CHAPTER 2

IN-OVO EXPOSURE TO INSECTICIDE FORMULATIONS: SIGNS OF TOXICITY DURING EMBRYONIC DEVELOPMENT

INTRODUCTION

It is remarkable that in the process of organismal development, one single cell undergoes transformations and reorganizations which lead to a final arrangement where many different cells, tissues and organs work together in a cooperative and orchestrated way and make a complete organism. On the contrary, awe and distress arise when these remarkable and carefully manoeuvred developmental sequences go digressed on the slightest of intrusion by certain extraneous agents, and lead to ugly consequences of developmental anomalies and put the survival of the organism at stake. A birth defect surveillance study by **Patel and Adhia (2005)** showed that the congenital malformations effect 2.5% of infants and are responsible for 15% of perinatal mortality. The Ci induced developmental anomalies described in the preceding chapter show a resemblance to those reported in the human populations, i.e. the craniorachischisis by **Naveen *et al.*, 2010 (Figure 2.1a and 2.1b)**, vertebral deformities by **Wynne-Davies, 1975; Altunkas and Sarikaya, 2009 (Figure 2.2a and 2.2b)**, craniofacial malformation (**Fries and Katowitz, 1990**). The etiological factors of these birth anomalies in the human are often elusive, though predisposed to environmental factors or aberrations in the genetic makeup (**Wynne-Davies, 1975; Wallingford, 2005**).

Among the environmental factors, the pesticide exposure has growing concerns over their potency of inflicting teratogenic effects. Investigations on human populations (**Shaw *et al.*, 1995; Blatter *et al.*, 1997; Rull *et al.*, 2006**) showed that maternal proximity to agricultural fields sprayed with pesticides increases the risk of congenital malformations. Experiments on the avian and rodent models (**Barone *et al.*, 2000; Levin *et al.*, 2002; Icenogle *et al.*, 2004; Tian *et al.*, 2005; Ahmad and Asmatullah, 2007; Slotkin, *et al.*, 2008**) also accentuate on the teratogenic potential of the pesticides.

Further, laboratory experiments hitherto have emphasized that mutant rodent of certain signalling molecules, express dearth or deviation in the regular development process, which then leads to a developmental malformation. **Ingham and McMahon, 2001** substantiated that the *sonic hedgehog (Shh)* activity is crucial for the survival of the cranial neural crest cells and ventral neural retinal cells. In various other studies (**Chiang *et al.*, 1996; Milenkovic *et al.*, 1999; Litingtung and Chiang, 2000**) the mice knocked out for *Shh* or other components of the *Shh* signal transduction, most commonly show abnormalities in the neuronal development and neural tube defects like holoprosencephaly and exencephaly. In another study by **Murdoch *et al.*, 2001** in *loop tail (Lp)* mutant mice, a novel gene *Lpp1* which is negatively regulated by the *Shh,* was found to be associated with the craniorachischisis. All these evidences suggest that signalling molecules play a very critical role in precisely regulating the development of an organism.

The results of chapter 1 make it obvious that certain pesticides (Ci here in this study) are teratogenic and are capable of inducing developmental anomalies; and the earlier investigations (listed above) have implications towards the early developmental processes falling prey through altered signalling mechanisms, leading to malformations. Hence, it was considered worthwhile to monitor few of these early developmental processes which might probably give clues to how the test chemical has intervened with the patterns of the chick embryonic development and led to the malformations.

However, before proceeding with the actual experiments, a concise briefing about few of the important early developmental events (which were hypothesized to have been targeted by the pesticide treatment) and their significance as described in the treatise is discussed below.

The early embryonic development

The process of development starts with fertilization and proceeds as morphogenesis, i.e. blastulation, gastrulation, histogenesis, organogenesis, and functional maturity. The early cell movements during this process of development are complex, interwoven and follow a strict spatiotemporal pattern. The developmental progression takes the following sequence:

(1) Right after the fertilization, the zygote is formed and cleavage begins. At this stage, the egg undergoes an extremely rapid series of mitotic divisions, leading to a cluster of cells called the morula. At the end of cleavage, the cells now called blastomeres, tend to form a hollow sphere known as the blastula. Its interior is called the blastocoel.

(2) The subsequent process of cell rearrangement is called gastrulation. The cells migrate and lead to an invagination, forming the gastrula, a bilaterally symmetric three-layered structure. These germ layers are responsible for the origin of all tissues, organs and systems of the embryo. The outer layer, the ectoderm, fabricates the cells of epidermis and the nervous system. The inner layer, the endoderm gives rise to the lining of digestive tube and its associated organs. The middle layer, the mesoderm, gives rise to several organs (heart, kidney, and gonads), all kinds of connective tissues (bone, cartilage, lymph, and blood), muscles etc, during further embryogenesis.

(3) The next step is organogenesis. After the formation of the three layers, the cells interact with each other producing the organs. During organogenesis, certain cells can undergo long migrations from their original place to their final location.

(4) The final stage is growth and maturation, where most of the organs and systems, already formed, acquire their functional capabilities. At this point, another very important cell differentiation takes place, when a portion of egg cytoplasm is set apart to originate the germ cells, the precursors of the gametes. All the other cells are called somatic cells. The germ cells typically migrate to the gonads, where they differentiate into gametes in a process called gametogenesis. The production of the gametes is only completed when the organism reaches its sexual maturity, usually after its birth.

Among the stages a fertilized egg undergoes until reaching its final shape, gastrulation represents the first step in breaking its initial symmetry. This process is of enormous importance in establishing the embryo's sagittal symmetry plane or dorso-ventral axis (**Castro-e-Silva and Bernardes, 2005**). As a result of the cellular movements during gastrulation, cells are brought into new positions, allowing them to interact with cells that were initially not near them. This paves the way for inductive interactions, which are the hallmark of neurulation and organogenesis. Neurulation involves the formation of a flat neural plate and its subsequent transformation into a hollow neural tube. This process is complex and regulated by many genetic and environmental factors. In chick, the process of neural induction begins before the onset of gastrulation, with competence being conferred by FGF signals emanating from the posterior of the embryo (**Muhr *et al.*, 1999; Streit *et al.*, 2000; Wilson *et al.*, 2000; Wilson and Edlund, 2001; Wilson *et al.*, 2001**). Neurulation involves a precisely orchestrated set of morphogenetic movements, which as an intrinsic processes in the neural plate include elongation and shaping of the neural plate by cell rearrangement, cell division; elevation and apposition of the neural folds; and fusion of the

folds at the dorsal midline (**Jacobson and Gordon, 1976; Schoenwolf and Alvarez, 1989; van Straaten *et al.*, 1993; Davidson and Keller, 1999**).

Neural tube Closure

The formation of a neural tube from an initially flat sheet of neuroepithelial cells is among the first and most critical events in the development of the central nervous system in the vertebrates. Under normal circumstances, the spinal cord and brain are surrounded and cushioned by cerebrospinal fluid and are protected by outer layers of tissue called the meninges. The brain is further protected by the skull, and the spinal cord by the vertebral column formed of serially arranged vertebrae, which form flexible protective armour of bone. A failure in the process of closure of the neural tube leads to neural tube defects (NTDs), which are among the most common human birth defects (**Murdoch, 2001**). NTD leaves one or more gaps in the brain or spinal cord's protection. This can affect the brain's development and can leave the spinal cord vulnerable to damage. Because the spinal cord contains the nerves that control body activities and movement, any damage that occurs can paralyse or weaken associated muscles and organs. These disorders arise early in foetal development and can cause life-long complications of varying severity. These debilitating conditions affect thousands of families each year. It is estimated that out of every 1000 pregnancies, one suffers a neural tube defect (**Murdoch, 2001**). The main NTDs are anencephaly, encephalocele, and spina bifida (including meningocele and meningomyelocele). Folic acid supplementation has dramatically reduced the frequency of these defects, but roughly 30% of NTDs appear to be resistant to folic acid, and may in many cases be the result of genetic lesions (**Wallingford, 2005**).

The high frequency of closure defects in human embryos (**Nakatsu *et al.*, 2000**) indicates the vulnerability of this process, and has triggered numerous investigations into the fundamental aspects of neurulation (**reviewed in Gordon 1985; Sadler 1998; Colas and Schoenwolf 2001; Copp *et al.*, 2003**). Apart from the direct gene knockout studies in mice (**Brouns *et al.*, 2000; Hamblet *et al.*, 2002; Curtin *et al.*, 2003**), the maternal exposure to various environmental chemicals including agricultural pesticides were found to be associated with the neural tube closure defects (**Shaw *et al.*, 1995; Blatter *et al.*, 1997; Rull *et al.*, 2006**).

Neural crest cell movement

The formation of neural crest is a key feature of vertebrate development. The neural crest cells arise from the dorsal margin of the neural tube by delamination shortly after the fusion

of the neural folds and migrate ventrally and laterally along specific pathways to form the neurons and glia of the peripheral nervous system as well as the facial skeleton (**Weston, 1970; Le Douarin, 1982; Tucker, 2001**). It has been indicated that the development of neural crest cells is under the influence of micro-environmental signals (**Dupin *et al.*, 1993; Stemple and Anderson, 1993; Bronner-Fraser, 1994**). The induced neural crest cells express *slug* which is a characteristic of cells that break away from an epithelial layer and subsequently migrate as mesenchymal cells. The migration of neural crest cells involves the loss of cell-to-cell adhesion molecules. This includes the loss of cadherins, which use calcium ions to attach to like cells. The migrating neural crest cells travel to the basal lamina. They can either penetrate through the basal lamina or travel along it. Neural crest cells may also travel along extracellular matrices that are present in the area using integrins (**Wakamatsu *et al.*, 1997**).

