PESTICIDE INDUCED DEVELOPMENTAL MALFORMATIONS: A MECHANISTIC STUDY IN CHICK EMBRYO

Synopsis of Ph.D. Thesis

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Introduction

India is an agriculture-based country, where more than 50% of the population is dependent on agriculture and it accounts for approximately 18% of India's gross domestic product **(Madhusudhan,2015)**. India is now the second largest manufacturer in Asia after China and twelfth globally in terms of consumption **(Mathur, 1999)**. Agricultural development continues to remain the most important objective of Indian planning commission, particularly the production of pesticides and fertilizers. Since 1952, the production of pesticides in India have become an integral part of the agriculture sector by reducing losses from the weeds, diseases and insect pests that can markedly reduce the amount of harvestable produce. The Food and Agriculture Organization has defined pesticide, as any substance or a mixture which is used with an intention of preventing, destroying, or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals, causing harm or nuisance during the production, processing, storage, transport, or marketing of food, agricultural commodities, feedstuffs, or substances that may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies.

These man-made chemicals cover a wide range of chemical and biological compounds that are mostly classified as per the target organism they act upon for e.g. Insecticides- insects, fungicides- fungi, rodenticides- rodents etc. Due to the usage of these chemicals and fertilizers in modern agriculture against the pests, diseases, competition from weeds and pests, the agricultural productivity has significantly increased. The developing countries on the other hand face an obstruction of crop losses due to insects followed by pathogens and weeds. Therefore, the use of insecticides becomes the first-choice of farmers during crop production. In India, the insecticides dominate the industry but with an unscientific pattern of application. Amongst the pest control chemicals, insecticides dominate the industry with 76% of consumption, followed by fungicides (13%), herbicides (10%) and others (1%). **Figure A** depicts the scenario of pesticide consumption in India compared to the global scenario of 44%. It is majorly due to spraying on crops like cotton, wheat and paddy and to control vector borne diseases such as malaria **(Devi *et al.*,2017; Choudhary *et al.*,2018)**.

With such a broad perspective, it is necessary to introduce some focus. The pesticides cover a wide range of compounds which are grouped chemically as: Organochlorines (OC), Organophosphates (OP), carbamates and pyrethroids.

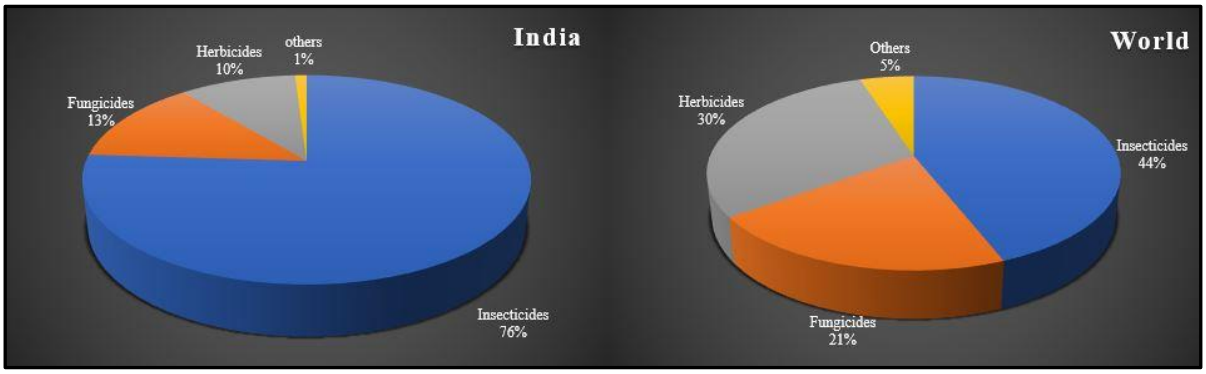

Figure A: Consumption pattern of pesticides **(Mathur, 1999)**

Organochlorines are the chlorinated hydrocarbons which act on the peripheral nervous system by electrophysiological modifications in the energy of the stream of Na+ and K+ through the film of the nerve cell **(Coats, 1990; Connel*et al.*,1999)**; resulting in the spread of multiple actionpotentials for each stimulus **(Kamrin, 1997)**, causing symptoms such as seizures and acute poisoning leading to death from respiratory arrest **(Tordoir and Van Sittert, 1994)**. It includes DDT, Chlordane, Deldrin and heptachlor.

