Chapter 5

General considerations

The embryonic period is a critical phase of an organism's life since that involves a whole gamut of cellular interactions that too in a short span of time. The embryonic development begins with fertilization and formation of zygote (a single diploid cell), which further divides in quick succession through mitosis to produce a cluster of cells that later differentiates and sculpts the whole body. In embryogenesis, the embryo undergoes rapid cell divisions (cleavage) with no substantial growth, giving rise to cells of the same size called as blastomeres. The cleavage stops when the cells reach the blastula stage (blastoderm surrounding a blastocoele) and precedes further towards gastrulation, giving rise to three germinal layers *viz*. ectoderm, endoderm and mesoderm. A significant amount of activity in the form of cell proliferation, cell fate specification and differentiation occur in the early embryo to establish cell polarity as well as axis formation and are determined by the nested action of a multitude of genes (Lynch et al., 2009; Le Dreau and Marti, 2012). In the initial stages, all the cells present in the embryo are totipotent but as the development progresses, the cells begin to interact with their surroundings and differentiate to form specialized tissues/organs and body parts, the phenomenon known as organogenesis.

Nevertheless, the limitation of this embryonic development is that, an exposure of any extraneous chemical or environmental agent to the embryo during the critical phases of development results in a marked increase in incidences of abnormal outcomes. In one of the much acclaimed monographs "Environment and Birth defects" Wilson (1973) was the first to affirmatively establish the relationship between environmental factors and deviant development. He suggested that the living organisms, especially at the early developmental stages are more vulnerable to environmental factors that cause abnormal morphology. Additionally, it has been well perceived that a combination of two or more environmental agents can induce alteration in the meticulous designs of development at a relatively low level. Hence, even subtle toxicity due to the environmental contaminants can severely hamper the normal development that may result in abnormalities, retardation in growth and/or death (Landrigan et al., 2002; Sandhu et al., 2013).

In an embryo, all the cellular processes (replication, apoptosis, differentiation) occur simultaneously, in a kaleidoscopic fashion and this way the organisms continue to develop even after their adulthood and progress up till their death. Herein, the developmental processes have been designed in such a way that they achieve two major milestones: 1) to generate cellular diversity and order within each generation, and 2) to ensure the continuity of life from one generation to the next (Gilbert, 2006). Therefore, any kind of disturbance created by a toxicant to an embryo during the initial phases of development, may persist throughout its life and in worst scenario, it might as well pass onto the upcoming generations.

However, the developmental mechanisms taking place in an organism are characterized by the intricate regulation and instrumentation of the signalling molecule cascades such that the cells proliferate, differentiate, migrate and form designated tissues/body parts at the correct time and place. The cellular processes are the results of the interaction between various signalling pathways that spatially and temporally get expressed to intercommunicate with the cells leading to organogenesis (Baker et al., 2004; Perry et al., 2011; Tebourbi et al., 2011). A set of key signalling molecules and pathways operate in a concentration dependent manner, at defined intervals of time in different regions of the embryo, to elicit diverse cellular responses. However, any failure in the expression of these sculpting signals can lead to a catastrophic consequence in the development of an organism. Moreover, the early developmental stages are naïve with respect to their ability to withstand any insult due to extraneous agents (Sandhu et al., 2013).

Brent and colleagues (1990) documented a varied range of teratogenic agents to be toxic to the developing embryo, 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). Out of these, pesticides are of a major concern as they pose a serious threat not only to humans but also the ecosystem. Among the four predominant classes of pesticides, insecticides hold a major share of 76 % of the consumption in India, followed by fungicides (13 %), herbicides (10 %) and others (1 %) ((Arbuckle and Sever, 1998; Devi et al., 2017). The insecticides have been further grouped into various classes *viz.* organophosphates, organochlorines, pyrethroids and carbamates. Even though these insecticides are designed to target specific species of organisms through a unique mode of inhibition, their specificity is often arguable. Though the new generation insecticides

tend to have a shorter shelf-life and accumulate less in the organisms, they exhibit undesirable harmful effects on non-target organisms such as humans and wildlife populations. Interestingly, the humans are much larger in size when compared to the pests themselves, yet they may get affected even by the smaller amounts of these chemicals. Subsequent to many human catastrophes and imposition of safety regulation, it is but mandatory to study the toxic effects of these environmental chemicals prior to marketing. Additionally, the continuous usage of these chemicals has caused resistance in the pests and to counterstrike this, combination of insecticides is applied in the fields. These commercial formulations are required to be tested for their toxic manifestations as compared to their active ingredients. This is because the inert ingredients in the formulations are often known to enhance the toxicities of active ingredients, also known as synergism (Lorenz, 2009; Kalluri and Weinberg, 2010; Hernandez et al., 2013).

