *et al.*, 2005**), X-rays (**Davidkova *et al.*, 2007**), ionizing radiation (**Stap *et al.*, 2008**),

toxins (**Arbillaga *et al.*, 2007**), chemicals (**Chye *et al.*, 2008**), and reactive oxygen species (**Li *et al.*, 2008**).

The pesticide toxicity screening often includes an evaluation of the genotoxic potential of the pesticides. **Dearfield *et al.* (1993)** have demonstrated that pesticide components like the pyridine, halogenated pyridines and halogenates when tested for mutagenicity by a variety of *in vitro* and *in vivo* assays (in mice), were found to induce chromosomal aberrations, micronucleus formation, and sister chromatid exchanges. Increased frequency of micronuclei in the erythrocytes of both bone marrow and peripheral blood were reported in chick after chronic exposure to monocrotophos (**Jena and Bhunya, 1992**). The organophosphorus pesticides exposure showed positive results for chromosomal aberrations in bone marrow cells (**Kurinny, 1975**) and also showed increased sister chromatid exchanges (**Tezuka *et al.*, 1980**) in mammalian cells. Chlorpyrifos depicted positive results for DNA damage in prokaryotic systems (**Waters *et al.*, 1980**), significant increase in frequency of sister chromatid exchanges in human lymphoid cell cultures (**Sobti *et al.* 1982**), and significantly increased values of chromosome aberrations in rats (**De Hondt *et al.* 1983**). In few other studies, an increased rate of chromosomal aberrations in the spleen cells of mice and in the bone marrow cells of rats treated with chlorpyrifos has been observed (**Soheir *et al.*, 1982; Soheir *et al.*, 1992; Soheir *et al.*, 1996**). Previously, many reports showed that chlorpyrifos has cytogenetic toxicity *in vitro* and *in vivo* (**Sobti *et al.*, 1982; Amer and Aly, 1992; Waters *et al.*, 1980**). Other organophosphates viz. Malathion, Methyl parathion, Parathion, Dimethoate, Dursban, Phorate, Phosdime and Viozene also increased sister chromatid exchanges in human lymphoid cells (**Sobti *et al.* 1982**). The pyrethroids too were reported to have genotoxic potential (**Chauhan *et al.*, 2007**). According to **Rudek and Rozek (1992)**, high concentrations of Fastac 10 EC (a pyrethroid) have clastogenic activity and/or damage the mitotic spindle, as manifested by a significant increase in the frequency of the micronucleated red blood cells of tadpoles of *Rana temporaria* and *Xenopus laevis*. Cypermethrin is also known to cause DNA damage, thereby leading to micronucleus formation (**Campana *et al.*, 1999; Çelik *et al.*, 2005, Sharaf *et al.*, 2010**). According to **EPA (1997)**, Spinosad has no mutagenic activity and the literature offers no additional data in this respect. However **Mansour *et al.* (2008a)** reported that Spinosad induced significant decrease in mitotic activity and increased micronucleated polychromatic erythrocyte in rat's bone marrow cells, even when administered at a safe dose.

A basic requirement of genotoxicity testing is an adequate activation of promutagens which *in vivo* generally occurs in the liver (**Wolf and Luepke, 1997**). Most of the reactive metabolites are formed in phase I reactions by cytochrome P-450 (CYP-450) dependent monooxygenases, whereas phase II activity leads to the formation of polar conjugates which then are eliminated. For some classes of compounds phase II reactions can result in the formation of ultimate mutagens, too (**Bresnick, 1992**). The avian embryo in early stages of embryonal development provides a wide range of metabolic activities, probably related to the early differentiation of the avian liver and the resulting autonomy of avian embryos (**Sinclair and Sinclair, 1992**). Several authors have extensively examined different isozymes of chick embryo CYP-450 and their induction by several inducers, such as phenobarbital type inducers (**Hamilton and Bloom, 1986; Lorr and Bloom,1987**) in the embryonic liver, the whole embryo, in primary cell cultures, or yolk (**Heinrich-Hirsch *et al.*, 1990**). Phase II-reactions have also been extensively examined: Reactions like glucuronidation (**Banjo and Nemeth, 1976**), sulfation (**Spencer and Raftery, 1966**), acetylation, conjugation with ornithine (**Wolfe and Huang, 1959**), and methylation (**Ignarro and Shideman, 1968**) have been demonstrated. All these studies showed that the avian embryo is able to detoxify and excrete xenobiotics into the allantoic sac, where the products of the kidneys are accumulated (**Romanoff, 1960**). Due to the complex metabolic competence, the chick embryo is therefore predestined for serving as a model system for genotoxicity testing, owing to its capability of covering metabolic activation and deactivation of xenobiotics.

Biomarkers such as micronucleus test (MNT) and the comet assay are widely accepted for testing the genotoxicity. The micronucleus test provides a simple and rapid indirect measure of induced structural and numerical chromosome aberrations and is scientifically and regulatory accepted by supranational authorities like the Organization for Economic Cooperation and Development (OECD), International Conference on Harmonization (ICH) and European Union (EU) (**Wolf *et al.*, 2002**). Micronuclei in cells are considered as a biomarker of damage to the DNA (**Saleh and Sarhan, 2007**). The formation of micronuclei (MN) is extensively used in molecular epidemiology as a biomarker of chromosomal damage, genome instability, and eventually of cancer risk. The occurrence of MN represents an integrated response to chromosome-instability phenotypes and altered cellular viabilities caused by genetic defects and/or exogenous exposures to genotoxic agents (**Iarmarcovai, *et al.*, 2008**).