The migratory pathway taken by the neural crest cells is determined by the positional value the cells take along the anterior-posterior axis of the embryo. According to **Bronner-Fracer (1998)**, the fate of the neural crest cells along the rostro-caudal axis of the embryo would be as follows:

Cranial neural crest cells: Connective tissue and skeletal contributions to the face, Schwann cells, ciliary and cranial sensory ganglia.
Vagal neural crest cells: Enteric nervous system.

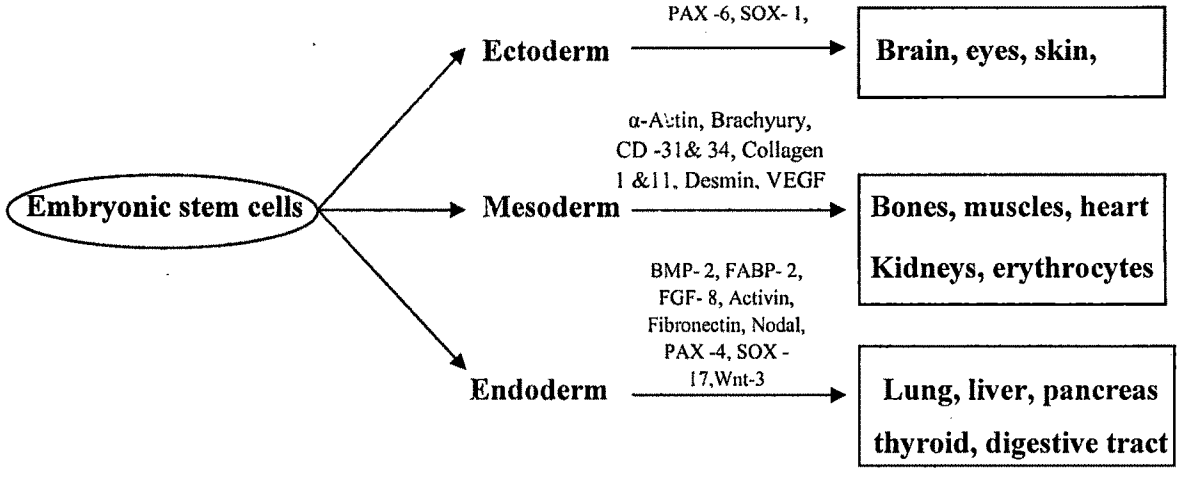
Trunk neural crest cells: Melanocytes, sensory and sympathetic ganglia, Schwann cells, adrenomedullary cells.
Lumbo-sacral neural crest cells: Enteric nervous system

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. Therefore, 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**).

There are ample evidences to believe that *Shh* is important for the survival of facial skeletogenic neural crest cells migrating to the first branchial arch (**Ahlgren and Bronner-Fraser, 1999, Jeong *et al.*, 2004, Brito *et al.*, 2006**). The Possible candidates for the source of *Shh* for facial skeletal development are: prechordal plate, foregut endoderm, the floor plate, the notochord and the facial ectoderm. Hence a failure of the expression in the *Shh* as well might lead to the craniofacial malformations.

The early gene expression - Sonic hedgehog

The embryonic stem cells are pluripotent, and are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm when suitably stimulated by the co-ordinated sequential patterns of various gene expressions.



Shh, a member of the Hedgehog (Hh) family of secreted signalling proteins carries out diverse functions during vertebrate development. *Shh* encodes a signal that is implicated in both short and long range interactions that pattern the vertebrate central nervous system (CNS), somites and limbs. Immunostaining of chick embryos indicates that *Shh* peptides are expressed in the notochord, floor plate and posterior mesenchyme of the limb at the appropriate times for their postulated patterning functions (**Martí, 1995**). Temporal expression of *Shh* peptides is consistent with 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**). In the limb, a signalling centre localized in posterior mesenchyme, the zone of polarizing activity (ZPA), controls anterior-posterior polarity (**Saunders and Gasseling, 1968**). Several lines of evidence suggest that the ZPA is

the source of a diffusible factor or factors whose concentration determines polarity: high concentrations for posterior structures, low concentrations for more anterior limb elements (**Tickle, 1981**).

The cell death during embryonic development

The programmed cell death (apoptosis) is one of the important events occurring during the embryonic development and does embrace critical 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**). Apoptosis is involved at all stages of development examined: removing cells before formation of the primitive streak; at sites of active growth/invagination such as the anterior neural tube, otic and optic placodes, branchial arches and tail bud primordium; at sites of morphogenesis as in neurulation, branchial arch and lens formation; in association with active cell migration of neural crest and sclerotomal mesenchymal cells and in closure of the body wall.

Vertebrate limb development provides a paradigm for developmental apoptosis. Programmed cell death (PCD) occurs in well-defined domains and sculpts the shape of the limb by eliminating cells between the differentiating cartilages (**Hurle *et al.*, 1996**). In the early chicken limb bud, the most prominent sites of apoptosis are located in the anterior (ANZ) and posterior (PNZ) necrotic zones and in the apical ectodermal ridge (AER). Later, massive cell death takes place in the mesodermal web (interdigital necrotic zone, INZ) separating the digits (**Grotewold and Ruther 2002**). Bone morphogenetic proteins (BMP) have been identified as important signals triggering cell death in these zones (**Yokouchi *et al.*, 1996; Pizette and Niswander, 1999**). In sharp contrast, BMPs also promote the formation of bone (**Duprez *et al.*, 1996; Buckland *et al.*, 1998**). These opposing activities reside in close vicinity to each other in the developing limb, namely in the interdigits versus the digital rays (**Grotewold and Ruther 2002**).

OBJECTIVE

The developmental anomalies *viz.*, the neural tube defects, craniofacial malformations and deformities in the limbs observed in the early embryos as well as the hatchlings in the combination insecticide treated group (chapter 1) prompted one to investigate the possible mechanisms by which this class of insecticide exerts their neural and craniofacial

malformations. The following early developmental milestones were studied to unearth the answers for the above notion.

(i) Neural tube closure defects

(ii) Neural crest cell (NCC) migration

(iii) Sonic hedgehog expression

(iv) Cell death during embryonic development

The Spinosad even though did not show overt signs of similar malformations was also included in the current study owing to its reported neurotoxic nature.

MATERIALS AND METHODS

Egg injections and dosing

The egg injections were performed as per **Blankenship *et al.* (2003)** on day '0' of incubation, The Ci treatment group received 0.05µg/egg of Ci while Spinosad treatment group received 0.75mg/egg of Sp. Two different groups of vehicle controls were given corn oil (VC1) or 0.4% methylcellulose (VC2). The insecticide treated as well as the control embryos were subjected to the following observation and a detailed methodology for these experiments is described in materials and methods in the beginning of this treatise.

Neural tube closure

Scanning electron microscopy

Neural tube closure was studied by observing the stage-8 embryos under scanning electron microscope. Embryos fixed in 4% phosphate buffered paraformaldehyde were dehydrated and transferred to 100% acetone and critical point dried, mounted on Cambridge stubs and gold coated. Then they were observed and photographed under scanning electron microscope (JEOL, JSM-5600).

Neural crest cell migration

The neural crest cells were located in stage-12 chick embryo by immunohistochemical staining. The fixed and endoperoxidase activity blocked sections were incubated with serum containing primary antibodies against the neural crest cells and subsequently located with a secondary antibody (rabbit anti-mouse IgG-HRP), and viewed by staining with Streptavidin – HRP complex and DAB system.

Sonic hedgehog expression

The Sonic hedgehog expression was located in cryostat sections of stage-12 chick embryo by immunohistochemical staining. The fixed and endoperoxidase activity blocked sections were incubated with serum containing the primary antibodies, anti-*Shh*, and subsequently located with a secondary antibody (rabbit anti-mouse IgG-HRP) and viewed by staining with Streptavidin – HRP complex and DAB system.

Cytotoxicity

Cell death was detected by two methods: a vital staining method and a fluorescent staining method.

a) Nile blue sulphate staining

The whole embryos at stage-8, stage-25 and stage-34 were dissected in lactated ringer's solution and subjected to the Nile blue sulphate staining according to **Jeffs and Osmond (1992)**.

b) Acridine orange and ethidium bromide staining

Thin cross sections (4-6μm) of the embryo of stage-6 and stage-12 were taken on a cryostat and stained with acridine orange and ethidium bromide (**Ribble *et al.*, 2005; Natesan, *et al.*, 2006**). The stained sections were observed under a fluorescent microscope (Leica, DM2500). Acridine orange stains live cells and fluoresces green while ethidium bromide stains dead cells and fluoresces orange/red.

RESULTS

Neural tube closure defects

The results of scanning electron microscopy revealed that, the embryos of control group showed the neural tube completely fused at the levels of mesencephalon and metencephalon and posterior prosencephalon (**Figure 2.3**). The anterior neuropore was found narrowing (**Figure 2.3a**). A caudally directed wave of closure was observed. However, the closure was not initiated at the posterior neuropore and was found open. Groups of cells, presumably the neural crest cells were found to be migrating into the neural tube at various sites (**Figure 2.3b**).

The combination insecticide treated embryos showed neural tube closure defects. The scanning electron microscopy picture of the 0.1μg/egg of Ci treated embryo (**Figure 2.4**)

showed that the neural tube closure was initiated at the cranial region but only on one side (**Figure 2.4a**). And, the closure was not complete. However, migrating cells into the neural crest were observed at the somitic region, though the cell migration as compared to the control was less at the cranial end (**Figure 2.4b**).