Despite of all the efforts put in to make the pesticides safe for the environment, their unwarranted usage has been a nuisance nowadays and surprisingly have proven to be teratogenic with severe intoxication symptoms (Hernandez-Diaz et al., 2012; Jayaraj et al., 2016). In addition, the chronic exposure of pesticides is well-established to be genotoxic, causing DNA damage by production of free radicals and causing mutations that lead to the development of cancer and adverse health effects (Alleva et al., 2018). Likewise, the insecticides are also reported to act as endocrine disruptors and affect the function of hormones by blocking, mimicking, displacing or acting to disrupt their natural roles in living species (Bernardes et al., 2015). Several lines of evidences have noted traces of insecticides in the body fluids of pregnant women, foetuses and small children Barr et al., 2004; Bradman et al., 2005; Greenburg et al., 2008; Hernandez et al., 2013). Rull and colleagues (2006) have observed elevated risks of pesticides on inflicting teratogenic effects on human populations. Likewise reports on rodent and avian models also suggest developmental toxicity of the insecticides (Raees Ahmad, 2007; Slotkin, et al., 2008; Uggini et al., 2012; Khan et al., 2015; Sharma et al., 2018). Ngo and colleagues (2010) have reported the toxic effects of chemicals on the developing embryo and that too at a particular stage of development, where detoxification mechanisms have not been formed or are not fully functional. The teratogenic impact of such insecticides on living beings results into a variety of congenital structural malformations. It has been noted that approximately 10-15 % of developmental deformities are a result of the adverse effects of these environmental factors during early embryonic development (Alleva et al., 2018; Choudhary et al., 2018).

Against this background, in our lab, experiments were conducted on chick embryo by exposing it to a commercial combination of organophosphates and pyrethroids (chlorpyrifos 50 % + cypermethrin 5 %). The earlier studies from the lab revealed that, this combination insecticide (Ci) induced severe embryotoxic and teratologic manifestations in the axial and appendicular skeleton of the treated embryos. The abnormalities include craniorachischisis, microcephaly, hydrocephaly, agnathia, anophthalmia, umbilical hernia, micromelia, hind limb twist, sacral hygroma, drooping twist, and kinky tail (Uggini et al., 2012). Subsequently, these toxic manifestations were also observed to be passed on to the succeeding generation of the chicks (Khan et al., 2015). Largely the anomalies observed in both the studies were pertaining to defective neural tube and somite development. A careful observation of developmental events occurring in the early embryonic period in chick revealed that the insecticide hampers the events associated with the late phase of gastrulation and also organogenesis. For instance, obstacles in the neural tube closure and defective migration of neural crest cells have culminated into craniorachischisis and other craniofacial malformations. Many investigators have attributed these deformities to impromptu development at early embryonic stages explicitly during patterning of germinal layers and somitogenesis. Moreover, deranged morphogenetic movements and alteration in the signalling mechanisms that regulate the said developmental events have also been reported (Bretveld et al., 2006; Timofeeva et al., 2008; Voronova et al., 2012).

Furthermore, the organophosphates and pyrethroids are known to affect neurotransmission by inhibiting acetylcholinesterase or Na^+ channels respectively that provides neurotrophic input, regulates proliferation, differentiation and migration of its target cells (Hohmann, 2003; Mascarelli, 2013). Thus, at an early stage of cell development, this chemical messenger may stimulate the genes that regulate cellular processes ranging from replication to differentiation. Hence, any hindrance in the signalling pathways or inappropriate apoptosis during early embryonic development, would be catastrophic to the embryo and will later manifest as structural deformities. Therefore, the present study was designed wherein the combination insecticide induced changes in the signalling pathways that regulate the major developmental events of embryogenesis was evaluated in chick. The deviant signalling might have culminated into structural defects when the embryos were exposed to the combination insecticide. In this study, the mechanistic insight of two major defects induced by Ci were taken into consideration: 1) Ventral body wall defects; 2) Neural tube defects.

The effect of this combination insecticide was studied on fertile RIR eggs by injecting them *in ovo* (air sac method devised by Blankenship et al., 2003). The embryonic development studies in *in ovo* system offers various advantages over other traditional methods/systems for evaluating toxicity. Firstly, the developing embryo inside the egg provides multiple features, similar to the human systems (Stern, 2018). There are no maternal influences over development post-laying that enables one to investigate the embryotoxic potential of a toxic compound without sacrificing the mother. Moreover, as the test chemical is administered directly to the growing embryo, it will be protected from the influence of metabolic machinery of the mother. Additionally, the chick embryo has been recommended as a model for scrutinising the effects of toxicants that may alter the expression of genes that regulate differentiation and migration of cells in the central nervous system (Bjørnstad et al., 2015). Therefore, the developmental toxicity evaluation of the combination insecticide was 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. Uggini and colleagues (2012) had evaluated the effective dose range of this combination insecticide and from the data published, the sub-lethal dose of 0.05 µg/egg was selected for further toxicity studies. Nonetheless, the same dose (0.05 µg/egg) was tried in the present laboratory condition and validated for its effectiveness in inducing structural anomalies prior to the main study. The insecticide doses were injected by the air cell method (Blankenship et al., 2003) in volumes of 50µl/egg. The control group was injected with olive oil as vehicle alone. The fertile RIR eggs intoxicated by the combination insecticides demonstrated a variety of morphological and skeletal aberrations that includes anophthalmia, beak deformities, absence of cranium, omphalocele, haemorrhage, gastroschisis, deformations in the vertebrae, sternum and ribcage. These anomalies can be largely categorised in to two classes namely ventral body wall defects and neural tube defects.