Micronuclei are cytoplasmic chromatin-containing bodies that appear in the cell like a small satellite nucleus around the cell nucleus, due to chromosome fragments or entire chromosomes that are not incorporated in the main nucleus after cell division. Accordingly, the micronucleus test evaluates the frequency of micronuclei formation in a proliferating cell population. Micronucleus test has been well established in several systems *in vitro* (**Miller *et al.*, 1995; Garriott *et al.*, 2002**) as well as *in vivo*, and in various tissues i.e. ovary, bone marrow, peripheral blood, liver and in foetal cells of rodents and humans (**Heddle, 1990, Saleh and Zeytinoglu, 2001**). **Wolf *et al.* (2002)** have developed the hens egg test (HET) by performing MNT in chick embryos along with other methods for detection of mutations in the proliferating cell population, induced by genotoxic chemical agents.

Apart from the MNT, the DNA damage can be tested by the single cell gel electrophoresis. The comet assay or the single cell gel electrophoresis has emerged as a sensitive technique for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and cross linking. The advantages of the technique include (**Tice *et al.*, 2000**):

(1) Its demonstrated sensitivity for detecting low levels of DNA damage
(2) The requirement for small numbers of cells per sample
(3) Flexibility
(4) Low costs
(5) Ease of application
(6) The ability to conduct studies using relatively small amounts of a test substance; and
(7) The relatively short time period (a few days) needed to complete an experiment.

The comet assay has been extensively used to measure DNA damage in different mammalian systems exposed to various physical and chemical agents (**Rydberg, 1975; Singh *et al.*, 1988**). It is a sensitive and rapid method for the detection of DNA strand breaks in individual mammalian cells after exposure to any physical or chemical agent. This method allows measurement of the migration of DNA, which uncoils due to the formation of single strand breaks. Hence, owing to their precision in analysis and widely accepted application in genotoxicity testing, the micronucleus test and the comet assay were performed in the present study to evaluate the genotoxic potential of the tested insecticides.

OBJECTIVE

The outcomes of chapter 1 and chapter 2 showed that the combination insecticide has induced teratogenic effects, cell death and loss of integrity in the embryonic developmental period, which ascertain the need to look into the genotoxic infliction. Therefore, these results prompted towards evaluating the genotoxic potential of the concerned insecticides. The following tests were performed to evaluate the same:

(i) Micronucleus Test

(ii) Comet Assay

MATERIALS AND METHODS

The genotoxic effects were studied trough the micronucleus test and comet assay in the vehicle controls and insecticide treated chick embryos after 11 days of incubation. A detailed description of the following methods is given elsewhere under the broad title materials and methods.

Micronucleus test

The test was performed as per **Chaubey *et al.* (1993)** on the blood smears of 11 day old chick embryos. The identification of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) was based on the criteria described by **Wolf and Luepke (1997)**. For classifying the different abnormal cell morphologies, the classification scheme of **Lucas and Jamroz (1961)** was followed.

Comet assay

Comet assay (**Malladi *et al.*, 2007**; **Singh *et al.*, 1988**) was performed on the 11 day embryonic tissues i.e. blood, liver and brain. The results were analysed on the TriTek Comet Score (Version 1.5.0) software. The software quantifies the comet length, comet height, comet area, comet tail length, percentage of the DNA in the tail and tail moment (**Figure 5.1**). The DNA damage was scored by comparative analysis of tail moment (product of percent DNA in comet tail and tail length) of the treated embryonic cells and the control embryonic cells.

Tail moment = %DNA in comet tail x tail length

The tail moments of control and insecticide treated embryos were considered for the statistical evaluation of damage to the DNA.

RESULTS

Micronucleus Test

The erythrocytes of both the vehicle control birds (corn oil as well as methylcellulose) were oval shaped and uniform in size with a single normal nucleus and showed no abnormalities in their morphology. The blood smears of 0.01μg/egg Ci treated embryos showed mironucleated cells, but were in comparable numbers to the control embryos. The PCE/NCE ratio was in normal range relative to the control embryos (**Table 5.1**). The embryos dosed with 0.05μg/egg Ci had a significantly higher number of mnPCE ($p \leq 0.05$), while the mnNCE were not present in comparable numbers and also the total number of micronucleated erythrocytes were insignificant (**Table 5.1**). The PCE/NCE ratio was also in normal range. The embryos which received 0.1μg/egg of Ci were found to have higher numbers of mnPCE ($p \leq 0.001$) as well as mnNCE ($p \leq 0.05$). The total number of the micronucleated erythrocytes also showed a significant ($p \leq 0.01$) increase (**Figure 5.2a, 5.3 and 5.4**). A significant decline ($p \leq 0.01$) was noticed in the PCE/NCE ratio in the blood of 0.1μg/egg of Ci treated embryos (**Figure 5.2b**). Apart from the micronucleated erythrocytes, the blood smears of 0.05 and 0.1μg/egg Ci treated embryos showed various abnormal cells i.e. dacryocytes (tear drop cells), microcytes, erythroplastids, squashed/notched nuclei, nuclear segmentation and spindle shaped erythrocytes (**Figure 5.5, 5.6, 5.7**) The primitive erythroblast cells too were found retained in the blood of the 11 day old 0.1μg/egg Ci treated embryos.

The Spinosad treated chick embryos at a concentration of 0.15 and 0.75mg/egg did not induce any significant micronuclei formation and also the PCE/NCE ratio was normal when compared to the control embryos (**Table 5.1**). However, 1.5mg/egg Sp has lead to the formation of significant numbers of mnPCE ($p \leq 0.01$). The NCE showed no significant micronucleus formation at this dose. Nevertheless, the total number of micronucleated erythrocytres was found to be in an elevated number ($p \leq 0.01$) and also the PCE/NCE ratio got significantly ($p \leq 0.05$) lowered (**Figure 5.8a and 5.8b**). Also, at this high dose, few abnormal cells were sighted like squashed RBC and fragmented nuclei.