Organophosphates are the esters of phosphoric acid, widely used for prevention of bugs and as miticides. They impart toxicity by inhibiting the enzyme acetylcholinesterase at synaptic junctions which is required for proper functioning of the nerve cells. They are utilized for spraying on vegetable products, grains, cotton and sugarcane among numerous others **(Fukuto, 1970)**.

Carbamates are the organic compounds derived from carbamic acid. They operate through inhibiting the enzyme acetylcholinesterase allowing acetylcholine to transfer nerve impulses indefinitely and causing a variety of symptoms such as weakness or paralysis **(Kamrin, 1997; Perry *et al.*, 1998)**.

Pyrethroids are naturally derived insecticides from pyrethrum extract obtained from chrysanthemum flowers, known as pyrethrins. They were subsequently obtained synthetically. Their mode of operation is through bringing changes in the flow of the Na^+ in the film of the nerve cell, making it extend its opening time, drawing out sodium current over the layer in both bugs and vertebrates **(Perry *et al.*,1998; Soderlund *et al.*, 2002)**.

PESTICIDES AS TERATOGENS AND EMBRYOTOXICANTS

A large part of the insecticides directly squirted on the crops are ultimately degraded by microbial or chemical pathways. However, a significant fraction of the applied amount gets

dispersed in the environment, contaminating the ecosystem. These insecticides are ideally designed to target specific species of organisms through a particular mode of inhibition. But their unwarranted use poses a serious threat that may result in the loss of biodiversity. Pollution of environment with these harmful chemicals is a growing concern to the human society as it might inadvertently lead to many health-related diseases or disorders in non-target organisms, not sparing the humans **(Tebourbi *et al.*, 2011)**.Many evidences have come forward indicating the serious threats posed by pesticides to various life forms and their ecosystem irrespective of acute or chronic exposure.**(Sulbatos *et al.*,1994; Helfrich *et al.*, 1996; Hodgson and Levi, 1987; Lorenz *et al.*, 2009)**.

Acute toxicity is referred as pesticides' ability to cause damage to an individual or an animal from a single exposure for a short duration. The chronic effects are generally determined by exposing the test animal to the pesticide for a longer duration. The harmful effects that occur from small repeated doses over a period are termed "chronic effects." Some of these adverse effects show up as immune-suppression, hormone-disruption **(Bretveld *et al.*,2006)**, diminished intelligence **(Landrigan *et al.*, 2002; Mascarelli, 2013)**, reproductive abnormalities **(Sanborn *et al.*,2007; Perry *et al.*, 2011)**, and even cancer **(Greenburg *et al.*,2008; Lynch *et al.*,2009; Gilden *et al.*,2010)**.

The numerous negative health effects mentioned above occur undeniably due to pesticides, regardless of the mode of exposure through skin, inhalation or congestion. Moreover, several evidences have already stated these chemicals to be teratogenic and carcinogenic in nature, culminating into a variety of congenital aberrations **(Stenberg, 1979; Durham and Williams, 1972; Garcia *et al.*,2001)**. **Brent (1990)** documented that approximately 10-15% of developmental deformities are a result of the adverse effects of these environmental factors on prenatal development. This means that approximately 1 in 250 infants have structural defects caused by an environmental exposure and, presumably, a larger number of children have growth retardation or functional abnormalities resulting from non-genetic causes, in other words, from the effects of teratogens. These pesticides induce developmental deformities through several mechanisms of cellular signalling and causing oxidative stress **(Eaton *et al.*,2008; Ngo *et al.*, 2010; Uggini *et al.*,2012; Alleva *et al.*, 2018)**. A representative diagram (**Figure B**) portrays how a pesticide may cause congenital defects and cancer through oxidative damage and altered signalling pathways.