The congenital deformities *viz*., omphalocele, gastroschisis, distorted ribcage and sternum, belong to ventral body wall defects (VBWDs) and occur with a prevalence of 3 in every 10,000 births (Prefumo and Izzi, 2014). During the neonatal development, the lateral body folds and somites play a major role in covering the embryo from the ventral side and as Duhamel (1963) postulated, any insult to these folds leads to VBWDs. The gastrulation phase of development marks the differentiation of the mesodermal layer into three different regions: Paraxial mesoderm, that differentiates into a whorl of cells called somites. These blocks lay foundation in the formation of the skeletal muscle and dermal layer covering the back whereas, intermediate

mesoderm forming urinogenital system and lateral plate mesoderm (LPM) gives rise to membranes covering the major organs such as heart and lungs. Also, coastal cartilages are derived from LPM (Sadler, 2010).

Subsequently, the lateral plate mesoderm splits into somatic and splanchnic layers cover the embryo and fuse at the midline on the ventral side (Christison-Lagay et al., 2011; Prefumo and Izzi, 2014). This fusion of the body wall folds involves important cellular processes like programmed cell death (apoptosis), formation of specialized cell-to-cell contacts, cell migration, and are required for the closure of ventral body wall (Copp et al., 2000; Colas and Schoenwolf, 2001). Moreover, their coordination is governed by various important molecules such as SHH, WNT, BMP and PITX2. In order to gain better clarity of how the combination insecticide inflicted toxicity in the chick embryos and caused ventral body wall defects, certain stages of embryonic development were selected. In addition, the expression patterns of key molecular signals as mentioned above were also taken into consideration so as to gain further insight on embryonic events like cell proliferation and patterning since aberrations in these events might have led to the ventral body wall defects.

The stages selected for the study were H-H stage 13 (day 2), H-H stage 24 (day 4) and H-H stage 36 (day 10). The sub-lethal dose of pesticide in the eggs caused a steep rise in the mortality rate as the development progressed. The embryos opened on day 2 showed abnormal disposition of somite (33 %) upon pesticide intoxication. The malformations observed on day 4 were microphthalmia and stunted growth. Further, the tissue architecture was studied with the help of differential staining that displayed well-developed myelencephalon and optic cup in control group embryos on day 4. Also, the heart was well formed with clear demarcation between atrium and the ventricle. Quite the opposite was noted in the treated samples with abnormalities in the form of stunted growth, distorted optic cup and reduction in the somite numbers.

The above deformities can be attributed to the subdued mRNA expression levels of sonic hedgehog in Ci exposed embryos which is one of the early expressed pattern forming genes playing a significant role in the process of somite formation and somitogenesis. Additionally, SHH coordinates its activity with BMPs to regulate the patterning of the somites in developing embryos. Moreover, inhibition of BMP4 by SHH controls its temporo-spatial expression, thereby holding the LPM from extending medially and ventrally (Marcelle et al., 1997; Gilbert, 2003). The BMPs are known to have a dual role in organising the event of body wall closure wherein,

expression of BMP4 in dorsal neural tube, promotes muscle differentiation and its ectopic expression in the paraxial mesoderm, inhibits myotome formation. Consequently, a proper myotomal development would also require the BMP to be inhibited at the paraxial mesoderm in a specific temporal manner (Marcelle et al., 1997). The mRNA expression analysis on day 4 showed that the Ci treated embryos had a downregulation of *SHH* and upregulation of *BMP4* when compared to the control embryos. This dysregulation in the expression of *SHH* and *BMP4* must have created the initial disturbances in the paraxial mesoderm, thereby disrupting the somite patterning and subsequently it must have caused anomalies in the myotome differentiation too in the embryos.

Further, under normal conditions there is a differential expression of PITX2 on the embryonic left and right sides resulting the axis formation, which is controlled upstream by selective inhibition of BMP4 and FGF8. Though the changes in *FGF8* expression remained insignificant, the increased expression of *BMP4* and *PITX2* in the Ci exposed embryo indicates a disparity. Contrary to our observations with regards to *PITX2*, according to Kitamura et al. (1999), its deficiency caused a failure of ventral body wall closure in mice, and they have also observed that lack of this molecule was influenced by a different genetic cascade, other than the FGF8-derived one. However, in another study by Fung et al. (2012) upregulated PITX2 is indicated during oncogenic progression, where PITX2 might be contributing towards the growth and migration of cells. Also, there is an isolated report (Tao et al., 2016) indicating that elevated levels of PITX2 step-up free radical scavenging by promoting the gene expression of antioxidants. Therefore, an increased expression of *PITX2* here might be an embryonic response to induce the formation of antioxidants against the stress induced by the Ci exposure.