Comet Assay/ Single Cell Gel Electrophoresis

Genotoxic indications were absent in embryonic tissues of embryos dosed with 0.01μg/egg Ci. No statistically identified differences were noted in DNA migrations in the cells of blood, liver or brain. With a dose of 0.05μg/egg of Ci the embryos were prone to DNA damage only in the livers ($p \leq 0.001$). Though the mean of tail moment in blood and brain seemed to be

higher than in the controls, they were not statistically significant. The results of single cell gel electrophoresis (**Table: 5.2**) revealed that the DNA damage occurred in the blood, liver and brain of 0.1μg/egg of Ci dosed embryos, as evident by the increase in tail moment (**Figure 5.9**) where the blood ($p \leq 0.001$) and liver ($p \leq 0.001$) seemed to be more affected than the brain ($p \leq 0.05$).

The Spinosad at the concentrations of 0.15 and 0.75mg did not cause any DNA damage as analysed from the results of the Comet assay (**Table: 5.3**). Nevertheless, with a dose of 1.5mg of Spinosad, *Tracer* caused DNA damage in the blood ($p \leq 0.05$) and liver ($p \leq 0.05$), but not in the brain (**Figure 5.10**).

DISCUSSION

Micronucleus Test

The circulating blood of the chick embryo consists nearly exclusively of erythroid cells which do not expel the nucleus during their whole life span. The occurrence of other cell types is sporadic except for the thrombocytes. The latter constitutes 2% of all blood cells throughout the complete incubation period after incubation day 6. The population of erythrocytes shows two distinct cell lines. The first erythroid cell line, consisting of the so called primitive erythrocytes, appears at about 36hr of incubation in the blood islands of the area vasculosa and constitutes the only recognizable erythroid cell type of the chick embryo until about day 5. These primitive erythrocytes approach maturity by 6 to 7 days of incubation so that all primitive erythrocytes are mature and appear normochromatic by day 11. Bone marrow erythropoiesis starts by the 10th day of incubation and it releases only definite erythrocytes in the blood circulation. The erythrocytes from the bone marrow also enter the peripheral circulation by 10–12 days of incubation. The spleen does not contribute to the pool of blood erythrocytes up to 11 days of incubation and even if it does, it is quite less (**Bruns and Ingram, 1973; Wolf and Luepke, 1997**). Hence, most of the erythrocytes observed in the embryos by the 11^{th} day of incubation are mostly of the yolk sac origin, which is the most metabolically active tissue (**Wolf *et al.*, 2003**). It has been reported earlier that by the day 11 of incubation, the spleen is not a functional organ. If the micronuclei are formed in the erythrocytes, the cells get accumulated in the blood as the spleen is yet not functional to clear them off the blood. When a treatment evokes the formation of micronucleated erythrocytes, the actual numbers affected can be directly observed without much aberrance in

the analysis. Therefore, the 11 day old embryo which carry mature as well as the other developing stages of the erythroid series were selected for the experiments.

The embryonic treatment with 0.05 and 0.1µg of Ci was capable of inducing micronuclei in the erythrocytes and the effect increased with the dose. Micronuclei are formed as a result of acentric chromatid or chromosome fragments and sometimes even the whole chromosome, which lag behind due to spindle malfunctioning, and hence they are used as an indicator of chromosomal damage (**Davidkova *et al.*, 2007**). Moreover, micronuclei could also be formed by the loss of whole or portions of chromosomes from daughter nuclei at anaphase and exist separately from the main nucleus of the cell (**Choy *et al.*, 1989**). Impaired function of topoisomerase-II can be involved in MN formation (**Müller and Streffer, 1994**). The PCE/NCE ratio indicates haematopoietic cell proliferation rate and a decrease in the ratio would mean hampered haematopoiesis or cytotoxicity. The Ci at 0.1µg/egg led to a decline in the PCE/NCE ratio, which might be due to the cytotoxic nature of the insecticide.

The blood picture of day 11 embryo under normal condition presents early, middle and late PCEs; definitive NCEs, primitive NCEs and very few thrombocytes. Apart from micronuclei, the Ci treatment induced poikilocytosis in the circulating embryonic blood. This blood picture clearly indicates a cytotoxic nature of the Ci. In addition to the common erythroid cell types cited above, the high dose groups presented large undifferentiated cells i.e. the erythroblasts and proerythroblasts. They can be discriminated from cells of the definite line by their greater size, the lower nucleus–cytoplasm ratio and their circular outline. The average life span of this cell type is stated to be 8 days. The production of primitive erythrocytes ceases after day 7 of incubation. However, under normal conditions these cell types are not present at this stage (11 day embryo) of embryonic development in the peripheral blood. As per **Wolf *et al.*, (2002),** at this stage of embryonic development, the proerythroblasts and erythroblasts would be observed only after a cytotoxic treatment. Thus, apart from inducing aberrance in the nuclear material, the Ci has a potential to bring about cytotoxic changes which inhibit erythropoiesis and cell differentiation. Similar observations were made by **Sharaf *et al.*, (2010)** in broilers treated with cypermethrin, where the treated birds showed micronucleated erythrocytes and various other morphological alterations like spindle and pear shaped erythrocytes along with nuclear segmentation.

The Spinosad treatment did not cause any genotoxic effects at 0.15 and 0.75mg/egg. Nevertheless, a dose of 1.5mg/egg of Sp induced micronuclei in the polychromatic erythrocytes and also lead to a decline in PCE/NCE ratio. Also, at this high dose, fragmented nuclei were observed in the embryonic blood smears. These results are in agreement with those of **Mansour *et al.* (2008a)**, who showed that Spinosad can causes mutagenic and reproductive effects on male albino rats. This cytogenetic activity could be attributed to the Spinosad's chemical structure and/or certain impurities in the commercial formulation; a matter, which needs further elucidation (**Mansour *et al.*, 2008a**).

