GENERAL CONSIDERATIONS

The organism makes a humble beginning as a single fertilized cell – the zygote, which then divides mitotically to produce all the cells of the body. The fate of embryonic development involves a multifaceted coordination of cell proliferation, cell fate specification and differentiation that are determined by the actions of a legion of genes (**Motoyama and Aoto, 2006**). It undergoes vast and complicated mechanisms of developmental sequences and finally shapes up into the whole new complex multicellular organism with various specialized functional capabilities. Nevertheless, the limitation to these meticulous designs of embryonic development is that, they are vulnerable to the slightest of the perturbations caused by any extraneous chemical or environmental agent. In his much acclaimed monograph - the environment and birth defect **Wilson (1973)** for the first time postulated that the living organisms are at a greater risk to the adverse influences of the environment during their early developmental stages than at any other time in their life cycle. A relatively smaller dose of a toxic substance can induce alterations in the embryonic developmental processes by defeating the embryonic defences, interfering with the hormones that control the developmental decisions and inducing maladaptive developmental responses (**Hamdoun and Epel, 2007**). These alterations due to subtle intoxication could therefore, severely hamper the normal development resulting in malformation, growth retardation or even death.

Most organisms never stop developing since development does not stop at birth, or even at adulthood, it is a continuum from fertilization till the death of the organism (**Gilbert, 2006**). Development accomplishes two major objectives: it generates cellular diversity and order within each generation, and it ensures the continuity of life from one generation to the next. Therefore, a hassle created by an embryotoxicant in an organism during the period of early development, might as well be carried throughout its own lifetime as well as the succeeding generation too.

The highly maneuvered orchestration of developmental mechanisms has been mainly deciphered through the identification of conserved families of signaling pathways, which show a very well defined spatially and temporally controlled pattern of expression (**Aulehla and Pourquié, 2008**). It is becoming clear that each of these discrete events is controlled by a

distinct set of molecular signals. An accurate process of embryogenesis involves a highly organized interplay of these molecular signals and a failure or faulty expression of any of these can lead to disastrous consequences in the animal development. Nevertheless, they might fall an easy prey to extraneous agents since their susceptibility is high and the repair mechanisms are naive and yet developing (**Hamdoun and Epel, 2007**). A wide range of agents were identified to be inducing embryotoxic effects, for example, the drugs (Accutane, aminopterine, thalidomide, androgenic hormones, penicillamine, tetracyclines, diethylstilbestrol, nitrazepam, etc.), ionizing radiations, maternal infections (Cytomegalovirus, herpes, rubella, syphilis, toxoplasmosis, etc), environmental chemicals: heavy metals (e.g. lead, mercury), ethidium bromide, certain pesticides and narcotics (e.g. heroin, methadone, opiates, cocaine). Numerous varieties of the active ingredients are being introduced to the pesticide market. Many of the pesticides used as herbicides, insecticides, and fungicides are known to be increasingly linked to immune suppression, hormone disruption, diminished neuron functions, reproductive abnormalities and cancer (**Abhilash and Singh, 2009**).

Though the pesticides work miraculously in field of pest management, their limitations in the form of hazardous side effects to the non-target species cannot be overlooked. Of late, there has been an increased awareness regarding the health and environmental issues created by the pesticide usage. Yet, the annual pesticide consumption is getting increased year after year (**Abhilash and Singh, 2009**), and the new varieties introduced from time to time are major issues which need a regular monitoring. Moreover, the commercial formulations which are actually been used by the end user need to be tested for its toxic manifestations rather than the much practiced method of testing the active ingredients of the pesticide for its ill effects. This is because the otherwise inert ingredients in the pesticide formulations are often known to enhance the toxicities of active ingredients (**Cox and Surgan, 2006; Mansour *et al.*, 2008a**).

Moreover, the fact that a maternal exposure which might end up symptomless at both physiological or biochemical level to the recipient is still be able to potentiate toxicity to the developing conceptus is of grave concern (**Slotkin, 1999, 2004**). Therefore, the present study was focussed towards understanding the effects of two different commercial formulations of insecticides on the embryonic development. The first insecticide was a commercial combination of chlorpyrifos and cypermethrin marketed under the trade name *Anaconda 505*^{TM} and the second one was Spinosad, a naturalyte insecticide traded under the generic

name *Tracer*. The effect of these insecticides on the embryonic development was studied on the fertile RIR eggs by injecting them *in ovo*. The study in an *in ovo* system offers advantages of eliminating the maternal influences over development. Moreover, the dose of toxicant reaching the embryo is a critical determinant of developmental toxicity, and is likely to be a key factor responsible for interspecies variability in response to many test substances (**Carney, 2004**). Therefore, the *in ovo* studies enable one to understand the causative dose of embryotoxicity and direct effect of the test chemical on the embryonic development rather than by the metabolic by-products which happen to form from the dosed parent chemical in an adult organism as a part of metabolic disposition mechanisms (**Kotwani, 1998**). Therefore, the developmental toxicity evaluation of the combination insecticide and Spinosad were conducted on the fertile RIR egg.