However, an overburden of the extraneous agent when not efficiently dealt by the clearance pathways might culminate into dire consequences like renewal of teratogen targeted cell population by inducing apoptosis (Torchinsky, 2005). Our study shows a highly significant increase in the expression of *CASPASE 3* activity in day 4 embryos which can be correlated to the large-scale apoptosis occurring in the Ci treated embryos.

Parallelly, the studies were extended to day 10 where the pesticide intoxicated embryos displayed the ventral body wall defects with a malformation rate of 60.6 % in the live embryos. The histological picture of day 10 Ci treated embryos showed less developed musculature and the internal organs were left uncovered. Absence of sternum in the treated embryo was a crucial

observation. At the same time, the levels of *PCNA* were found to be downregulated indicating reduced rate of cell proliferation in Ci exposed chicken embryos. Also, activation in the SHH signalling pathways is seen under conditions of oxidative stress and could regulate cell proliferation and apoptosis (Heine and Rowitch, 2009; Xia et al., 2012). In concordance with the above statement, we too observed an upregulated expression of the *SHH* in the day 10 Ci treated embryos as compared to that of controls and this could be a result of Ci induced oxidative stress as opined by Xia et al., (2012).

Furthermore, the somitogenesis, LPM formation and movement of lateral body folds are governed by cellular activities like programmed cell death, intercellular crosstalk, cell migration and cell proliferation (Copp et al, 2000; Colas and Schoenwolf, 2001). In the developing embryos, the migrating cell is highly polarized and is regulated by a complex set of signals. The differentiating new cell types depend upon a finely balanced and coordinated regulation of gene expression with precise interaction amongst their neighbours. We therefore, sought to identify few such molecular signals that regulate the said processes. It has been reported that the expression of CDH1 during embryonic development happens quite early where they play a role in adhesion and compaction of the blastomeres (Bahm et al., 2017). Moreover, CDH1 also signals the controlled epithelial-to-mesenchymal conversion and regulates developmental processes like cell migration and proliferation (Fleming et al., 1992; Barth et al., 1997; Kalluri and Weinberg 2009; Bahm et al., 2017). In addition, it is well documented that the migration of mesodermal cell is favoured by downregulation of the CDH1 and loss of cell adhesion (Ciruna and Rossant, 2001; Basson, 2012). Our results have shown that levels of *CDH1* were found to be upregulated in both day 4 as well as day 10 Ci treated embryos, giving clear evidence that the cell migration was delayed and/or hampered. Further, the CDH1 is known to be the downstream target of SHH (Xiao et al., 2010), which suggests that an inhibition of *SHH* would result in decreased *CDH1* expression. However, our results contrastingly have shown that the *SHH* was downregulated while *CDH1* was upregulated in 4 day treated embryos, while in day 10 treated embryos, both were found to be upregulated. This indicates that *CDH1*, though regulated by *SHH*, might as well be under the control of some other upstream signal. A negative correlation between the SHH and CDH1, nevertheless, was reported during metastasis (Karhadkar et al., 2004; Sun et al., 2017). Further, the Paxillin (*PXN*), which is a multifunctional focal adhesion adaptor protein, was found to be upregulated here. PXN expression has its significance not only during embryonic development and cell movement, but its elevated levels are also found in pathological conditions like oxidative stress and metastatic cancers (López-Colomé et al., 2017).

An elevated *PXN* observed in day 10 treated embryos could as well be related to the condition of oxidative stress caused by the Ci exposure. Earlier studies by Ray et al., (2010) in neonatal rats has also shown that chlorpyrifos administration leads to differential expression of genes like PXN involved in cell adhesion and migration.

Subsequently, studies were extended to understand the pattern of WNT expression. The WNT signalling plays a significant role in abdominal myogenesis and formation of secondary ventral body wall. Likewise, WNT11, a non-canonical WNT member, has a role in cell adhesion and movement. The WNT11 and WNT6 are cited to be pivotal in maintaining the epithelial nature of the dorso-medial and ventro-lateral lips of dermomyotome and a deficit here would lead to defects in the ventral musculature formation (Zhang et al., 2014). However, our study has shown a significant upregulation of *WNT11* and *WNT6* in the Ci treated embryos on day 10. Nevertheless, such aberrant upregulation of the WNT signalling pathways has been reported after a chronic exposure to cadmium in mouse (Chakraborty et al., 2010) and also in many cases of malignant human cancers (Loh et al., 2013).