Comet Assay

Occupational exposure to pesticides has been associated with several neoplastic diseases like the multiple myeloma, lymphoma and sarcoma (**Hardell and Erikson, 1988; LaVechia *et al.*, 1989; Brown *et al.*, 1990; Cullen *et al.*, 1990**). A certain number of field studies have been done and reported an association between the occupational exposure to pesticides and the presence of chromosomal aberrations as a factor which increases the risk of cancer (**Rita *et al.*, 1987; Rupa *et al.*, 1988; Garaj-Vrhovac and Zeljezic, 1999**).Several of the authors have demonstrated the genotoxic potential of various pesticides through Comet assay (**Ribas *et al.*, 1995, Clements *et al.*, 1997, Sasaki *et al.*, 1997, Vigreux *et al.*, 1998**).

In general, migration of DNA fragments from the nucleus of a damaged cell, during the single cell gel electrophoresis is dependent on the extent of the damage caused, and increases with the increasing doses of the genotoxin (**Chaubey *et al.*, 2006**). However, it has been observed that exposure to certain chemicals may not result in proportional increase in tail length with increasing dose level since most of the DNA has migrated from the nucleus to the tail region. In such situations measurement of only tail length might be erroneous. The tail moment, which is a product of percent DNA in comet tail and tail length, would instead give a more precise calculation to the extent of DNA damage. Therefore, the tail moments were calculated and statistically analysed between the control and different groups of insecticide treatment in the present study.

The next experiment performed for evaluating the genotoxic effects was the comet assy. Data obtained from comet assay clearly demonstrated a significant increase in DNA strand breaks in all the tested tissues i.e. the blood, liver and brain of Ci treated chick embryos with 0.1μg/egg. However, with a dose of 0.05μg/egg, the damage was significant only in the liver. The lowest damage was observed in the brain. The Spinosad too induced DNA damage,

nonetheless only at a high dose of 0.75mg/egg. The DNA damage was more prominent in liver and blood than in the brain in both the group of insecticides. The higher susceptibility of liver to the genotoxins could be due to fact that liver is the first internal organ to receive the toxicants that need to be metabolized. Scientific literature provides evidence that the avian embryo unlike in mammals provides a wide range of metabolic activities related to the early differentiation of the avian liver (**Sinclair and Sinclair, 1992**). The chick embryonic stage relevant to the present study is also shown to be having functional isozymes cytochrome-P450 (CYP). It has a high concentration of the xenobiotic metabolizing enzymes (XME) particularly isoforms of cytochrome P450 (CYP). These enzymes, while primarily involved in detoxification also carry out many activation reactions, the products of which may cause hepatotoxicity or toxicity in other organ systems after being distributed throughout the body. Moreover, the CYP-450 induction is associated with DNA damage or formation of DNA adducts (**Shu and Hollenberg, 1996; Dubois *et al.*, 1997; Shaw *et al.*, 2002**). The liver is therefore highly susceptible to the genotoxic disposition of the xenobiotics. These discussed reasons might account for the high levels of genotoxicity observed in the embryonic livers of Ci treated embryos as compared to the blood and brain.

SUMMARY

Both the tests employed for the genotoxic assessment i.e. the micronucleus test as well as the comet assay clearly indicate that the Ci can induce DNA damage in the developing chick embryos when treated with concentrations as low as 0.05 and 0.1µg/egg; while Sp induced genotoxicity only at a high dose of 1.5mg/egg. From the current study it could be summarized that the Ci is a potential inducer of DNA damage to developing chick embryos when compared to the Sp. Further, it could be reasonably surmised that the genetic lesions induced by the Ci treatment might have also involved certain deleterious mutations which might have lead to the array of teratological malformations described in chapter 1.

TABLE 5.1 Micronucleus test in control and insecticide treated embryos

Treatment	Attribute			
	%mnPCE	%mnNCE	%mnEs	PCE/NCE
VC1	0.161±0.07[@]	0.077±0.04	0.117±0.04	0.927±0.02
0.01Ci	0.536±0.17	0.232±0.13	0.392±0.13	0.953±0.07
0.05Ci	0.971±0.29↑*	0.296±0.11	0.620±0.17	0.776±0.03
0.10Ci	1.736±0.29↑***	0.657±0.25↑*	0.958±0.21↑**	0.626±0.06↓**
VC2	0.179±0.06	0.127±0.06	0.116±0.05	0.901± 0.02
0.15Sp	0.223±0.05	0.143±0.02	0.181±0.04	0.884±0.02
0.75Sp	0.469±0.12	0.248±0.07	0.350±0.09	0.867±0.04
1.50Sp	0.997±0.22↑**	0.191±0.04	0.523±0.09↑**	0.742±0.06↓*

[@] Values are expressed as Mean ± SE, n = 6

VC1: corn oil; VC2: methyl cellulose

Ci: combination insecticide; Sp: Spinosad

*p≤0.05; **p≤0.01; ***p≤0.001

↓: significant decrease; ↑: significant increase

mnPCE : micronucleated polychromatic erythrocytes

mnNCE: micronucleated normochromatic erythrocytes

mnEs : micronucleated erythrocytes

PCE : polychromatic erythrocytes

NCE : normochromatic erythrocytes

TABLE 5.2 DNA damage evaluated by Comet assay in the vehicle control (VC1) and combination insecticide (Ci) treated chick embryos

Treatment	Tail Moment		
	Blood	Liver	Brain
VC1	0.711 ± 0.19[@]	0.851 ± 0.28	0.938 ± 0.30
0.01 µg/egg	1.032 ± 0.36	1.894 ± 0.43	0.939 ± 0.31
0.05 µg/egg	1.494 ± 0.50	7.757 ±1.74↑***	1.366 ± 0.33
0.10 µg/egg	8.055 ± 2.37↑***	9.088 ±1.99↑***	2.948 ± 0.75↑*

[@] Values are expressed as Mean ± SE, n = 6

*p≤0.05; **p≤0.01; ***p≤0.001

↑ : significant increase

VC1: corn oil

Ci : combination insecticide (µg/egg)

TABLE 5.3 DNA damage evaluated by Comet assay in the vehicle control (VC2) and Spinosad (Sp) treated chick embryos