The detailed procedures of the experiments, evaluations made, and the materials used were described in the section **Materials and Methods**. The first set of the experiments were done to evaluate the effective dose range of the two different classes of insecticides used in the study and their effects on morphological and skeletal development, and were described in **Chapter 1.** Different doses of the combination insecticide (0.001 to 0.8µg/egg) diluted in corn oil as well as Spinosad (0.05 to 3.5mg/egg) diluted in 0.4% methylcellulose were tested for their embryo lethality. The insecticide doses were injected by the air cell method (**Blankenship et al., 2003**) in volumes of 50µl/egg.

In the present study, the median lethal dose of combination insecticide (Ci) was worked out to be 0.109µg/egg, implying that the Ci is highly toxic to the developing embryos. Spinosad (Sp) on the other hand was lethal to half of the dosed embryos at 1.54mg/egg, which is quite higher than that of the Ci dose. This gives a clear picture that Spinosad is relatively less toxic to chick embryos in terms of lethality while the Ci exerted substantially higher toxicity. Subsequently the doses which were equivalent to $LD_{50}/10$, $LD_{50}/2$, and LD_{50} were selected for further study i.e. the Ci were dosed in concentrations of 0.01, 0.05 and 0.1µg/egg; while Sp was dosed in concentrations of 0.15, 0.75 and 1.5mg/egg. Controls were injected with their respective vehicles alone. Sham injections were given to ascertain if there was any stress on the embryo due to the egg injections.

The Ci treatment of RIR eggs (0.05 and 0.1µg/egg) lead to an alarming expression of various types of morphological and skeletal malformations which include crooked limbs, micromelia, missing phalanges, beak deformities, deformations in the vertebrae, sternum and ribcage,

craniorachischisis, etc. All these malformations were observed either in the hatchlings or in the unhatched/dead embryos. More or less similar malformations have been reported by various investigators (**Rao *et al.*, 1992; Anwar, 2003; Ahmad and Asmatullah, 2007**), while working with avian and murine models, treated with organophosphates or pyrethroid insecticides. In addition, the early embryonic observations of the Ci treated embryos (during the stages 10, 13, and 20) revealed that the somite number, size and pattern were inappropriate when compared to the control embryos. Many workers (**Moe *et al.*, 1978; Barnes *et al.*, 1996; Yusuf and Saberi, 2006**) while addressing the grotesque development in animal models have opined that the defects in embryonic somite formation and patterning could be responsible for such structural anomalies observed during the later phase of the life. Therefore, it is logical presume that the currently observed axial and appendicular skeletal anomalies in the Ci treated embryos and hatchlings could also be well associated to the somite irregularities observed during the early period of embryonic development.

Many other investigators however, have ascribed the vertebral defects to the decrease in the AChE or disrupted cholinergic system during the embryonic development (**Greenberg and La Ham, 1970; Walker, 1971; Landauer, 1975; Meiniel, 1978**). The appropriate synthesis and functioning of the cholinesterases during the embryonic development is of profound importance because, at an early stage of development these neurotransmitter molecules act as signalling molecules and provide the neurotrophic input and thereby regulate the proliferation, differentiation and migration of the target cells (**Slotkin, 2005**).

Further, craniorachischisis was observed in the Ci dosed embryos, which is an indication of failure of neural tube closure at the start of neurulation (**Smith and Schoenwolf, 1997; Murdoch *et al.*, 2001**). **Murdoch and co-workers (2001)** proposed that *Lpp1* (a novel gene) and *Shh* (which acts as a negative regulator of *Lpp1*) allow a precisely controlled midline bending of the neural tube closure. Consequently, it was hypothesize that a flaw in the expression of either of these genes might have resulted in the defects of neural tube closure in the Ci treated embryos.

Therefore, it could be summarized that the regulatory genes which play a role in somite formation, differentiation and neural tube closure; and the impediments to the AChE synthesis and/or functioning (refer chapter 3) could be envisaged as the probable candidates for the induction of the vertebral deformities in the Ci treated chicks.