The Spinosad induced no significant defects in the closure of the neural tube. The migration of the cells was comparable to that of the control embryos.

Neural crest cell migration

At the cranial level, the cross section of the control embryo showed dense population of the neural crest cells (cranial) all along the section, mainly concentrated along the regions of myelencephalon, notochord, the aortic arches, pharyngeal lining and the heart primordium (**Figure 2.5a**). At the level of anterior somites, the neural crest cells (vagal) were populated along the neural tube, the notochord, somites and the mesoderm (**Figure 2.5b**).

In the cross sections of 0.05μg/egg 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 (**Figure 2.6a**). At the level of somites, the staining for neural crest cells was very meagre and was found dispersed along the neural tube, notochord and somitic region (**Figure 2.6b**).

The embryos treated with Spinosad, when cross sectioned, showed dense population of the neural crest cells, concentrated along the regions of myelencephalon, notochord, the aortic arches, pharyngeal lining and the heart primordium (**Figure 2.7a**). At the level of somites, the neural crest cells were populated along the neural tube, the notochord, somites and the mesoderm (**Figure 2.7b**).

Sonic hedgehog expression

In stage-12 control embryos, the sonic hedgehog expression was found all along the developing brain i.e. the mesencephalon, metencephalon and myelencephalon. The head mesenchyme did not show the expression. A differentiating otic cup also showed the expression of sonic hedgehog protein (**Figure 2.8a**). At the level of somites, floor plate of the spinal cord and notochord showed a high expression of the protein (**Figure 2.8b**).

The cross sections at the cranial level of the embryos (stage-12) treated with 0.05μg/egg of Ci treatment showed an improper differentiation of the brain. These insecticide treated embryos after 48hr (stage-12) of incubation seemed akin to an earlier stage i.e. a lag in development

was observed. It showed the mesencephalon and the notochord surrounded by the head mesenchyme, while the rhombencepahlon was not differentiated. The *Shh* expression was found all along the mesencephalon and the notochord (**Figure 2.9a**). In the embryo cross section at the somite level, the sonic hedge expression was absent in the neural tube and notochord (**Figure 2.9b**).

In embryos treated with Spinosad (0.75mg/egg), the expression was similar to the control. At the cranial level the section showed *Shh* expression along the mesencephalon, metencephalon, myelencephalon and the otic capsule (**2.10a**). At the level of somites, the section showed the expression of sonic hedgehog protein akin to that of the control (**2.10b**).

Cytotoxicity

a) Nile blue sulphate staining

Vital staining of the stage-8 whole embryos with nile blue sulphate has shown that the cell death occurred in the control embryos along the closure of the neural tube. The cell death occurred more intensively along the neural crest in the Ci treated embryos when compared to the controls, while the Spinosad treated embryos showed cell death similar to the controls.

The NBS staining of the control group of embryos at stage-25 showed cell death along the margins of the wing bud and limb bud and along the outer margin of the eye (**Figure 2.11**). In the 0.05µg/egg of Ci treated group at stage-25, the cell death could not be located along the margins of the limb buds and wing buds (**Figure 2.12**). The cell death in the 0.75mg/egg Spinosad treated group of embryo at stage-25 occurred as in the control group. However, two of the six embryos observed showed haemorrhages in the cardiac region (**Figure 2.13**).

In stage-34 control embryos, contours of webs between digits and toes are concave and arched and were similar to the descriptions of **Hamilton and Hamburger (1951)**. The cell death at this stage was prominent and took place along the inter-digital region of the hind limb buds (**Figure 2.14a**) as well as the wing buds (**Figure 2.14b**). The intensity of apoptosis seemed alike in both the left and right limbs of the embryo. By stage-34, the 0.05µg/egg of Ci treated embryos showed cell death which was either more or less than in the control. **Figure 2.15a** shows an eight day old embryo, where the hind limbs differed in the intensity of the inter-digital cell death. One limb had cell death in the inter-digital region at a higher rate and also cell death was spotted on the dorsal side of the limb; while the other limb had a low frequency of cell death. Haemorrhages were also located on the hind limb regions of these embryos. The wing bud also showed a patch of restricted cell death (**Figure 2.15b**).

In the stage-34 Sp treated chick embryos, no abnormalities were sighted in the interdigital region of the wing buds and limb buds and were similar to that of the control counterparts.

b) Acridine orange and ethidium bromide staining

The cryostat sections of the control embryos at stage-6 showed minimal cell death along the neural crest region (**Figure 2.16a and 2.16b**). In the combination insecticide treated embryos (0.05µg/egg), the cell death was much higher along the neural folds in the stage-6 embryos (**Figure 2.17a and 2.17b**).

By stage-12, the cell death could be located along the somites and the neural tube (**Figure 2.18**) in the cross section of control embryos. In the combination insecticide treated embryos, the cell death was much higher in stage-12 embryos and appeared to be predominant along the neural tube which also failed to close (**2.19a**). The lining of the foregut, at the level of pharynx and the truncus arteriosus also showed cell death (**Figure 2.19b**).

In Spinosad treated group, no abnormal cell death was located in the stage-6 and stage-12 embryos' cross sections. The cell death which appeared along the neural crest was assumed to be the normal PCD occurring during the embryonic development and was similar to that of the control embryos.

DISCUSSION

Neural tube closure

Neural tube formation (neurulation) is a highly complicated biological process controlled by a number of genes, growth factors, adhesion molecules and receptors (**Padmanabhan, 2006**). The study of human NTDs reveals distinct classes of these birth defects. For example, anencephaly defines a failure of the rostral neural tube closure, while spina bifida defines a failure of caudal neural tube closure. On the other hand, craniorachischisis refers to a total failure of closure along the entire length of the neuraxis (**Wallingford, 2005**). A review by **Wallingford (2005)** emphasizes on the mechanism of morphogenetic movements i.e. apical constriction (elevation and apposition of neural folds) and convergent extension (narrowing of the tissue in one axis while elongating in perpendicular axis) as being vital phenomenon for the closure of the neural tube. A multitude of gene expressions with discrete functions during the neural tube closure were identified like the *p190RhoGAP* and *shroom* which control apical constriction (**Brouns *et al.*, 2000; Martin, 2004**) and the *Dishevelled* (*Dvl*) and *flamingo/Celsr-1,* (**Smith and Stein, 1962; Hamblet *et al.*, 2002; Curtin *et al.*, 2003**) which

control convergent extension. Any of these genes when targeted, led to neural tube defects in the experimental animals. Therefore there are distinct mechanisms which drive the competent closure of the neural tube and any disturbance to these sensitive mechanisms would lead to the NTDs.

The NTDs are often positively correlated with the maternal exposure or proximity of residence to the agricultural pesticide usage (**Shaw *et al.*, 1995; Blatter *et al.*, 1997; Rull *et al.*, 2006**). Elevated risks of NTDs and anencephaly or spina bifida subtypes were also associated with exposures to chemicals classified as amide, benzimidazole, methyl carbamate or organophosphorus pesticides, and chlorpyrifos being one among them (**Rull *et al.*, 2006**). Further, chlorpyrifos has also been associated with inhibition of mitosis of neuroepithelial cells during neurulation in some animal studies (**Slotkin, 2004**); and cell division is one amongst the important phenomenon that occur during the intrinsic process of neurulation (mentioned in the introduction of this chapter).

The failure of neural tube closure in the Ci treated chicks (with a dose of 0.05 and 0.1μg/egg) is consistent with the above reports that certain pesticides are potent inducers of neural tube defects and the mechanism of this teratogenic effect could be due to the inhibition of the morphogenetic movements by restricting the signal molecules, or by inhibiting the cell division or by inflicting cell death. The Spinosad treatment showed no indications of a similar teratogenic effect unto the dose of 1.5mg/egg, thus disclosing a safety rather than meddling up with the early mechanisms of neurulation. The control embryos of stage-8 in the present study showed the closure of the neural folds at the level of the mid brain, which is consistent with description of **Hamburger and Hamilton, 1951**.

Neural crest cell migration

Apart from the cranial sensory ganglia and Schwann cells, the cranial neural crest cells contribute to the formation of facial skeletal structures and connective tissue, as well as the visceral skeleton; while the vagal neural crest cells contribute the enteric nervous system (**Bronner- Fraser, 1998; Kulesa and Fraser, 2000**). The neural crest cell migration in the control embryos, at the cranial as well as the vagal region, as observed in the present study were in accordance with earlier reports (**Tan and Morriss-Kay, 1986; Kulesa and Fraser, 2000**), and so did their normal development into healthy hatchlings. Considering the above facts regarding the contribution of the neural crest cells to the development of various embryonic structures, the strewn nature of the neural crest cells in the cranial as well as the

vagal regions in the Ci treated embryos gives an unambiguous explanation to the anomalies in the formation of the facial and visceral skeletal structures as observed in Chapter 1. Therefore, the Ci at a concentration of 0.05μg/egg imposed interruption to the migration and patterning of the neural crest cells in the cranial and vagal regions and thereby created the malformations in the axial skeletal structures. 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. In addition, the viability of these NCCs is depended on presence of the sonic hedgehog signalling, whose failure might have reduced their competency for survival. The Spinosad dosed at 0.75mg/egg, however, showed the neural crest localization similar to that of control embryos indicating that it creates no hindrance to the migration and patterning of cranial and vagal neural crest cells.