Treatment	Tail Moment		
	Blood	**Liver**	**Brain**
VC2	0.953 ± 0.16[@]	0.846 ± 0.24	0.908 ± 0.29
0.15mg/egg	0.891 ± 0.22	1.285 ± 0.35	1.101 ± 0.42
0.75mg/egg	1.157 ±0.24	1.439 ± 0.46	1.129 ± 0.43
1.50mg/egg	2.256 ± 0.61↑*	2.776 ± 0.86↑*	1.301 ± 0.24

[@] Values are expressed as Mean ± SE, n = 6

*p≤0.05

↑ : significant increase

VC2 : methyl cellulose

Sp : Spinosad (mg/egg)

FIGURE 5.2a Percentage of micronucleated cells in the vehicle control (VC1) and combination insecticide (Ci) treated chick embryos

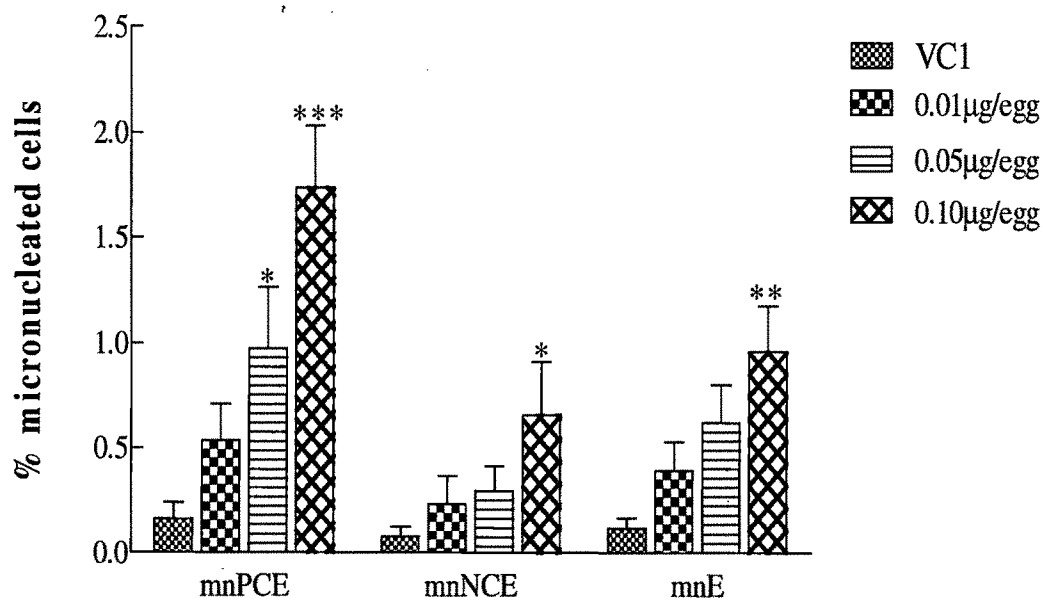
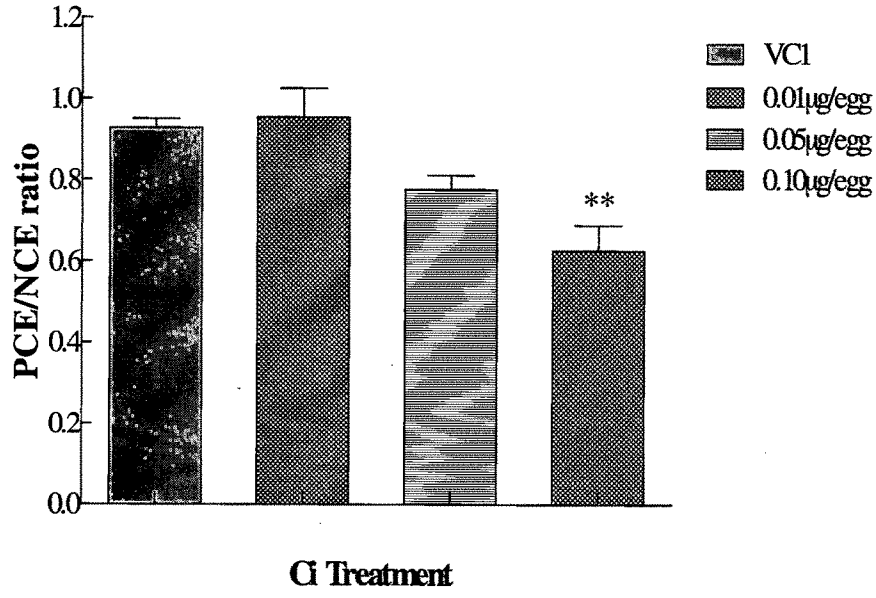


FIGURE 5.2b PCE/NCE ratio in the vehicle control (VC1) and combination insecticide (Ci) treated chick embryos



*p≤0.05; **p≤0.01; ***p≤0.001

FIGURE 5.8a Percentage of micronucleated cells in the vehicle control (VC2) and Spinosad (Sp) treated chick embryos