Tracer, on the other hand, exhibited a toxicological profile which was relatively benign. Under the highest tested dose, i.e. 1.5mg/egg, only hydrocephaly and edematic condition could be noticed. No axial or appendicular deformities were observed at the tested dose levels of Spinosad. **Mansour *et al.* (2008b)** have demonstrated that Spinosad could inhibit AChE activity in albino rats. However, in this study, Sp if at all inhibited the AChE activity, it might have happened either at an innocuous magnitude that is beyond detection or inhibited at a later stage of development thus waiving of the possibilities of skeletal malformations.

The results of chapter 1 have shown that Ci can induce a variety of morpho-skeletal anomalies right from a decrease in hatchability and hampered growth to more serious conditions of skeletal malformations. It is likely that the action of Ci on the embryonic development involves more than just a cholinesterase inhibition. It could be because of multiple impairments *viz.*, biochemical or molecular lesion or interrupted cell signalling, inappropriate apoptosis, and/or defective closure of neural tube. Hitherto, the evidences discussed earlier indicate that the flaws in regulation of certain early embryonic events might lead to an erroneous development in the future organism. It was therefore, considered worthwhile to study certain early embryonic developmental events like the neural tube closure, neural crest cell (NCC) migration, sonic hedgehog (*shh*) expression and cell death during embryonic development (**Chapter 2**).

The rationale behind selecting these events was that, the craniorachischisis and defects of spinal cord are an indication of inappropriate neurulation or neural tube closure. Therefore, the morphologic and histologic profiles of embryos at stage-8 were studied through a combination of scanning electron microscopy (SEM) as well as light microscopy. The SEM results revealed that both the control as well as Spinosad treated embryos showed neural tube closure akin to that of standard references (**Brouns *et al.*, 2005**). However, in the 0.1µg/egg of Ci treated embryos the closure of neural tube was either initiated (along one side at the cranial region) or the closure was relatively incomplete.

The next important developmental process studied was the neural crest cell migration. The neural crest gives rise to migratory cells that colonise a wide range of embryonic tissues and later differentiate into neurones and glial cells of the peripheral nervous system (PNS), pigment cells (melanocytes) in the skin and endocrine cells in the adrenal and thyroid glands. In the head and the neck, the neural crest also yields mesenchymal cells that form craniofacial cartilages, bones, dermis, adipose tissue, and vascular smooth muscle cells (**Weston, 1970;**

Le Douarin, 1982; Tucker, 2001). The neural crest migration is therefore, a model system to study cell diversification during embryogenesis and phenotype maintenance in the adult.

Among the cranial neural crest cells, those arising from the posterior diencephalon and anterior mesencephalon give rise to the frontonasal skeleton, whereas those exiting from the posterior mesencephalon and form rhombomeres 1 and 2 colonize in the first branchial arch, to form the skeleton of the maxilla and mandible. The posterior rhombomeres yield neural crest cells, which participate in the formation of the medial and posterior parts of the hyoid cartilage (**Gilbert, 2006**). Hence, any defect in patterning, proliferation, migration or differentiation of the cranial neural crest cell population would contribute to the craniofacial malformations (**Dixon *et al.*, 2006**).

Immunohistochemical staining of the neural crest cells at the cranial level cross-section of the control embryos showed dense population of the neural crest cells all along the section, mainly concentrated along the regions of myelencephalon, notochord, the aortic arches, pharyngeal lining and the heart primordium. At the level of anterior somites, the neural crest cells (vagal) were populated along the spinal cord, the notochord, somites and the mesoderm. However, in the cross sections of Ci treated chick embryos, at the cranial level the neural crest cells were found scattered along the myelencephalon, and sparsely located along the aortic arches and the heart. At the level of somites, the staining for neural crest cells was very weak and was found dispersed along the spinal cord, notochord and somatic region. The absence or sparse population of the cranial and vagal neural crest cells might certainly explain the anomalies in the formation of the cranio-facial and visceral skeletal structures as reported in Chapter 1. Impediment to these migrations could be due to the disturbances created in the process of neurulation as discussed above, which might have further led to loss of the micro-environmental signals which guide the NCC migration. The Spinosad treatment, however, showed no deviation in the pattern of the neural crest localization compared to that of controls indicating that it creates no hindrance to the migration and patterning of cranial and vagal neural crest cells.