Sonic hedgehog expression

Sonic hedgehog expression as reported by **Marti *et al.* (1995)**, in stage-10 embryo shows graded activity. The *Shh* is localized at the prechordal plate mesoderm and ventral forebrain, when observed in a cross section through the forebrain. At the level of the 1st somite, the *Shh* is present in the notochord and floor plate. Further, towards the posteriorly placed somites, the *Shh* expression becomes very weak in the floor plate and finally at the level of caudal unsegmented mesoderm, it is present only in the notochord. By stage-18, *Shh* is restricted to the notochord, caudal to the forelimb buds. Along the developing central nervous system, sonic hedgehog expression in stage-15 chick embryo is restricted to the ventral midline in most of the brain, with exception to the dorsal diencephalon. By stage 20, the *Shh* undergoes a dorsal expansion at the zona limitans intrathalamica, a major pathway of axonal migration that lies at the boundary between prosomeres 2 and 3 in the diencephalon **(Figdor and Stern, 1993)**.

The results of the *Shh* expression in the stage-12 control embryo were found akin to the previous report by **Marti *et al.* (1995) and Figdor and Stern (1993)**. The *Shh* was clearly localized in the ventral line of the brain i.e. along the mid brain and hind brain of the CNS and at the somite level it was located along the notochord and slightly at the floor plate.

The expression patterns in the Ci treated embryos differed with that of the controls. At the cranial end, the brain was defectively differentiated, but the *Shh* expression could be localized. As per **Hayhurst and McConnell (2003)**, there are two pathways involved i.e. *Shh*

and *Nodal*, both of which can induce ventral cell fates in a mirror-symmetrical manner on either side of the midline of the brain. Therefore, it could be derived that though *Shh* signalling could be located, the magnitude and gradient of expression and/or one or more of the other signalling sequences could have been disturbed, and might have led to defective differentiation in the brain. And ultimately the disruption of pathways for ventral brain development might have resulted in anomalies sighted as craniofacial malformations in chapter 1.

Earlier investigation by **Chiang *et al.* (1996)** reported that targeted disruption of the *Shh* gene lead to defective patterning of the axial skeletal structures. The results of *Shh* localization of Ci treated embryos also showed a mosaic pattern of *Shh* along the notochord and floor plate, which differed from the controls. The sections through the anterior somites exhibited the *Shh*, though only in the notochord. Towards the caudal end, even before the ending of segmented region, the *Shh* expression withered away both in the floor plate as well as the notochord. 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 consistent 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).

Cytotoxicity

Programmed cell death is a regular and vital process during normal embryonic development. The developmentally programmed cell death occurs in a specific temporo-spatial pattern. 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, abnormal development can result (**Maccabe and Noveroske, 1997**).

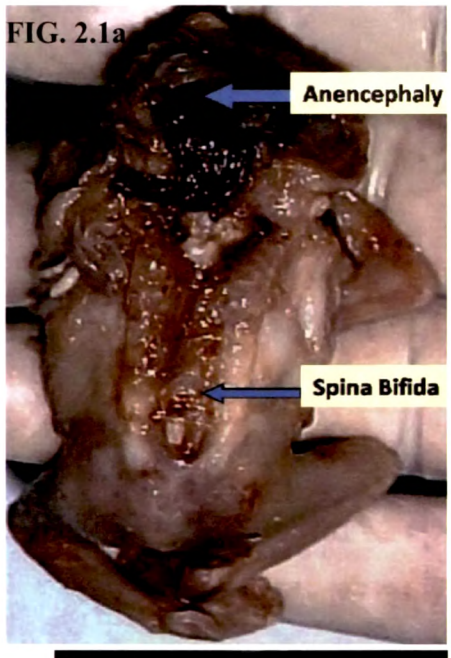
Vital staining and also staining with fluorescent dyes, showed controlled apoptosis along the axis of neural tube closure and later along the digital patterning in the control embryos. However, in the Ci treated embryos, the cell death occurred more intensively along the neural crest and at irregular magnitude during the patterning of the limbs when compared to the controls. The Spinosad treated embryos showed cell death similar to the controls.

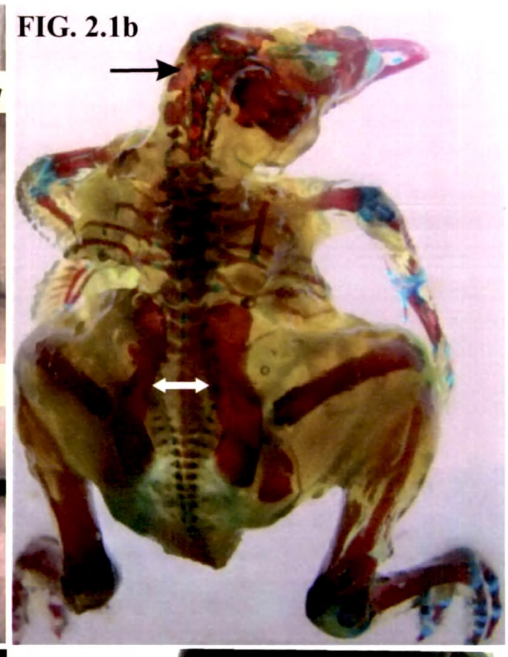
PCD is required for the cell rearrangements that occur in the epithelial sheets when the neural folds fuse, and may in a similar way, be required wherever epithelial sheets fuse during animal development (**Weil *et al.*, 1997**). During spinal cord development, there is evidence for programmed cell death initially in neural crest precursors and the floorplate region at early stages (**Homma *et al.*, 1994**) and subsequently in ventral motor neuronal populations (**Lance-Jones, 1982**). However, an inappropriately controlled 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. Further the interesting observation that, in the stage-25 embryos the cell death was not initiated, while the cell death appeared to be more intense by day 8 suggest that, Ci treatment had led to alterations in cell signalling cascade directed towards apoptosis, and thereby interrupted the control over the programmed cell death.

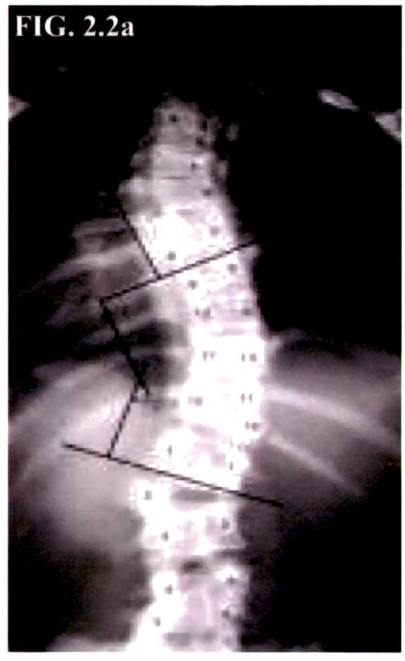
SUMMARY

A careful observation of certain events occurring in the early developmental period in chick revealed that the Ci treatment induced a sequence of signs of toxicosis. A dose of 0.05µg/egg of Ci had induced cell death/uncontrolled apoptosis during late phase of gastrulation (stage-6, 8 and 12) and also during organogenesis (stage- 25 and 34). This uncharacteristic 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 deranged morphogenesis and altered upstream signalling mechanisms might have created obstacles to the neural tube closure and the migrations of neural crest cells. The teratogenic anomalies like craniorachischisis, craniofacial malformations, malformations in the visceral skeletal structures 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 mechanisms of development i.e. the neural tube closure, NCC migration and the sonic hedgehog expression, and hence were structurally similar to the control embryos. In the light of the present findings it could be concluded that the Ci is a potent teratogenic agent, while the Sp seems to be much safer in terms of inflicting embryopathy.

Comparison between axial deformities observed in human foetus and Ci treated chick embryo







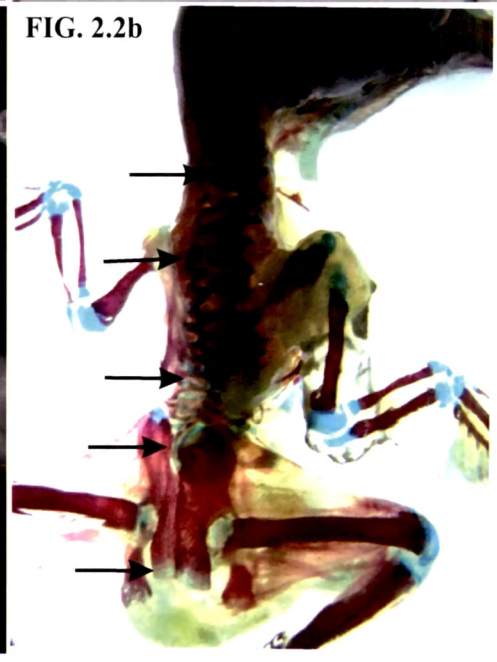


FIGURE 2.1a and 2.1b Craniorachischisis in human foetus and 0.1µg/egg Ci treated chick embryo

FIGURE 2.2a and 2.2b Deformed vertebral column in human showing a similarity to that of chick embryo treated with 0.1µg/egg Ci.