Apart from the signalling molecules discussed so far, BMPs lay a strong foundation in the embryonic development and skeletal patterning. A review by Wang et al., (2004) discusses the role of BMP4 in early developmental process, where, its absence leads to failure of mesoderm formation and at a later stage, BMP1 deficiency would lead to defects in ventral body wall closure. It was evident from the histological sections of the chick embryos that muscle formation and development has been apparently hampered in the Ci treated ones. In order to consolidate this visual observation, expression of marker of early myoblasts, *MYOD1*, was analysed. A relatively higher expression of the *MYOD1* in the treated embryos could be an indication that the myoblasts retained their naïve tissue state and failed to undergo further differentiation in the intoxicated embryos. Moreover, Voronova and colleagues (2013) have stated role of Sonic hedgehog in regulating MYOD1 expression to enhance the embryonic skeletal myogenesis. Additionally, an upregulation of *MYOD1* would have been subsequent result of *SHH* upregulation, as mentioned earlier for day 10 treated embryos. Thus, the signalling molecules discussed above provide possible clues about mechanism of Ci toxicity and their aberrant signalling led to defects in ventral body wall.

Further, the study was extended to understand the possible downbeats in the signalling pathways during the early embryogenesis that led to the craniofacial dysmorphism provoked by

combination insecticide exposure in chick embryo. Much of the embryonic dysmorphism observed here could be attributed to the lapses in the process of neurulation. The primary neurulation is of prime importance in the development of chick embryo, in which the two sides of flat neural plate begin to converge and involute at the midline and adhere to each other to form the neural tube. Any failure in this closure towards the rostral and/or caudal end, results in conditions like anencephaly and/or spina bifida respectively, and if the tube fails to fuse throughout the body it would lead to rachischisis. The present study reveals such defects in neural tube closure on day 2 in the pesticide treated embryos. Further the treatment group on day 2 exhibited many overt signs of toxicity such as underdeveloped heart, reduction in size of the whole embryo, absence of eye stalk, underdeveloped telencephalon and distorted cephalisation. Hence, we looked into the molecular regulators of these embryonic events.

The chick embryo expresses E-cadherin and L1-CAM throughout the ectoderm during the neurulation process (Taneyhill, 2016). These Ca^{2+} dependent cell adhesion molecules are the identity of the neuro-ectodermal plate and their expression regulates the fusion process. Due to the switch in gene expression, N-Cadherin comes into picture which marks the separation of ectodermal and non-ectodermal cell types (Taneyhill, 2016). Herein, our results showed significant reduction in mRNA expression levels of *CDH1*, *CDH2* and *L1-CAM* in the Ci treated embryos when compared to the control embryos on days 2 and 4. This dysregulation, especially in N-cadherin levels must have led to the improper closure of the neural tube, thereby disturbing the neurulation and causing anomalies in the rostral region of the embryos. Similar results were reported by Detrick and colleagues (1990) wherein deficiency of N-cadherin levels induces morphological defects in *Xenopus* embryos that gives credence to the present findings.

Further, as the surface ectoderm separates from the newly formed neural tube, a group of cells delaminates from its anterior most layer, undergo epithelial to mesenchymal transition and migration to further diversify into various cell types termed as cranial neural crest cells (CNC) (Mayor and Theveneau, 2013). These multipotent cells in the later stages of development give rise to forebrain meninges, craniofacial cartilage, bones of the jaw, neurons and glia in the vertebrate embryo (Meulemans and Bronner-Fraser, 2004; Mayor and Theveneau, 2013). The formation of neural crest cells is a complex process carried out under the influence of specific micro-environment signals that confer patterning and migratory capabilities to this cell population (Nikolopoulou et al., 2017). Many genes and transcription factors such as SHH, WNTs, BMPs, FGFs and PAX6 are secreted from the adjacent tissues to induce patterning in the

neural tube in a gradient fashion (Kulesa et al., 2010; Bragdon et al., 2011; Reid et al., 2011; Prasad et al., 2012; Yardley and García-Castro, 2012; Griffin et al., 2013). Depletion of neural crest cells or perturbations in the gene expressions during patterning process, have been reported in mouse models to cause neural tube defects, microphthalmia and reduced size or absence of jaw structures, suggesting their importance in the development of the future cranium (Wilde et al., 2014).

The neural tube undergoes patterning along its two major axes *viz*., dorso-ventral and anterior-posterior to form a functional nervous system under the influence of paracrine factors secreted from ventral floor plate and dorsal roof plate (Ahlgren and Bronner-Fraser, 1999). There are reports that SHH cooperates with BMP7, to play an important role in patterning the dorso-ventral axis of the nervous system (Ahlgren and Bronner-Fraser, 1999; Xavier et al., 2016). Mutations in the SHH gene in mouse and humans have suggested its role to be critical and targeted deletion of SHH causes significant craniofacial defects such as holoprosencephaly and cyclopia (Ahlgren and Bronner-Fraser, 1999; Xavier et al., 2016). Moreover, knockout studies of BMP7 in mice have clearly indicated its roles in the development of eye structures (Solloway and Robertson, 1999). In the present study, the mRNA expression analysis on day 2 and 4 embryos showed a relatively downregulated expressions of *SHH* and *BMP7* in Ci treated embryos, which might have contributed to the observed craniofacial deformities and defects in eye development.