Apart from the micro-environmental signals which guide the NCC migration, the viability of these NCCs is depended on presence of the sonic hedgehog signalling (**Ahlgren and Bronner-Fraser, 1999, Jeong *et al.*, 2004, Brito *et al.*, 2006**). Expression of *Shh* peptides is also important in the induction of sclerotome in somites and floor plate, and motor neurons in the CNS, as well as the regulation of anterior-posterior polarity in the limb (**Martí, 1995**).

Shh also regulates the proliferation and survival of oligodendrocyte precursors (**Davies and Miller, 2001**), and of neural tube and neural crest cells (**Ahlgren and Bronner-Fraser, 1999; Garg *et al.*, 2001**). Taking into consideration the significant role played by the Shh in the somite formation (sclerotome), neural tube and the neural crest cell survival and the fact that these embryonic developmental events are of considerable importance in paving way for the skeletal and neuronal development; and further considering the results of chapter one (craniofacial and skeletal deformities) and the flaws in neural tube closure and neural crest cell migration (chapter 2) in the Ci treated chicks, it was felt essential to monitor the localization of the Shh. Therefore, the stage 12 chick embryos of the control, 0.05µg/egg of Ci and 0.75mg/egg of Sp treated groups were cross sectioned on a cryostat and were subjected to the immunohistochemical procedures to localize the *Shh* protein expression. The results revealed that in the control embryos at the cranial level, the *Shh* expression was found all along the developing brain (ventrally) and along the differentiating otic cup. At the level of somites, floor plate of the spinal cord and notochord showed a high expression of the *Shh* protein. The results of the control embryos were similar to that of the previous report by **Figdor and Stern (1993) and Marti *et al.* (1995)**. Whereas, the cross sections at the cranial level of the embryos treated with 0.05µg/egg of Ci treatment showed an improper differentiation of the brain, wherein the rhombencephalon was not differntiated and so did the *Shh*, whose expression seemed inappropriate. At the somite level the sonic hedgehog expression was found in patches along the ectoderm, and was not expressed in the neural tube and notochord. Therefore, it could be construed that though *Shh* signalling could be located, the magnitude and gradient of expression and/or one or more of the other downstream signalling sequences might have been disturbed, and that could have led to the defective differentiation observed in the brain of Ci intoxicated embryos. And ultimately the disruption of pathways for ventral brain development might have resulted in anomalies sighted as craniofacial malformations in chapter 1. Also, evidences by **Chiang *et al.* (1996)** bring to light that targeted disruption of the *Shh* gene in mice would lead to defective patterning of the axial skeletal structures. Therefore, the *Shh* signalling cascade which plays a critical role in patterning of vertebrate embryonic tissues, including the brain and spinal cord, the axial skeleton and the limbs, is disturbed by the interference of the Ci treatment and thereby led to the teratogenic anomalies sighted in this study.

In the Spinosad treated embryos no deviations in the expressions of sonic hedgehog have been identified and the results were in concomitant with that of the control, indicating that

this insecticide does not interfere with the gene expression of *Shh* with the current dosage tested (0.75mg/egg).

The next parameter studied under chapter two was the pattern of cell death in the early embryonic development. The normal embryonic development involves a closely regulated (spatio-temporally) phenomenon of programmed cell death (PCD). This fundamental process helps in achieving essential functions like removal of damaged, misplaced, abnormal or excess cells; sculpting structures during morphogenesis; removal of structures as during metamorphosis; controlling cell number, etc (**Clarke, 1990; Ellis *et al.*, 1991; Jacobson *et al.*, 1997; Hirata and Hall, 2000**). Consequently, it is of critical importance to the embryo that cell death be carefully controlled, for, if it fails to occur, or if it occurs excessively and/or inappropriately, abnormal development can result (**Maccabe and Noveroske, 1997**). Therefore, cell death pattern was monitored in the control, Ci treated and Sp treated embryos by vital staining (in stage-8, 25 and 34 embryos) and fluorescent staining (stage-6 and stage-12 embryos) methods. The results revealed that controlled apoptosis took place along the axis of neural tube closure and later along the digital primordia in the control embryos. However, in the Ci treated embryos, the cell death occurred more intensively along the neural crest and at irregular magnitude during the patterning of the limbs when compared to the controls embryos. The Spinosad treated embryos showed cell death pattern similar to that of controls. These results therefore, indicate that the Ci's toxic mechanisms apart from causing cell necrosis in developing embryos might have also deranged the regulation of programmed cell death.