Scanning electron microscopy of control embryo (stage-8)

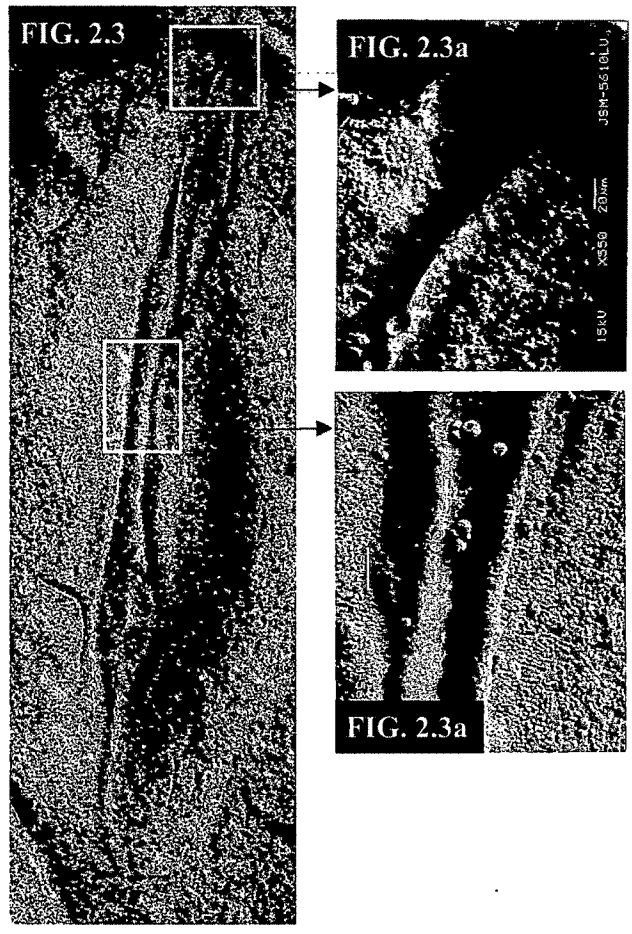


FIGURE 2.3 Neural tube completely fused at the levels of mesencephalon and metencephalon and posterior prosencephalon. The anterior neuropore was found narrowing (**FIGURE 2.3a**). Closure was not initiated at the posterior neuropore. Groups of cells were found to be migrating into the neural tube at various sites (**FIGURE 2.3b**)

Scanning Electron microscopy of 0.1µg/egg of Ci treated embryos

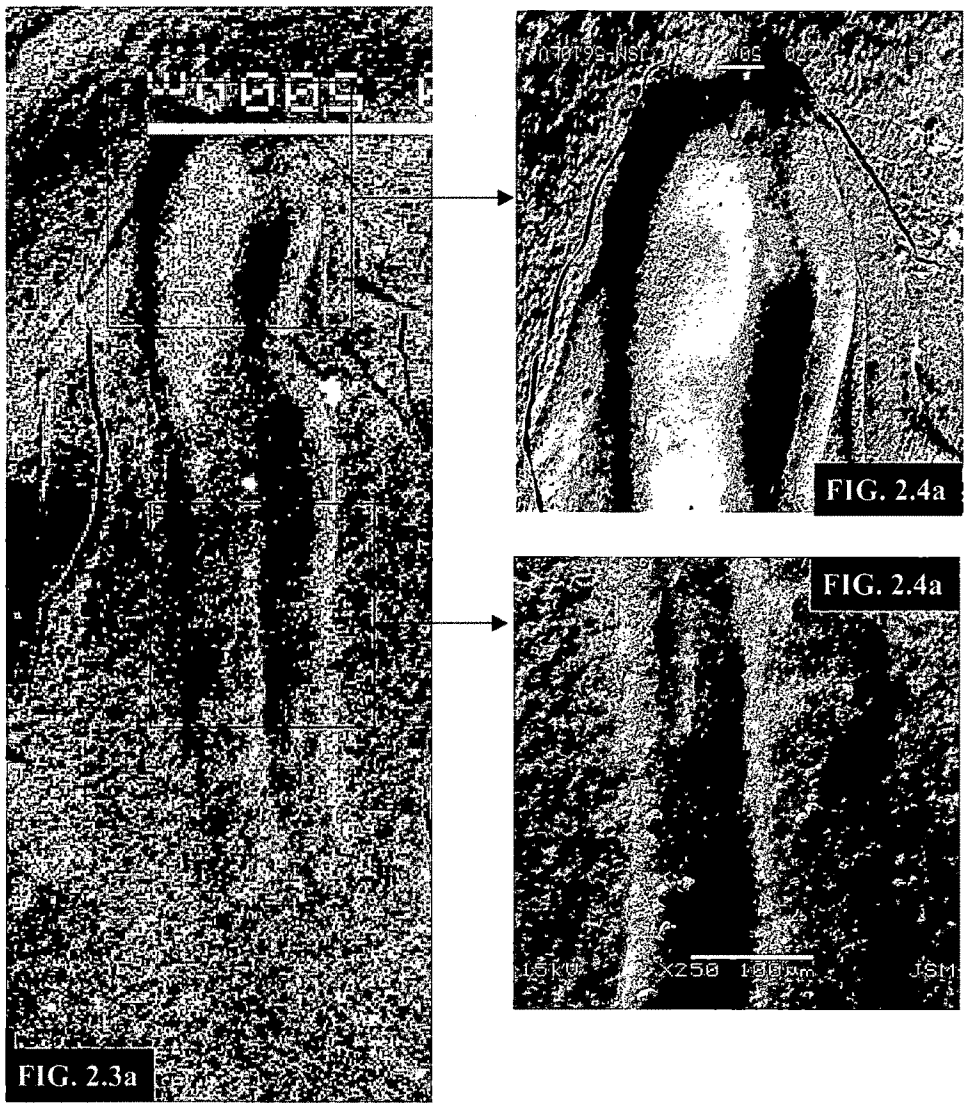
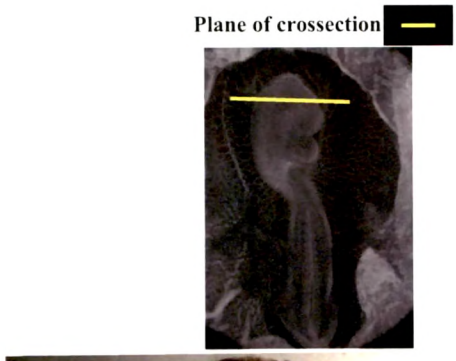
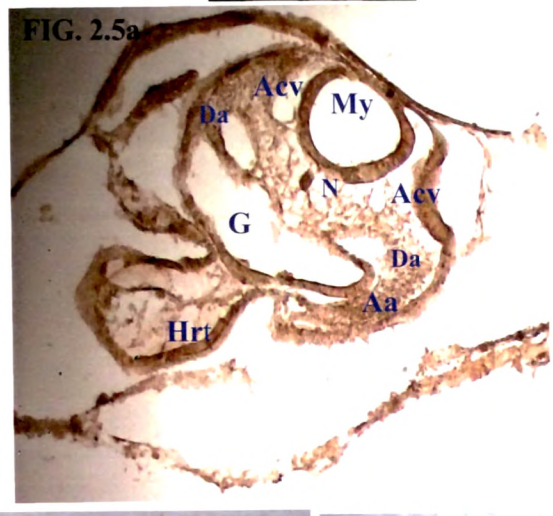
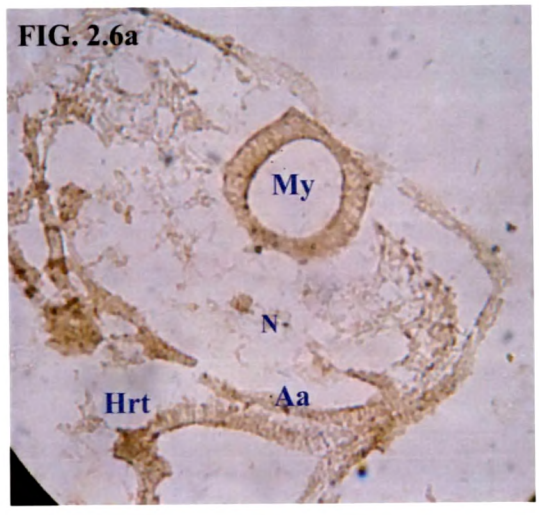


FIGURE 2.4 Scanning electron microscopy of 0.05µg/egg of Ci treated embryo showing neural tube closure initiated at the cranial region but only on one side (**FIGURE 2.4a**) and cells migrating into the neural crest at the somitic region (**FIGURE 2.4b**).

Immunohistochemical localization of Neural Crest Cell in the Cranial region of stage-12 embryo







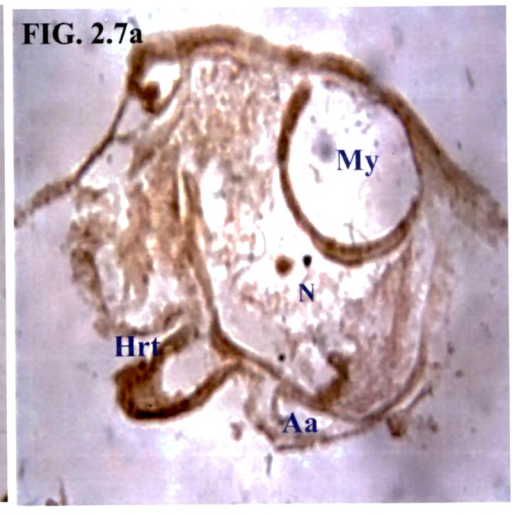
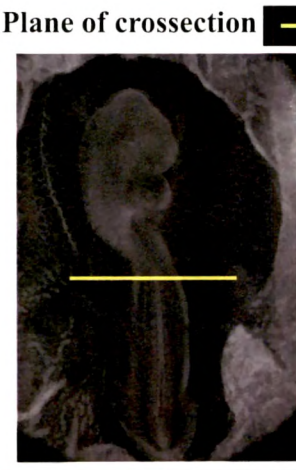


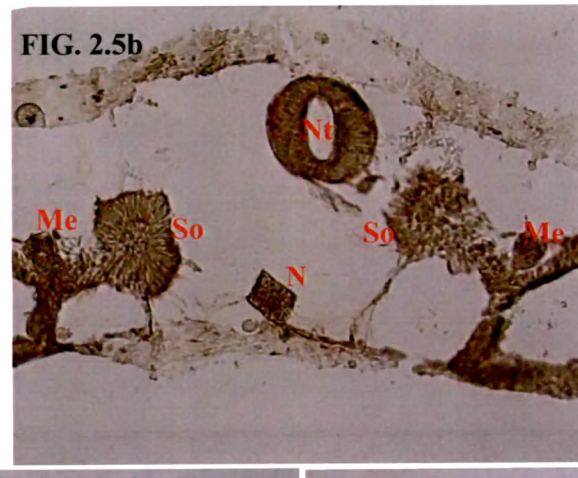
FIGURE 2.5a Neural crest cells (cranial) in control embryo, mainly concentrated along the regions of myelencephalon (My), notochord (N), the aortic arches (Aa), pharyngeal lining (G), dorsal aorta (Da) and the heart primordium (Hrt).