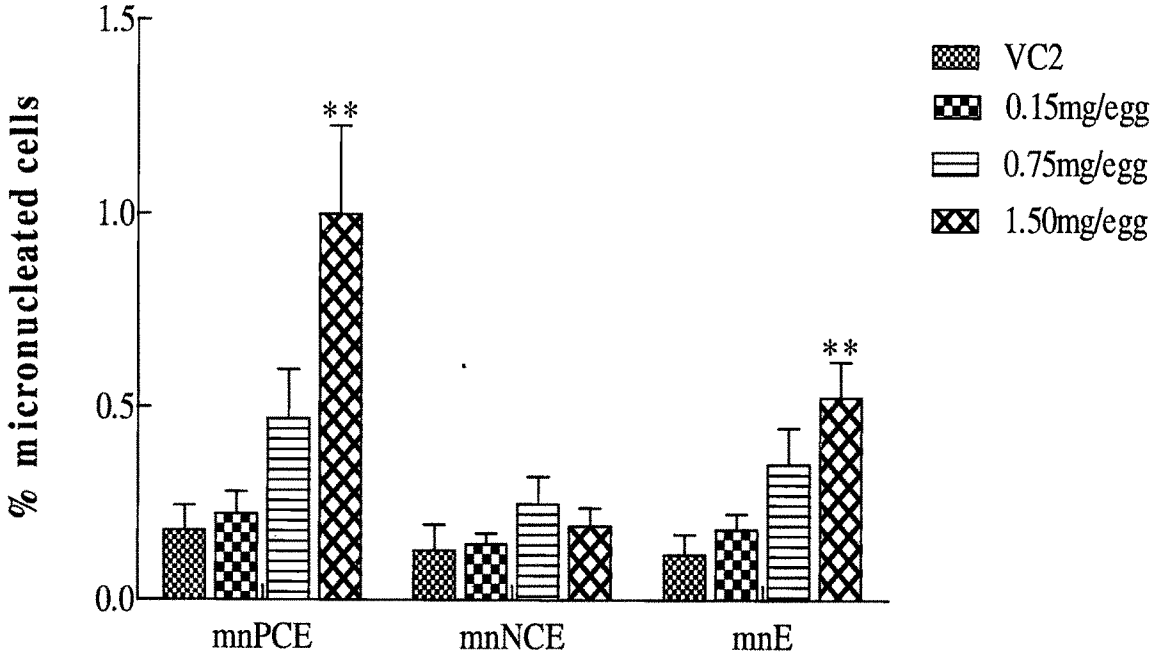
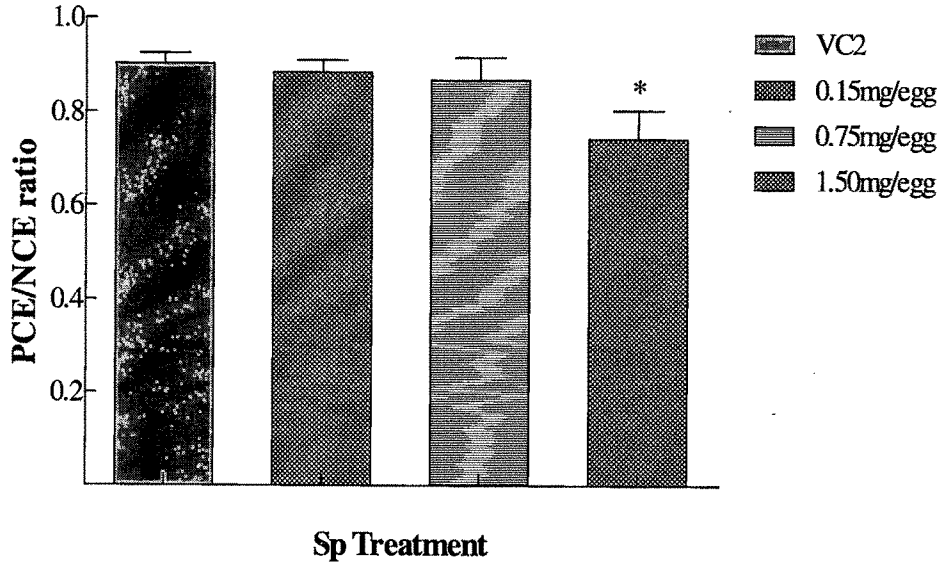
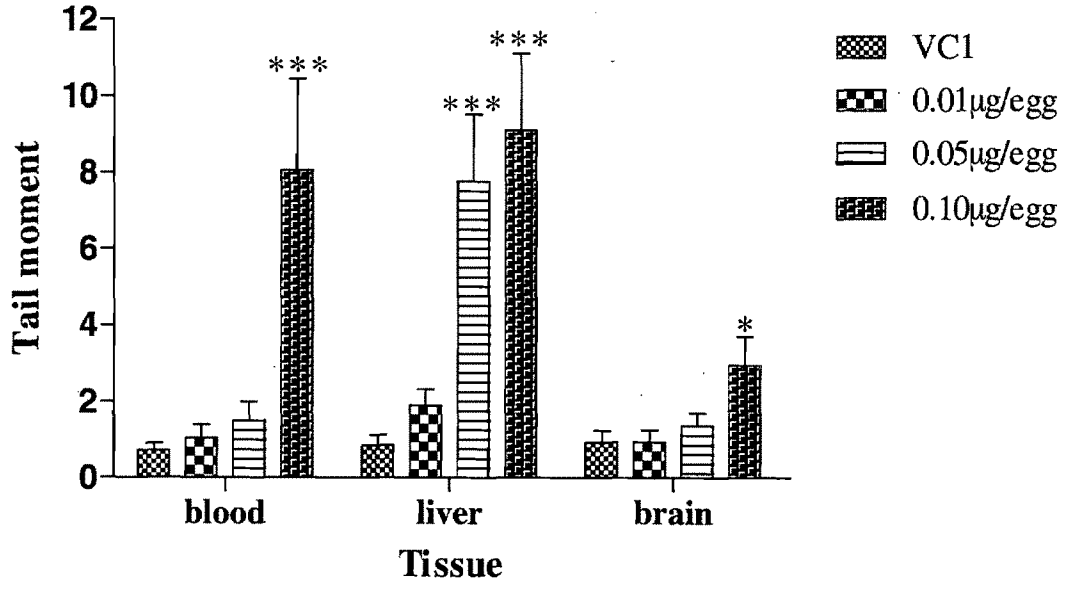


FIGURE 5.8b PCE/NCE ratio in the vehicle control (VC2) and Spinosad (Sp) treated chick embryos