Another important family of signalling molecule namely, the WNTs, is responsible for inducing proliferation in neural tube and thereby regulating multiple aspects of animal development and adult homeostasis (Megason and McMahon, 2002). One of the studies has shown the role of Wnt signals in the vertebrate nervous system for developing axonal guidance to synaptic region. In the developing vertebrate brain, WNT1, WNT5A and WNT7A are expressed with an overlapping spatiotemporal pattern, and any alteration in this signalling pathway leads to cranio-facial dysmorphism (Brault et al., 2001). Also, there are evidences suggesting that the WNTs are acting downstream of the BMPs (Wine-Lee et al., 2004). In the present study, the mRNA expression analysis of days 2 and 4 subjects revealed that the Ci treatment resulted in significant downregulation of *WNT1, WNT5A* and *WNT7A*. This dysregulation in their expression might be the consequence of downregulated mRNA levels of *BMP7*, which must have further worsened neural tube development and the cell proliferation.

Moreover, neural crest cells are known to be involved in patterning the head and require a synchronized regulation of cell number to tissue size. This probably occurs through a combination of events like cell proliferation and survival, which is controlled by SHH (Le Dréau and Martí, 2012; Xavier et al., 2016). It can be hypothesized that diminished levels of Shh signalling hampers the growth of the neural tube, primarily because of increased cell death, which leads to an overall reduction in head size in the pesticide intoxicated embryos. We checked the levels of *CASPASE 3* and *PCNA* and found respective hike and fall in their mRNA levels, in treated embryos. The decrease in head size could be an outcome of neural crest cell death and hence, it appears to be its primary determinant. In addition to the defects observed in the present study, we have noted occurrence of wavy neural tube due to intoxication of chlorpyrifos and cypermethrin in combination. Similar abnormality has been observed in mice with defects in platelet derived growth factor (PDGF) receptor, which is mediated through SHH (Suzuki et al., 2016).

The Sonic Hedgehog, through its downstream zinc-finger transcriptional regulators GLI, orchestrates two major functions namely patterning early on and cell proliferation during the neurulation process. However, these factors appear to play no role during migration of cranial neural crest cells (Xavier et al., 2016). Moreover, Aoto and collegues (2002) have documented involvement of GLI3 in suppressing Fgf8 during neural tube development of mouse embryo. We have observed similar incidences of *GLI3* downregulation, resulting in the up regulation of *FGF8* expression. Since FGF8 is essential for the control of pattern formation in the developing embryo, its elevated expression can affect the developmental programs of many tissues as reported by Yardley and García-Castro, (2012).

Further, in the embryonic development, the neural tube undergoes drastic changes to attain anterior-posterior identity. Towards the anterior region, the neural tube organizes itself into three, balloon like structures: the prosencephalon, mesencephalon and the rhombencephalon. These primary vesicles differentiate into secondary structures i.e. forebrain differentiates into telencephalon (forming the cerebral hemispheres) and the diencephalon (forming the optic vesicle for the development of eye), midbrain transforms into mesencephalon and the hindbrain develops metencephalon and myelencephalon (Gilbert, 2003). The boundaries of these vesicles are marked by the presence of SHH and FGF8. Both these paracrine diffusible factors are also involved in the patterning of CNC cells in a rostro-caudal manner. Several studies have supported the role of growth factors, including FGF, BMP and WNT as they regulate and define the

positional identity of neural crest cells and their derivatives (Sato et al., 2005; Suzuki et al., 2016). Interestingly, FGF8, a member of FGF family, is reported to be involved in the neural crest induction, patterning, and its migration by inducing the expression of various transcriptional factors (Sato et al., 2005; Yardley and García-Castro, 2012). FGF8 along with SHH marks the midbrain and hindbrain boundary, by silencing the HOX gene expression in the pre-migratory CNC cells (Irving and Mason, 2000).

Several lines of evidence have proved beyond doubt that overexpression of FGF8 results in severe developmental defects in telencephalon, mesencephalon and pharyngeal arches (Kimelman and Martin, 2012; Shao et al., 2015). Cell patterning in the anterior most part of the brain requires low levels of FGF8 for induction of HOX10A and HOX11A which confers positional identity to CNC cells in the brain. In the craniofacial study (Chapter 4), we observed an elevated expression of *FGF8* which could be a plausible reason behind downregulation of *HOX* genes and therefore leading to underdeveloped telencephalon in pesticide treated embryos. Another study in mice showed heightened FGF8 expression and its activation by Wnt in the neural crest cells, which led to severe craniofacial abnormalities, including exencephaly and anophthalmia (Prakash et al., 2006). Further, we checked the levels of transcription factor *PAX6* which is known to be involved in migration of cranial neural crest cells. The PAX6 is influenced by FGF to mediate morphogenesis of the CNC cell derivatives to form facial structures (Makarenkova et al., 2000). Normal expression of PAX6 is required to guide CNC cells migration for forebrain patterning. In the development of eye, role of Pax6 is of prime importance in the communication between cranial neural crest cell derivatives, required for lens induction as well as for corneal and retinal development (Nelms and Labosky, 2010). The observed low levels of PAX6 expression suggests its inefficiency to guide CNC cells to form optic stalk, leading to absence of optic cup and causing anophthalmia in the pesticide treated embryos.