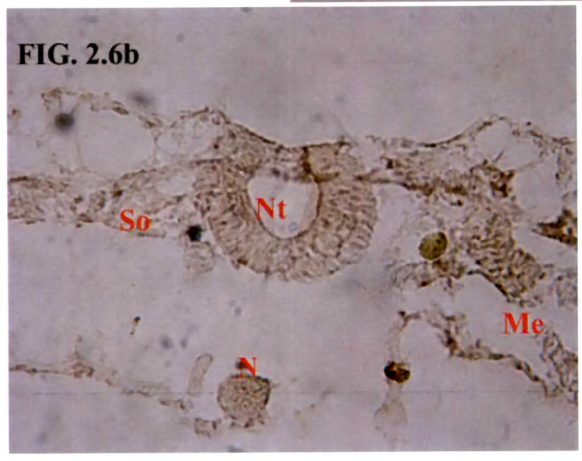
FIGURE 2.6a The cross sections of 0.05µg/egg of Ci treated chick embryos at the cranial level showing neural crest cells scattered along the myelencephalon (My) , and sparsely located along the aortic arches (Aa) and the heart (Hrt).

FIGURE 2.7a The embryos treated with spinosad, showed dense population of the neural crest cells, concentrated along the regions of myelencephalon (My), notochord (N), the aortic arches (Aa), pharyngeal lining (G) and the heart primordium (Hrt).

Immunohistochemical localization of Neural crest cells in the anterior somite region of stage-12 chick embryos







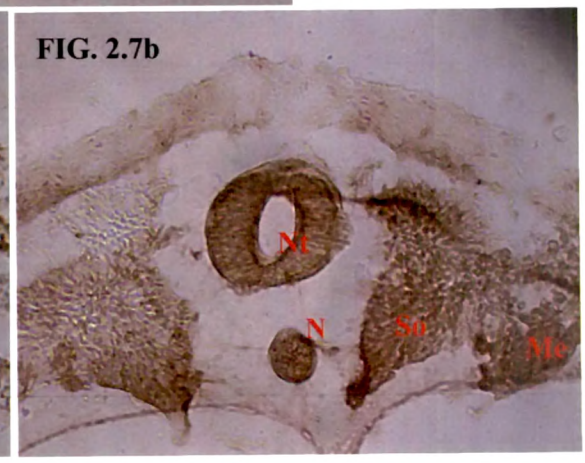
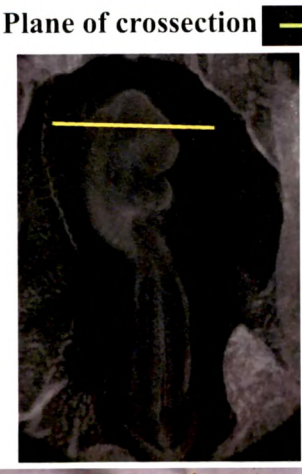


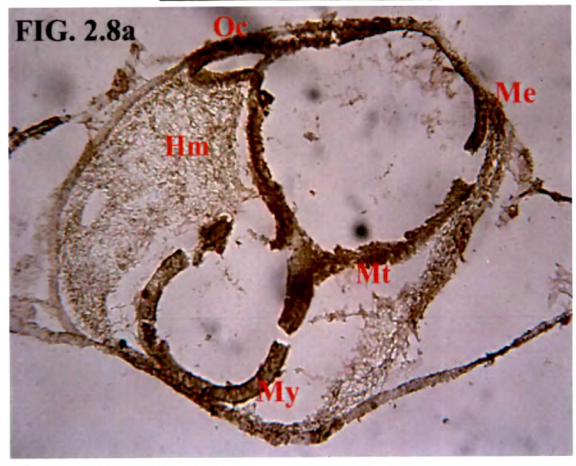
FIGURE 2.5b Control embryo cross sections at the level of anterior somites showing the vagal neural crest cells populated along the neural tube (Nt) , the notochord (N), somites (So) and the mesoderm (Me).

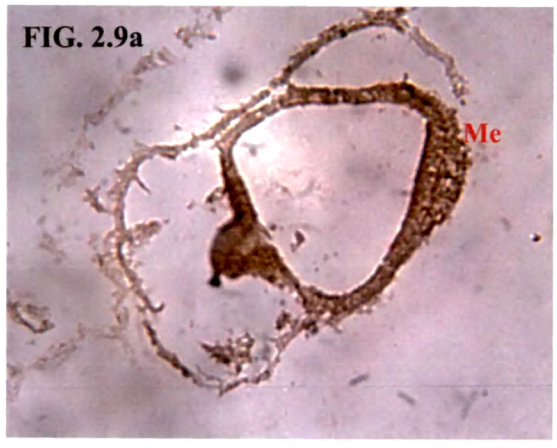
FIGURE 2.6b Ci (0.05µg/egg) treated embryo cross sections at the level of somites showing very meagre distribution of neural crest cells dispersed along the neural tube (Nt) and notochord (N). The somites were obscure.

FIGURE 2.7b Spinosad (0.75mg/egg) treated embryo cross section at the level of somites showing neural crest cells populated along the neural tube (Nt) , the notochord (N), somites (S) and the mesoderm (Me).

Immunohistochemical localization of Sonic hedgehog protein at cranial level in stage-12 chick embryos







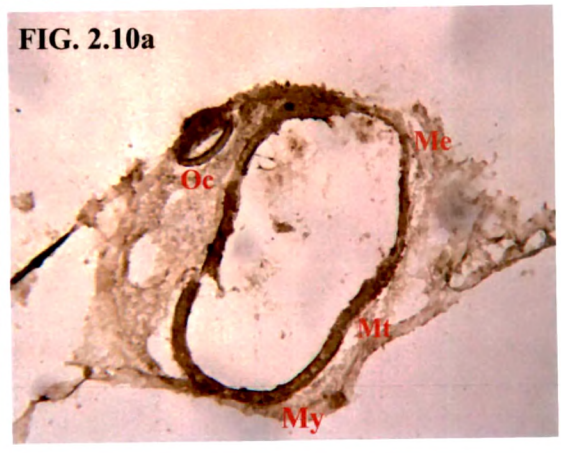
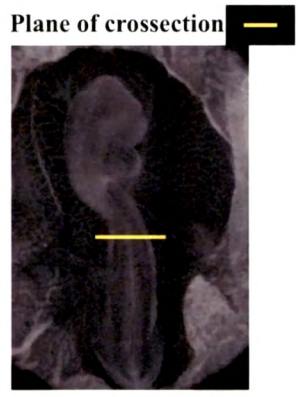


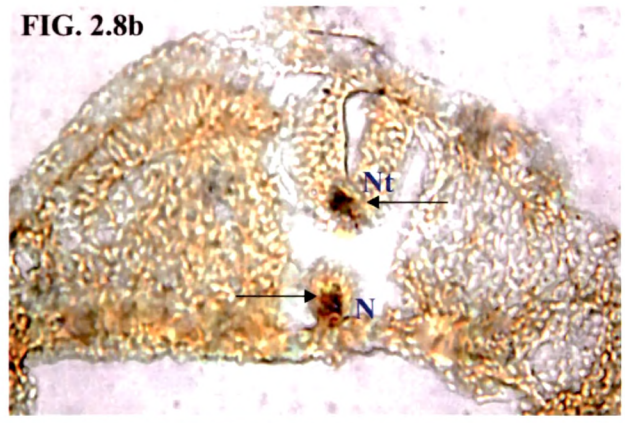
FIGURE 2.8a: In stage-12 control embryos, the sonic hedgehog (*Shh*) expression was found all along the developing brain i.e. the mesencephalon (Me), metencephalon (Mt) and myelencephalon (My). The head mesenchyme (Hm)did not show the expression. The differentiating optic cup also showed the expression of *Shh* protein.

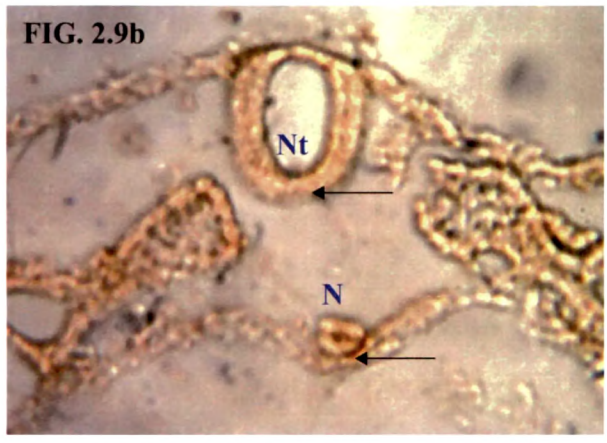
FIGURE 2.9a: The cross sections at the cranial level of the embryos treated with 0.05µg/egg of Ci showed *Shh* expression all along the mesencephalon (Me), while the rest of the cranial region showed an improper differentiation.