As per **Weil *et al.* (1997),** PCD is required for the cell rearrangements that occur in the epithelial sheets when the neural folds fuse, and also there is evidence for occurrence of programmed cell death initially in neural crest precursors and the floor plate region at early stages (**Homma *et al.*, 1994**). However, a misregulated PCD during the neural tube closure would lead to a defect in the process. Here in this investigation, the Ci seemed to have induced cell necrosis and also an uncontrolled apoptosis and this overt cell death might have resulted in a loss of signalling molecules required for the neural tube closure, NCCs migration and survival.

Putting together the results of experiments in chapter two, it could be imperatively summarized that the Ci when injected to chick embryos at a dose of 0.05μg/egg has the potential to derail certain very significant events in the development. The unusual cell death

along the neural crest at an early stage might hinder the normal pattern of morphogenetic movements of the rest of the neural crest cells, and also could hamper the formation and transmission of the molecular signalling cascades. This disturbed morphogenetic movements and altered upstream signalling mechanisms might have created hindrance to the neural tube closure and also the migrations of neural crest cells. The teratogenic anomalies like malformations in the axial skeletal structures (craniofacial malformations and craniorachischisis) and in the hind limbs observed in the Ci treated embryos/hatchlings (chapter 1) can be correlated to these early developmental incongruities induced by the treated class of pesticide. On the other hand, Sp treatment at a dose of 0.75mg/egg did not target the above discussed critical milestones of development i.e. the neural tube closure, NCC migration and the sonic hedgehog expression hence, the embryos in this group developed without exhibiting any apparent deviation from the normal pattern.

All the above discussed malformations clearly depict the toxic inflictions of the insecticide treatment in the developing embryos. However, after the hatch, the chicks which are either indisposed or normal looking might be under the stress of the xenobiotic, which might thereby lower their competence for survival. As a pilot study to assess the extent of toxicosis, the haematological and biochemical evaluations are often used. Therefore, in the succeeding **Chapter-3**, the hemogram and certain biochemical parameters were monitored. An evaluation of the haematological parameters can be related to the prevailing physiological condition including the immune status of the organism. The monitoring of various enzyme activities (like ALT, AST, ALP, BUN, etc) in the serum would reveal a normalcy or injury to the internal organs like the liver or kidney.

The haemato-biochemical analyses were performed on day old hatchlings. 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 haematological estimations made were: total red blood cell count (TRBC), total white blood cell count (TWBC), differential white cell count (DC), haemoglobin (Hb), packed cell volumes (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), calculated as per standard methods.

The biochemical parameters evaluated were glucose, serum albumin, serum globulin, total serum protein, activities of enzymes such as alkaline phosphatase (ALP), alanine

aminotransferase (ALT) or serum glutamate pyruvate transaminase (SGPT), aspartate aminotransferase (AST) or serum glutamate oxaloacetate transaminase (SGOT), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and blood urea nitrogen (BUN) were estimated in the plasma, liver and brain homogenates. Also, 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.

The haematological parameters in hatchlings of 0.05 and 0.1µg/egg of Ci treated groups varied significantly with that of the control chicks. There was a decline in total blood cell counts along with a decrease in PCV, while the MCV, MCH and MCHC were normal. This indicates that the anemic condition is due to hampered erythropoiesis, cytotoxicity and/or internal haemorrhages, caused by the insecticide treatment. Chlorpyrifos as well as cypermethrin toxicities were individually studied earlier in rats (**Barna-Lloyd *et al.*, 1991; Goel *et al.*, 2006; Ambali *et al.*, 2009**) and chicks (**Qadri *et al.*, 1987**) and were shown to lower the values of RBC, Hb and PCV. It was opined that both chlorpyrifos (**Ambali *et al.*, 2009**) 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 Ci treated groups indicates adversely effected lymphoid progenitors; while the heterophilia observed in 0.1µg/egg of Ci treated group, might be in 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 subjected organophosphate pesticide. The number of circulating lymphocytes in peripheral blood is an index of functional ability of lymphoid organs. A reduction in the number of total lymphocyte may therefore, be an indication of lowered immunocompetence in the Ci treated chicks. However, lower dose (0.01µg/egg) of Ci showed no adverse effect on the hatchling's haematological parameters.

The haematological parameters of the hatchlings were unaffected by the treatment of eggs with 0.15mg of Spinosad. Nevertheless, significantly reduced levels of Hb were observed in the 1.50mg/egg treatment group. This was recognized as macrocytic hypochromic anemia, 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 resulted 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 leucocytopenia 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.