As described by Knight and Schilling (2013), cranial neural crest cells are derived from the anterior most part of the neural plate and have the potential to form the skeletal tissue. In avian model system, little is known about the genetic regulation of cranial vault development. Most of the skeletal system undergoes endochondral ossification, to differentiate mesenchymal cells into chondrocytes, eventually leading to the bone formation (Kronenberg, 2003). We have observed severely compromised chondrogenesis, especially in the craniofacial region of the chicks due to pesticide intoxication. To get an insight into the extent of bone and cartilage formed by day 10 chick embryos, head region of control and treated groups were stained with Alcian Blue and

Alizarin Red. The pesticide in question delayed the bone formation in the dosed group as compared to control. The extent of cartilage condensation was checked biochemically, by estimation of hydroxyproline. Hydroxyproline is the amino acid found in the highest concentration in collagen. This fibrous structural protein is abundantly seen in cartilage. During early embryogenesis, the skeletal system is made up of cartilage from mesenchymal tissue which further differentiates into chondrocytes and is later replaced by endochondral ossification. The treated groups showed diminished level of hydroxyproline indicating less amount of cartilage formed as compared to control indicating the toxic manifestation of Ci.

However, there are ample evidences to prove the role of BMPs in inducing bone formation and regulating chondrogenesis (Kamiya and Mishina, 2011). The use of genetically modified mice has unearthed various signalling pathways activated by BMPs that control multiple aspects of chondrogenesis (Kugler et al., 2015). Herein we have chosen *BMP2* and *BMP7* for the study as they play a major role in chondrogenic and osteogenic differentiation. It was suspected that the Ci treatment might have hampered the BMP signalling and hence, hindered the process of cartilage formation and condensation. In order to test the above notion a mechanistic study, primarily focusing on the expression pattern of *BMP2* and *BMP7* was performed and the result revealed that the expression of both the BMPs remained significantly low in the pesticide treated embryos. The bone morphogenetic proteins target transcription factors that induce differentiation of mesenchymal cells toward ossification in which SOX9 and RUNX2 are important. SOX9 is a chondrocyte marker expressed during early differentiation stages. Gene expression studies in mouse have been documented, suggesting its role in activating chondrocyte specific markers such as COL10A1, COL2A1 and Aggrecan to form cartilage. On the other side, RUNX2 was needed to differentiate chondrocytes into osteoblasts. Further, its expression is suggested to be causing proliferation of osteoblast progenitors, thus regulating the first check point of chondrocyte maturation to osteoblasts (Kelly and Jacobs, 2010; Shen et al., 2010). The analysis of the result showed the toxic manifestations of the combination insecticide by way of compromised mRNA expression levels of *RUNX2*, *SOX9* and *COL10A1*. This derailment in the signalling pathway might have caused improper sculpting of the head and facial structures in the treated embryos.

The cranial vault in the chick embryo gets formed almost entirely by the endochondral ossification. DLX5, a transcription factor from distal, less homeodomain containing family, is expressed before osteoblast differentiation as well as in proliferating osteoblast precursors and

is required for craniofacial morphogenesis (Holleville et al., 2003). It is mainly expressed during the formation of structural elements such as cartilage. DLX5 null mutations in mice have testified its part in triggering skeletal defects in the form of deferred endochondral ossification and abnormal osteogenesis. Knockout studies have reported craniosynostosis syndromes and severed chondrogenesis (Holleville et al., 2003). Herein, pesticide intoxication led to high levels of *DLX5* expression in the treated embryos. Heightened expression of *DLX5 i*mpairs differentiation of chondrogenic precursors into osteoblasts of humans. *DLX5* transcription in the current study might have been upregulated by BMPs and were found expressed together at high levels in Ci challenged embryos.

Another major factor in skeletal development is the expression of WNTs, which are crucial signals for the regulation of chondrocyte and osteoblast differentiation (Day and Yang, 2008). Wnt5a has a crucial role in patterning the anterior-posterior axis by regulating of chondrogenic differentiation through SOX9 (Day and Yang, 2008). On the contrary, it suppresses hypertrophic chondrocytes by inhibiting RUNX2 expression (Bradley and Drissi, 2010). The pesticide treated embryos showed downregulation of *WNT5A* expression, thereby inhibiting the formation of chondrocytes by significantly suppressing the expression of SOX9.