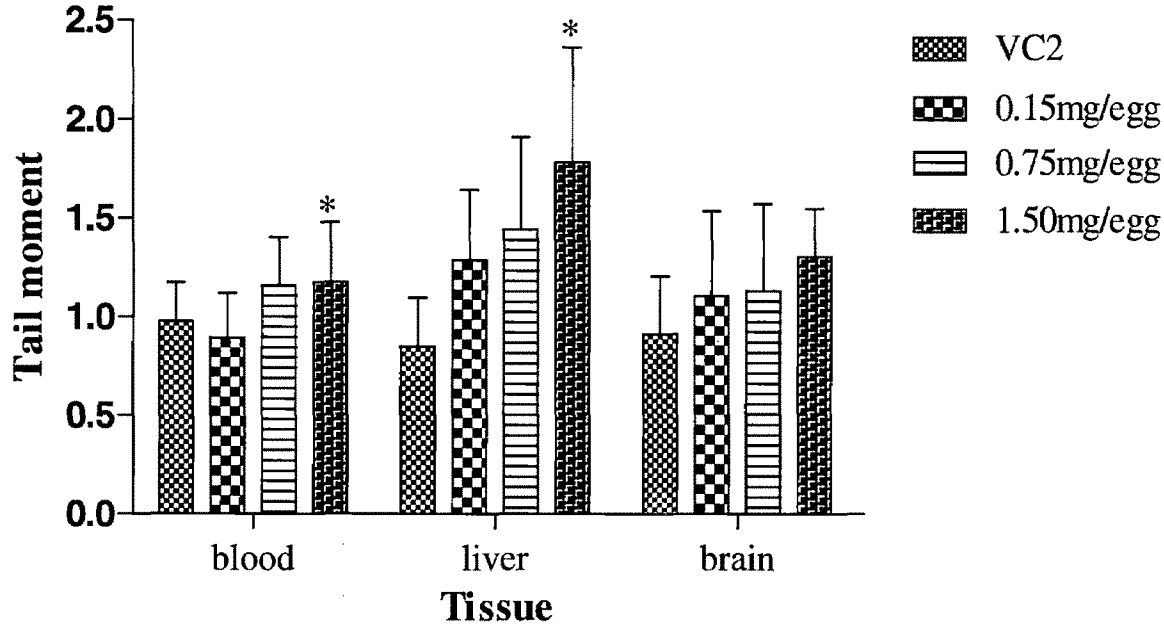
*p≤0.05; **p≤0.01; ***p≤0.001

FIGURE 5.9 Tail moment in the vehicle control (VC1) and combination insecticide (Ci) treated chick embryos



*p≤0.05;; ***p≤0.001

FIGURE 5.10 Tail moments in the vehicle control (VC2) and Spinosad (Sp) treated chick embryos



*p≤0.05

Comet dimensions analysed by TriTek Comet Score

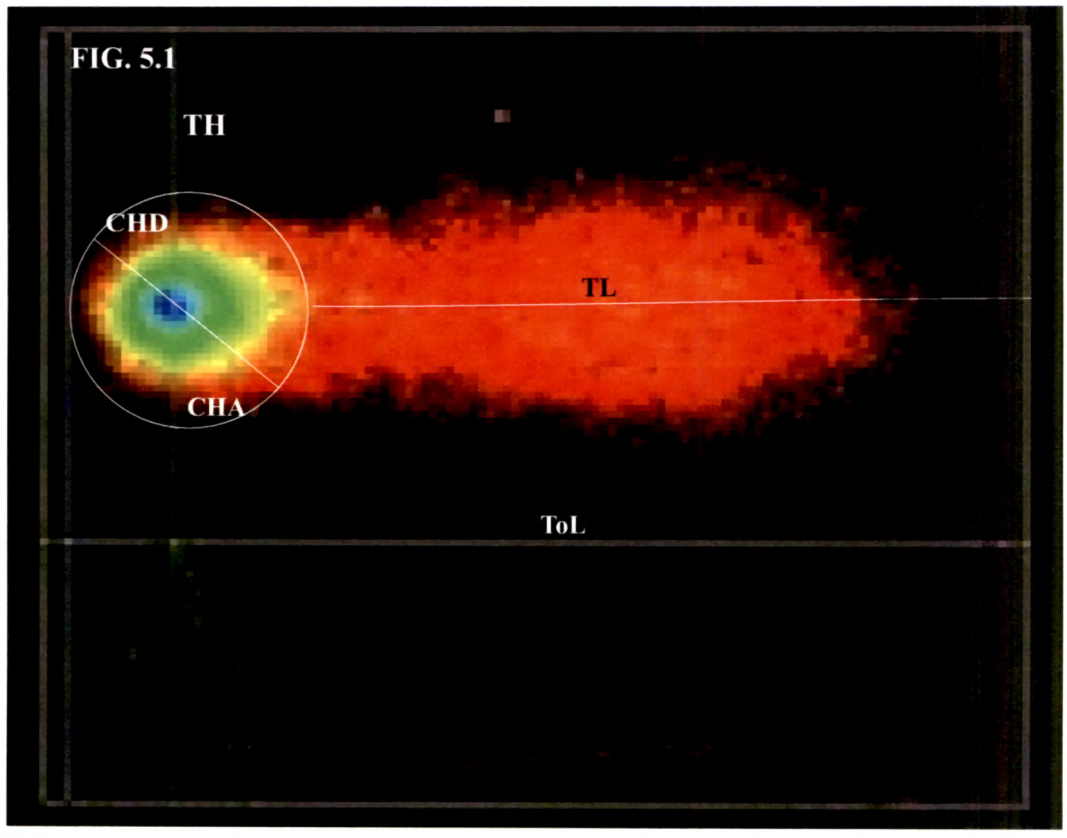


FIGURE 5.1 Total comet height (TH); Total comet length (ToL); Comet head diameter (CHD); Comet head area (CHA); Tail length (TL)

Blood smear of 11 day embryo treated with combination insecticide showing micronucleated cells