Analysing the results of the serum chemistry, increased glucose content was observed in 0.05 and 0.1µg/egg of Ci and 1.50mg/egg of Sp treatment group of hatchlings. In earlier studies it was discussed that pesticides could induce hyperglycaemia by several mechanisms including oxidative stress, inhibition of cholinesterase of the central or peripheral synapses that act in endocrine regulation of glucose metabolism and/or disturbances in metabolism of liver **(Matin and Siddiqui, 1982; Clement, 1985; Fletcher 1988; Kant *et al.*, 1988; Joshi and Rajini, 2009)**.

A significant decrease of albumin, globulin and total protein in the 0.1µg/egg of Ci treated groups and also a decrease of globulin and total protein in 1.50mg/egg of Sp treatment might be due to the damage to the liver or reduction in globulin synthesis by the plasma cells or could be because of decrease in number of plasma cells.

A marked increase in the activity of the enzymes ALT, AST and ALP has occurred in the present study due to the embryonic Ci (at a dose of 0.05 and 0.1µg/egg) treatment, which could be due to the insecticide induced toxicosis in the hatchlings' liver. The Spinosad treatment however, showed an increase of the above enzymes among the high dose group, though with a dose of 0.75mg/egg, only ALP activity was increased, while ALT and AST activities were comparable to the controls. 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, meaning that a damage to the renal function might occur at these doses.

The organophosphates, cypermethrins and a combination of these (Nurelle-D550, a Commercial formulation of chlorpyrifos and cypermethrin) have been shown to be cholinesterase inhibitors in the avian and mammalian systems as well (**Garcia-Cabero *et al.*, 1998; Abu-Qare *et al.*, 2001; Ashry *et al.*, 2002; Scharf, 2003**). 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 as well as BChE activities in serum, liver and brain. However, amongst the three tissues tested more pronounced decrease in AChE activity was observed in the brain of Ci treated animals. Sp treatment (0.75 and 1.5mg/egg) also led to a lowered AChE and BChE 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.

Subsequently, when the embryos were studied on the eight day of incubation, the Ci (at all the three tested doses) as well as Sp (1.50mg/egg) induce changes in the early AChE activities of the embryos, though the Ci has been highly potent inhibitor than Sp. This early stage inhibition of AChE would mean more than the disruption of the classical regulation of neurotransmission (**Koenigsberger *et al.*, 1997, 1998**). It might result in faulty axonogenesis or disrupt the fundamental process of brain development such as DNA synthesis (**Dam *et al.*, 1998; Whitney *et al.*, 1995**), alter the 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 current investigation, the Ci treatment at a concentration of 0.05 and 0.1µg/egg also showed significant disturbances in the synthesis of DNA and RNA in the brain, while the Spinosad showed lowered DNA and RNA quantity only at a higher dose. 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, the levels of nucleic acids could be sensitive indices to access the biochemical changes in response to the insecticide toxicity (**Pushpanjali *et al.*, 2005**).

Na^+-K^+ ATPase is an integral membrane bound hydrolytic enzyme concerned with immediate release of energy and also has a role 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**).

Analysing the results of the haematological and biochemical parameters in the early embryos and in the hatchling described in **Chapter 3** it is apparent that the Ci treatment induces an imbalance in the DNA and RNA content, thereby indicating a hampered protein synthesis, by creating a setback to the non-enzymatic functions of the cholinesterases. Even subtle changes in the activities these early molecules might lead to overt functional changes. The embryonic exposure to the insecticide in this study has occurred 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 *viz.*, cytotoxicity, and 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 far ahead of Sp in terms of inflicting the toxicity even at very low concentrations.

The results of three have shown the variations in the leucocytes and lymphocyte counts while looking into the haemogram of the insecticide treated group of chicks. This prompts one to further look into the immunological status of the chicks. Therefore, in the next chapter **(chapter 4)**, the insecticide treated birds were checked for the immune responses evoked after a challenge with a known antigen.

The defence mechanisms in a vertebrate act against a foreign antigenic invasion by evoking an appropriate immune response. These responses are quite sophisticated and function on an intricate balance. When a bacterial invasion is recognised in the blood circulation, the vertebrate immune system works to eliminate them by engulfing and later destroying them (through the phagocytic cells in the blood, neutrophils and monocytes). The mononuclear phagocytic system takes up the function of phagocytosis in the form monocytes in blood and macrophages in the tissues like spleen, liver and lymph nodes. The macrophages are unique in that they are crucial players in both innate and adaptive immune responses **(Qureshi, 2003)**. The heterophils which offer an innate immune response are also important phagocytic cells against microbial pathogens **(Stedman *et al.*, 2001)**.