Moreover, BMP2 is known to play a critical role in osteoblast function by mediating its activity through hedgehog signalling. Gli proteins play an essential role as transcriptional activator (GLI2) and repressor (GLI3) in embryonic development by regulating Shh target genes. Null mutation of GLI2 in mice has caused structural defects (Zhao et al., 2006). It has been reported that Sonic hedgehog signalling stimulates BMP2 activity in osteoblast and its effects are mediated by Gli2 (Zhao et al., 2006). In the present study, we observed reduction in mRNA levels of *SHH* and *GLI2*, thereby hampering the expression of *BMP2* and its downstream effectors. Both the signals (*SHH* and *GLI2*) together act as a powerful activator of *BMP2* expression a prerequisite for normal osteoblast differentiation. Not surprisingly, Gli3, a transcriptional repressor of SHH pathway, has been reported to trigger skeletal defects in mice and humans (Yip et al., 2019). Downregulated levels of *GLI3* observed in the current study support the concept of *FGF8* overexpression, causing abnormal apoptosis of the skeletal tissues. Similar reports also supported the defects in GLI3 and FGF8 signalling causing neural tube related defects in mice (Putoux et al., 2018).

Our study was further extended to understand the role of FGFs in skeletal development wherein FGF2 and FGF8 are the major candidates. The results vividly expressed that pesticide treatment induced downregulation of BMP2 and BMP7, possibly as a result of the increased expression of Fgf8 which is known to antagonize the action of BMP (Yoon and Lyons, 2004). Moreover, it has been documented that the upregulated expression of Fgf8 will activate the apoptotic pathway (Wan and Cao, 2005). To ascertain the extent of cell death, day 10 chick embryos were extracted, and *CASPASE 3* activity was checked. It was observed that *CASPASE 3* activity increased progressively by several times in treated groups compared to the control group animals. Overall it showed an unusual pattern of apoptosis due to diminished BMP signalling in treated groups. Kawane and colleagues (2018) have elaborated the role of FGF signalling in skeletal development, craniosynostosis, and the progression of some breast cancers. The osteoblast differentiation and proliferation of their progenitors requires a positive signal from RUNX2 (as discussed earlier) and Fibroblast growth factor 2 enhances its ability through MAPK pathway. We found contrary result in *FGF2* mRNA levels showing upregulation in the treated embryos and downregulation of *RUNX2* mRNA. Similar results were reported where elevated levels of FGF2 were localised in the non-differentiated mesenchyme, around the bones in the chick mesenchyme (Moore et al., 2002). These results suggest that other factors must be acting upstream of *RUNX2* in controlling osteogenesis and chondrogenesis.

Conclusively, *in-ovo* administration of sub-lethal dose of chlorpyrifos and cypermethrin in RIR eggs conjured a myriad of structural anomalies and hampered key developmental events by deranging various signalling pathways in the non-target species (herein, chick embryo). In addition, it was observed that certain regulators of morphogenetic movements like somitogenesis, neural tube patterning, neural crest cell movement, chondrogenesis and muscle differentiation got downregulated due to pesticide intoxication (Figure 5.1). There were several key features observed in the results obtained that possibly give clues to the observed structural deformities due to pesticide exposure. The list is as follows: 1) heightened cell death; 2) changes in proliferation rate where a drop in its rate turned out to be critical for the developing embryo; 3) disrupted cellular interactions; 4) hampered morphogenesis (Sharma et al., 2018); 5) damage to macromolecules including proteins, nucleic acids etc. and 6) mechanical disruption of ion channels or cell membranes (Uggini et al., 2012). As a closing remark, our observations when monitored at microlevel could be connected to not just isolated signalling molecules but also with a large number of such regulatory molecules, which sets an alarm on how devastating a toxic exposure during the phase of embryonic development could be; and that the process of

development which does not stop with the embryonic phase and continues throughout the animal's life. Therefore, a comprehensive knowledge on the toxicity of insecticide mixtures to non-target species is of great significance to design a better pest control programme for creating a safer environment.

Neural tube closure

BMP7

Dorsal

Ventral

SHH

Epidermal roof-plate

Neural crest cells

Neural tube

Notochord

Neural crest cells patterning

patterning

Anterior-Posterior patterning

FGF8

HOX10A

HOX11A

PAX6

Sculpting of head/skeletal tissue

Mesencymal cells

Condense to cartilage

Endochondral ossification

Chondrogenesis

Bone Formation

Mesodermal Derivatives

Paraxial Mesoderm

Lateral Plate Mesode

Somites

Somatic Mesoderm

Sp

Myotome dermis of dorsal body region

Dermis of ventral body region

Wa

Ci

ABNORMAL BODY WALL CLOSURE

Day 4

Delayed Somitogenesis

SHH

CASP3

BMP4

PITX2

E-CAD

Day 10

Hampered Epithelial ce

Abnormal increased

Derailed chondrog

PCNA

SHH

WNT11

WNT6

PXN

MYOD1

CASP3

Ci

SHH

IP7

GLI2

FGF8

CASP3

1