FIGURE 2.10a: In embryos treated with Spinosad (0.75mg/egg), at the cranial level the cross section showed *Shh* expression along the mesencephalon(Me), metencephalon(Mt), myelencephalon (My) and the otic capsule.

Immunohistochemical localisation of Sonic hedgehog protein at the anterior somite level in stage -12 chick embryo







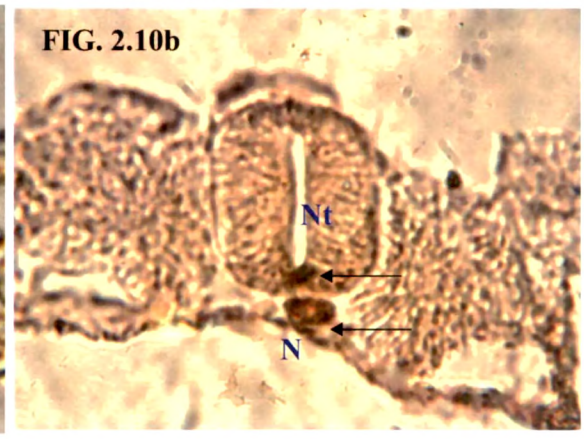
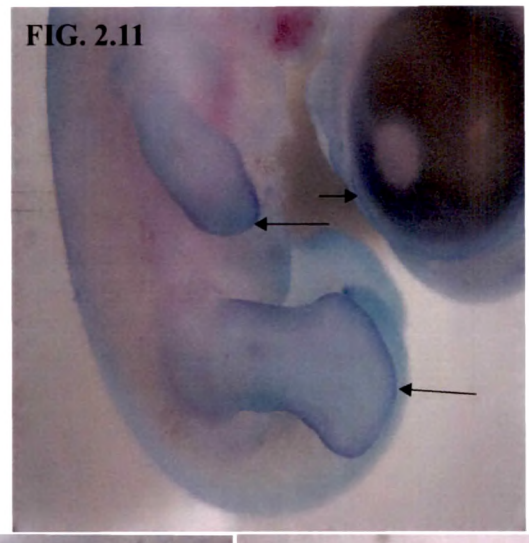


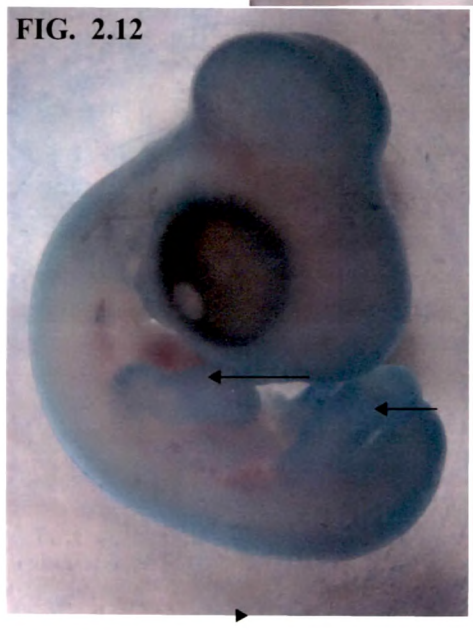
FIGURE 2.8b The control embryo cross sections showed the *Shh* expression in the floor plate (Nt) of the neural tube and notochord (N).

FIGURE 2.9b In the embryo with 0.05µg/egg of Ci treatment, the *Shh* expression was absent in the neural tube (Nt) and notochord (N) .

FIGURE 2.10b In embryos treated with 0.75mg/egg of spinosad, the cross section showed the expression of *Shh* protein in the floor plate of neural tube (Nt) and notochord (N).

Vital staining of control and insecticide treated embryos at stage-25





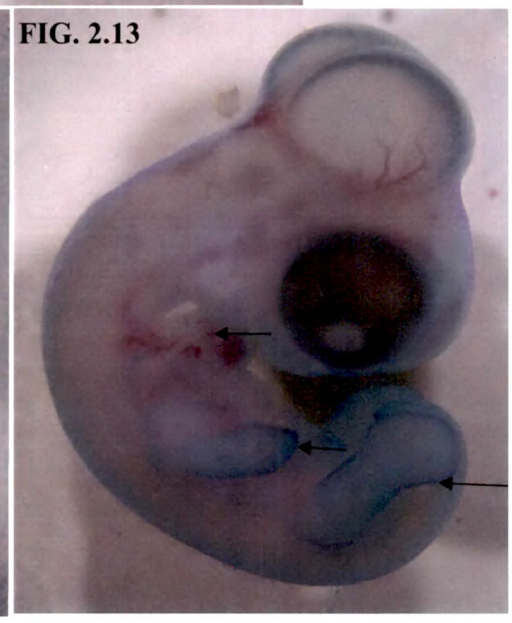


FIGURE 2.11: The NBS stained control embryos at stage-25 showed cell death along the margins of the wing bud and limb bud and along the outer margin of the eye.

FIGURE 2.12: In the 0.05µg/egg of Ci treated embryo, the cell death could not be located along the margins of the limb buds and wing buds after the NBS staining.

FIGURE 2.13:. The cell death in the 0.75mg/egg spinosad treated embryo located along margins of fore and hind limbs and with hemorrhages in the cardiac region.

Cell death localization by vital staining in the hind limb buds of control and Ci treated stage-34 embryos

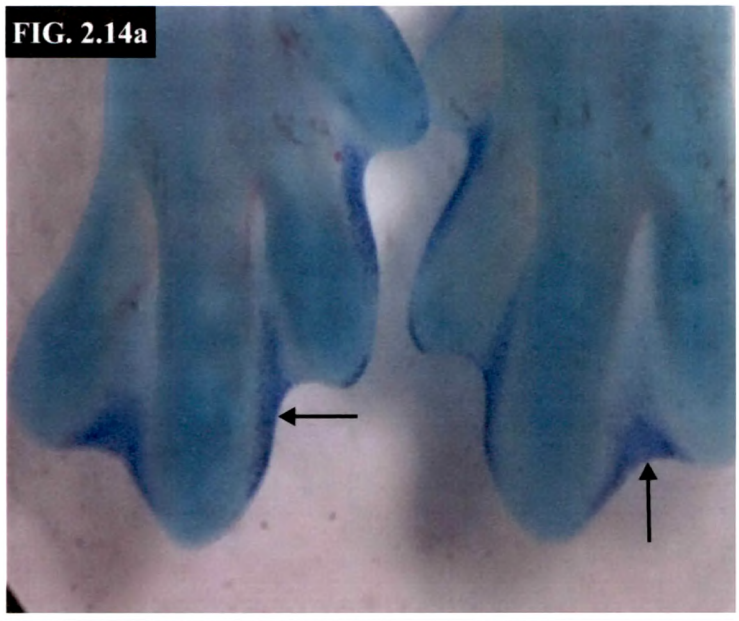


FIGURE 2.14a Control embryo showing cell death along the inter-digital region of the hind limb buds

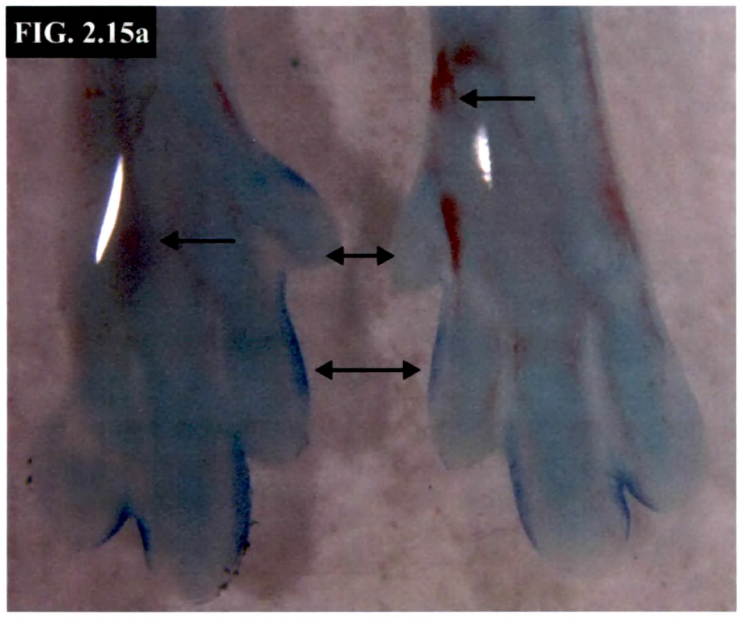


FIGURE 2.15a Ci treated (0.05µg) embryo showing hind limbs depicting altered pattern of cell death. Also seen areas of intense vascularization/haemorrhage

Cell death localization by vital staining in the wing buds of control and Ci treated stage-34 embryos