Bacterial clearance test was conducted to study and compare the immune responses evoked in the two groups of *in ovo* insecticide intoxicated chicks by employing an *Escherichia coli* challenge into their blood streams. After stipulated time intervals of bacterial inoculation, the blood and tissue samples were collected and the viable bacterial colonies were retrieved from

the same and counted. An appropriately evoked immune response should be efficient enough to clear off the invaders gradually over the time. Therefore, the functional capability of the immune response could be judged by rate at which the bacteria are eliminated from the body. That is, an aptly functioning immune system would leave lesser number of viable bacteria in the blood or other tissue over a changing period of time. The results of this experiment revealed significantly high numbers of viable bacterial colonies from the blood streams of 0.1 and 0.05µg/egg of combination insecticide treatment when compared to the control groups. Further, the liver and spleen homogenates of these two groups of Ci treatment showed higher numbers of uncleared bacterial colonies. This indicates a compromised ability of bacterial clearance, which might be a consequence of weakened phagocytic and lytic potential of the monocytes and/or heterophils in the blood and macrophages in the liver and spleen. On the other hand, the Sp treatment led a decrease in the bacterial clearance ability only toward the end of the observation period at the highest dose tested (0.75mg/egg).

Further, NBT-salt reduction test was performed to assess the functional ability of splenic macrophages. The spleen is an immunologic filter of the blood. It is made up of B cells, T cells, macrophages, dendritic cells, natural killer cells and red blood cells. Owing to their scavenging and phagocytic functions, spleen macrophages are regarded to be important in the induction and maintenance of both innate and acquired immune defence mechanisms. The results of the NBT test showed that, the percentage of active macrophages in the 0.1 and 0.05µg/egg of Ci intoxicated chicks was significantly lower than that of control. While no significant changes were observed in the Spinosad treated groups. The lowered number of active phagocytes in the Ci treatment group could be probably explaining an adversely modulated immunogenic potency of antigen recognising cell as well as the phagocytic ability of the splenic macrophages.

During the embryonic development, the programming of the immune function is under close coordination by neural input. The xenotoxins that interfere with the development of the nervous system elicit corresponding immunologic deficits (**Navarro *et al.*, 2001**). Through different mechanisms the organophosphates (**Rice and Barone, 2000; Landrigan, 2001; Qiao *et al.*, 2004**) and also the pyrethroids (**Shafer *et al.*, 2005; Farag *et al.*, 2007**) are known to induce developmental neurotoxicity. It is therefore, likely that the immune suppression in the combination pesticide dosed chicks could be due to disturbances in neural development. The results analysed in chapter three have shown that the combination pesticide treatment lead to a decline in the cholinesterases activity in the day old chicks as well as in

the 8day embryos. The present observations are in agreement with the earlier reports that the immune development is under the control of neural input (**Madden *et al.*, 1995; Felten *et al.*, 1998; Navarro, *et al.*, 2001**).

The clinical estimations in the Sp treated group showed a decline in the acetylcholinesterase activity only at the higher dose, both in the 8 day embryo as well as day old chicks. Spinosad was reported to be non- neurotoxic to rats in acute, sub chronic or chronic toxicity studies and had shown no developmental effects (**EPA, 1997**). Therefore, the perceived non neurotoxicity to the developing embryos at the current tested levels of Sp treatment could be correlated to the alterations in the immune system.

The weights of thymus, bursa of Fabricius and spleen can be used to assess the relative immune status in poultry (**Rivas and Fabricant, 1988**). In the present study the absolute body weight and also the relative weight of thymus and spleen were significantly low at 0.1μg/egg of combination insecticide treated chicks, while the relative weight of bursa showed no variation compared to control. These lowered weights might be associated to a direct necrotic effect of the insecticide on the lymphoid tissue and leading to lowered numbers of T cells and macrophages, and thereby causing a deficit in antigen recognition and phagocytosis. At 0.01 and 0.05 μg/egg neither the body weight nor the relative weight of lymphoid organs showed any significant changes. However, in none of the Spinosad treated groups changes relating absolute body weight or relative lymphoid organ weight were observed.

A comparison of both the pesticide treated groups in terms of the rate of bacterial clearance and splenic phagocytic activity, it becomes increasingly apparent that the combination pesticide is far more potentiated to induce immunotoxicity in developing chick at quite lower doses while Spinosad was relatively mild in terms of inducing developmental immunotoxicity in chicks.