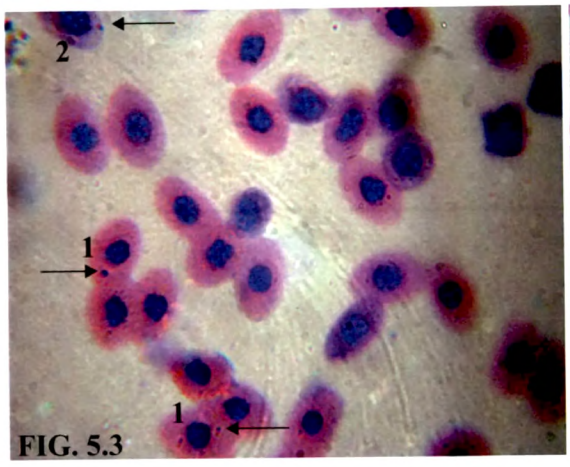


FIGURE 5.3 mnNCE (1), mnPCE (2)

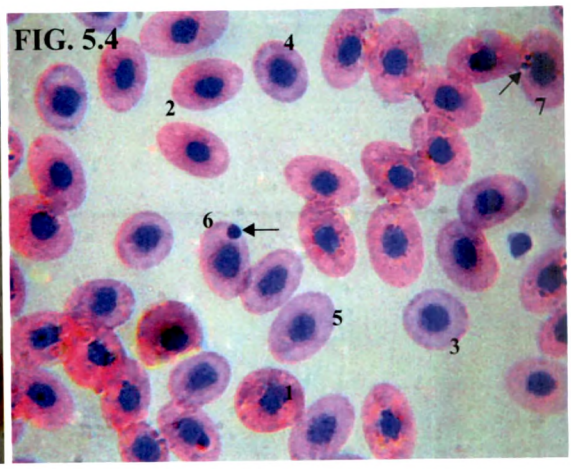
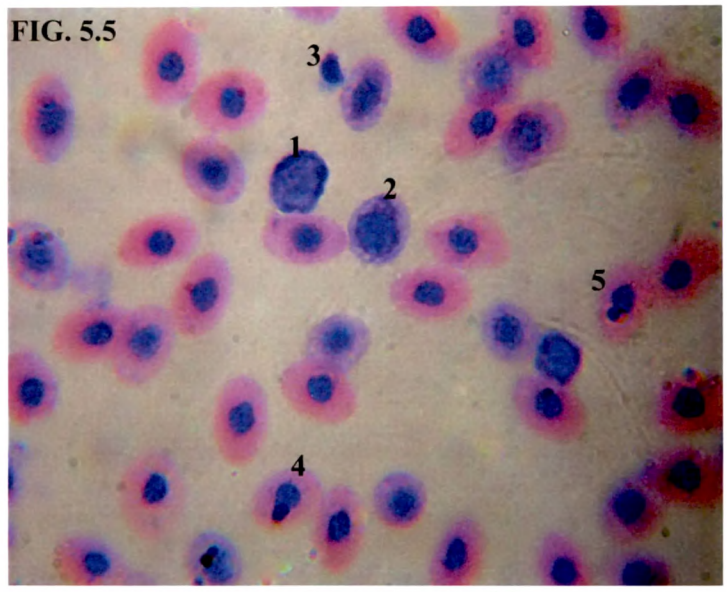
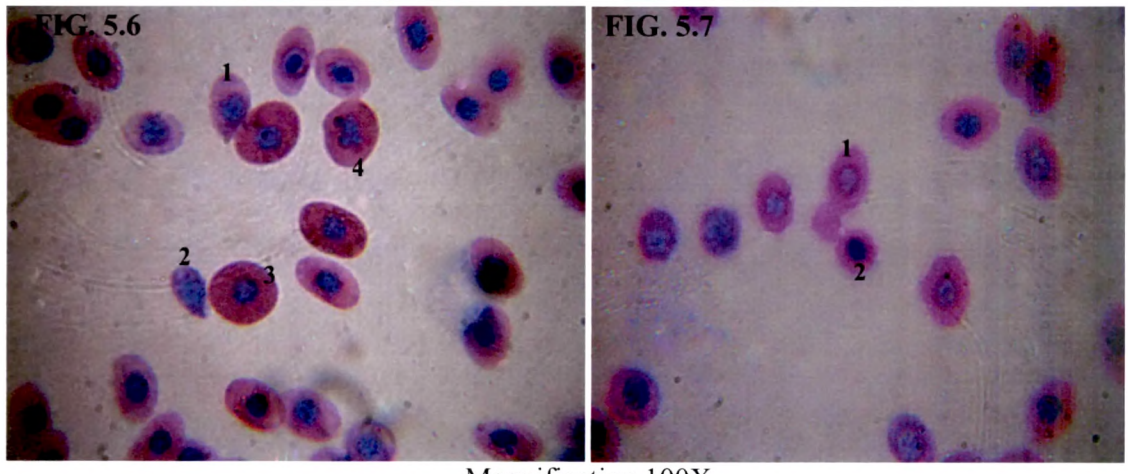


FIGURE 5.4 primitive NCE (1), NCE (2), early PCE (3), mid PCE (4), late PCE(5), mn PCE (6), mnNCE (7)

Magnification 100X
mnPCE : micronucleated polychromatic erythrocytes
mnNCE: micronucleated normochromatic erythrocytes
mnEs : micronucleated erythrocytes
PCE : polychromatic erythrocytes
NCE : normochromatic erythrocytes

Blood smear of 11 day embryo treated with combination insecticide showing abnormal cell morphologies





Magnification 100X

FIGURE 5.5 Thrombocyte (1), large lymphocyte (2), small lymphocyte (3), NCE with squashed nucleus (4, 5)

FIGURE 5.6 Dacryocyte or tear drop cell (1, 2), primitive NCE (3), primitive NCE with chromosomal aberrance (4)

FIGURE 5.7 Notched erythrocyte (1), microcyte (2)