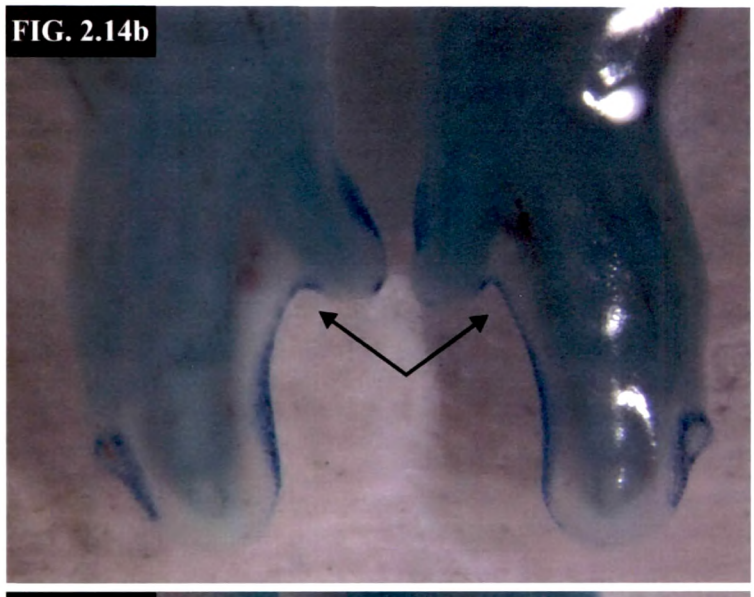


FIGURE 2.14b Wing buds of the control embryo showing cell death along the margin

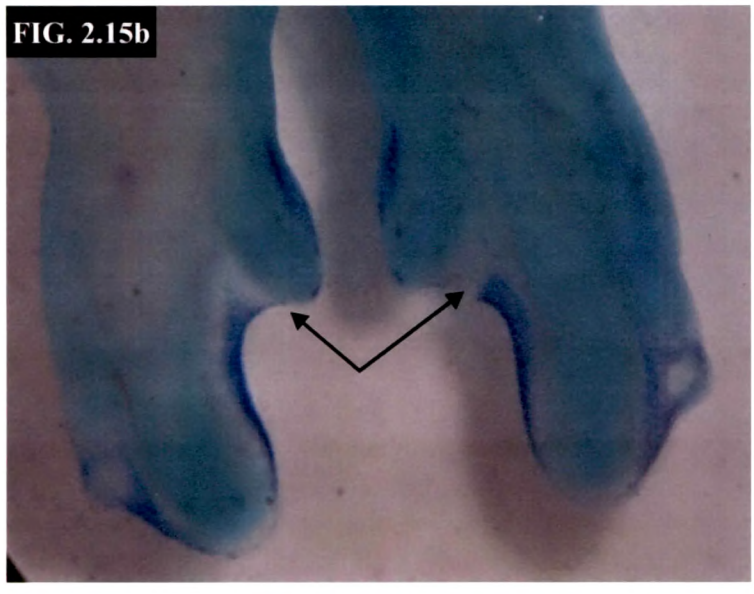
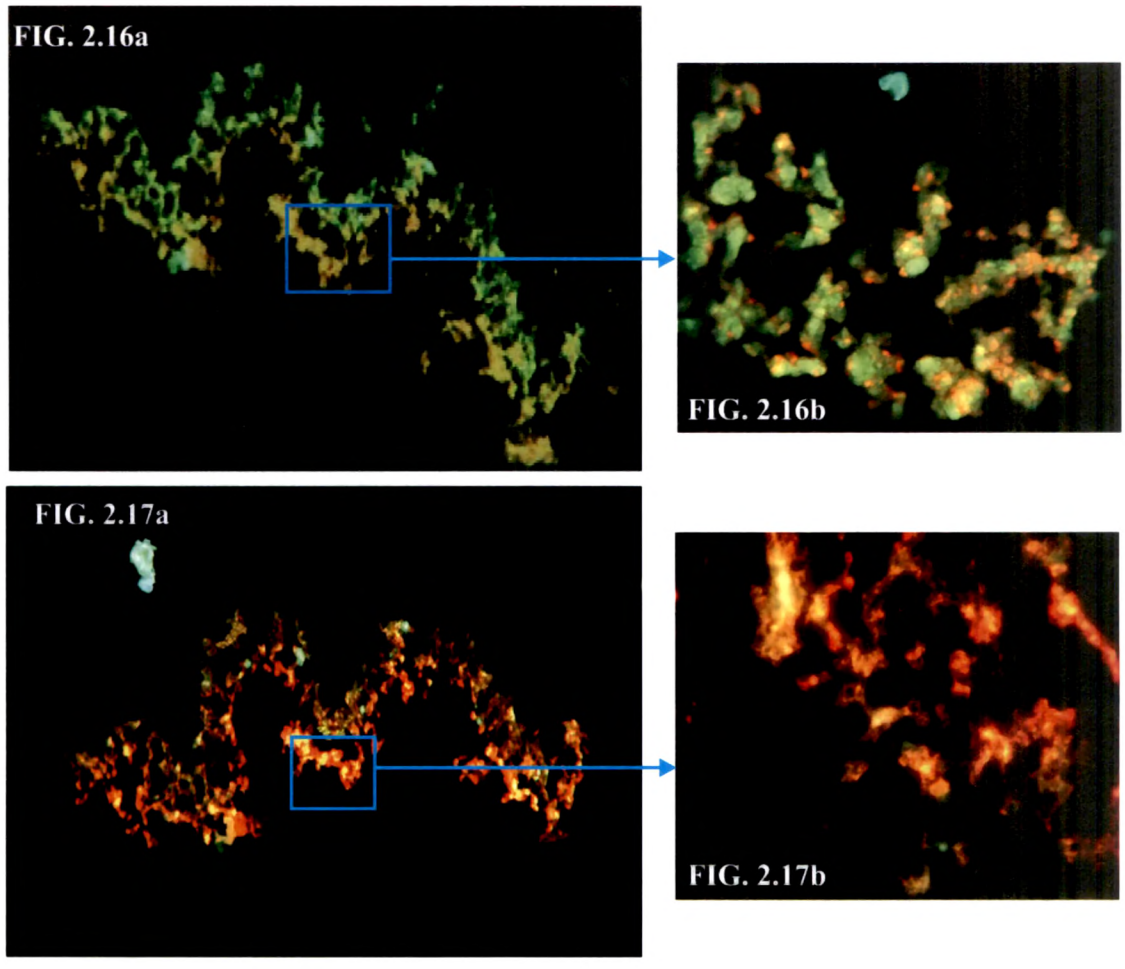


FIGURE 2.15b Ci (0.05µg) treated embryo showing absence of cell death at an area along the margin of one of the wing buds

Cell death localization by fluorescent staining of stage-6 embryo cross sections

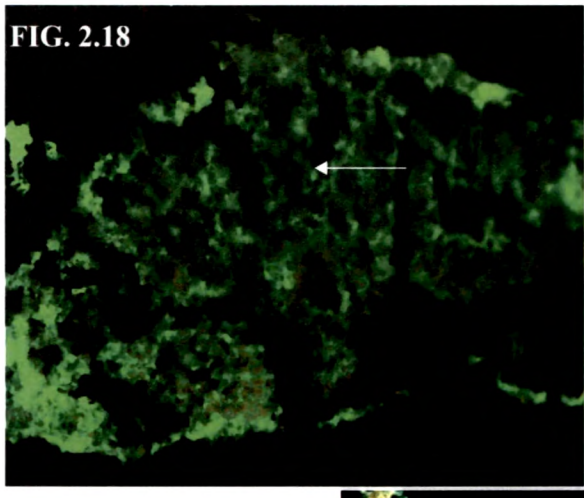


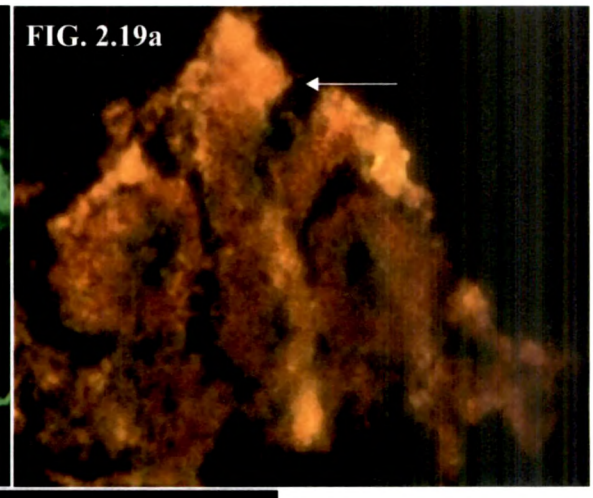
Magnification 40X
Red fluorescence: dead cells
Green fluorescence: live cells

FIGURE 2.16a and 2.16b Control embryo at stage-6 with minimal cell death along the neural crest region.

FIGURE 2.17a and 2.17b: Ci treated embryo (0.05µg/egg) showing a higher intensity of cell death along the neural fold.

Cell death localization by fluorescent staining in control and insecticide treated stage-12 embryos





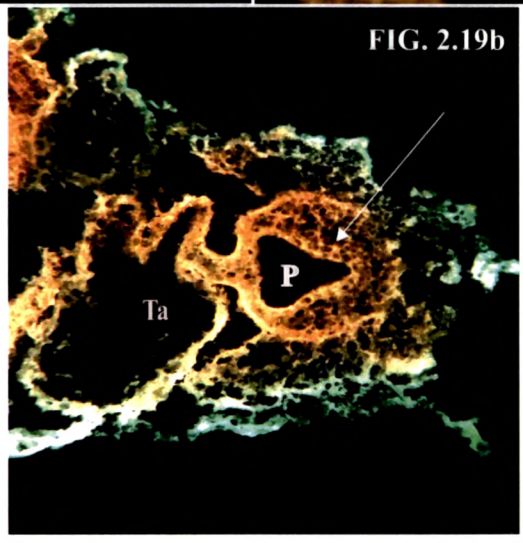


FIGURE 2.18 Control embryos showing minimal cell death (scattered) on the somites and neural tube which is considered to be a regular process during neural tube closure.The neural tube is completely closed

FIGURE 2.19a and 2.19b Ci treated embryo with open and bent neural tube. The lining of pharynx (P) and the truncus arteriosus (Ta) also showed cell death