Lastly, making an overall analysis, and specially looking into the teratogenic effects, the altered cell death mechanisms, and loss of integrity of certain embryonic developmental mechanisms like the deranged sonic hedgehog signalling and neural crest cell movement, it would be of interest to look into the genotoxic inflictions if any. The genotoxic evaluations highlight the harmful changes in the genetic material of the cell which might affect its function and integrity. The study is significant since it not only manifest the injury to the

animal but also a likelihood of transmitting the same to its descendents. Hence, in the last chapter (**chapter 5**), the intoxicated embryos were screened for genotoxic effects.

The genotoxicity was ascertained in the developing embryos after 11 days of incubation through the micronucleus test and the comet assay. The study was conducted in both the classes of insecticide treated groups.

The nucleated erythroid cells form a majority of the cells in the circulating blood of chick embryo, while the thrombocytes are about 2% of all the blood cells and the rest of cell types could only be found occasionally. The definite erythrocytes from the bone marrow 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**). 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**). 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. Therefore, when a treatment evokes the formation of micronucleated erythrocytes, the actual numbers affected can be directly observed without much aberrance in the analysis. Hence, the 11day old embryo which carry mature as well as the other developing stages of the erythroid series were selected for the experiments.

The results of the micronucleus test revealed that the embryonic treatment with Ci was able to induce micronuclei in the erythrocytes and the effect increased with the treatment dose. The induction of micronuclei could be a result of abnormal mitotic activity, mainly lagging behind of acentric chromatid or chromosome fragments or even the whole chromosome during anaphase and metaphase; or due to the interphase chromatin diminution, irrespective of karyokinesis (**Choy *et al.*, 1989; Stopper and Muller, 1997; Manskikh, 2006; Davidkova *et al.*, 2007**). Also, the Ci treatment at 0.1µg/egg led to a decline in the PCE/NCE ratio, which is indicative of diminished hematopoesis. The decreased PCE/NCE ratio may also be due the cytotoxic effects of the insecticide.

Apart from micronuclei, the Ci treatment induced poikilocytosis in the circulating embryonic blood i.e. dacryocytes (tear drop cells), microcytes, erythroplastids, squashed/notched nuclei, nuclear segmentation and spindle shaped erythrocytes. The 0.1µg/egg of Ci treatment also showed large number of undifferentiated erythroblasts and proerythroblasts. As per **Wolf *et al.* (2002)**, by day 11 of embryonic development, the proerythroblasts and erythroblasts

would have been transformed to definitive erythrocytes; however, their retention at this stage could be due to 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. Moreover, 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. Though Spinosad is known to be a pesticide with reduced risk, the cytogenetic activity could be attributed to the Spinosad's chemical structure and/or certain impurities in the commercial formulation (**Mansour *et al.*, 2008a**).

The analysis of the data obtained from comet assay revealed that the Ci treatment at higher dose resulted in a significant increase in DNA strand breaks in all the tested tissues of chick embryos with the liver being most affected. However, the damage was significant only in the liver at a lower dose of Ci, compared to the controls. 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. In contrast to mammalian embryos, the avian embryo in early stages of embryonic development provides a wide range of metabolic activities, probably 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). While CYP 450 is primarily involved in detoxification, they 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. Further, 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**). In the light of the above discussion, it is reasonable to construe that a similar activation of microsomal mixed function oxidase enzymes in the liver of the treated embryo should be the reason for the high occurrence of genotoxicity (evidenced by the extent

DNA damage) observed in the embryonic livers of insecticide treated embryos as compared to the blood and brain.

Lastly, in the light of the present study of in-ovo embryotoxicity screening of two pesticide formulations, it could be concluded that the combination insecticide (chlorpyrifos 50% and cypermethrin 5%) is a very potent embryotoxin and if administered even at a very low dose it would evoke a myriad of malformation, hamper growth and derange key physiological functions in a non targeted species. In addition, it was observed that certain modulators/events of early embryonic development likes the sonic hedgehog signalling and neural crest cell movement, immunotoxicity and genotoxicity too got downregulated in the event of said pesticide poisoning. However, the Spinosad, the naturalyte class of insecticide, even when administered at a much higher dose, showed only subtle signs of embyotoxicity. The current results gain significance considering the fact that the chemical pesticide is an all important component of any Integrated Pest Management programme and no intense pest control programme yield success but for a suitable insecticide. Therefore, a comprehensive knowledge of relative toxicity of pesticide formulations to non-targeted species gain significance since that will help one better design a pest control programme which is more environmental friendly.