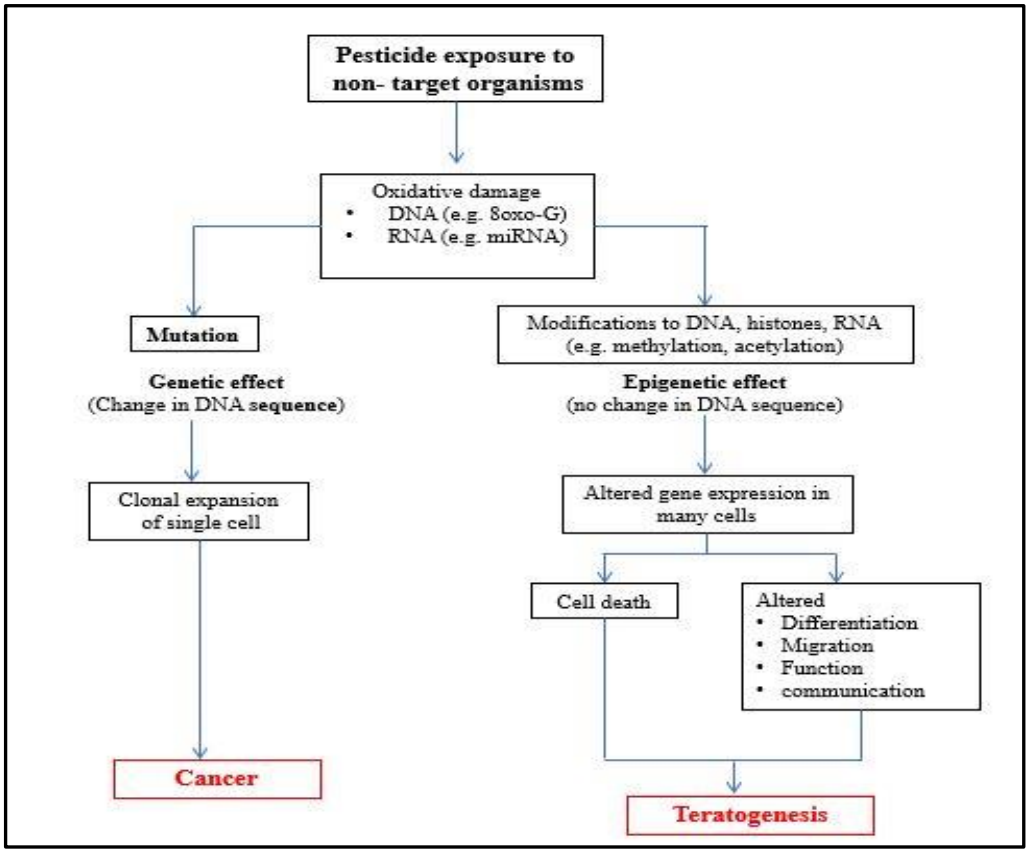

Figure B: Teratogens causing oxidative damage culminating into malformations.

Most of the pesticides (Organophosphates and organochlorines) alter the expression of genes as they are generally nervous and/or endocrine disruptors. The structural abnormalities caused by teratogenic exposures occur during the embryonic period, which is when critical developmental events are taking place and the foundations of organ systems are being established. Therefore, it is mandatory to understand the early developmental processes which might probably give clues to how the pesticide exposure can disturb the patterns of the embryonic development and lead to the malformations.

EARLY EMBRYONIC DEVELOPMENT

The process by which a fertilized egg undergoes changes to form the embryo is called embryogenesis. After fertilization, cleavage takes place and cells divide mitotically to form a ball of cells called the blastula. Each cell in the blastula is known as a blastomere. This event is followed by gastrulation wherein cells undergo cellular movements like invagination, evagination, ingression and involution, thereby landscaping a bilaterally symmetric three-layered structure,*viz.*, ectoderm, mesoderm and endoderm.

Further development proceeds as germ layers undergo restructuring to craft the different organs in the body and this process is called organogenesis. These germ layers are responsible for the origin of all tissues, organs and systems of the embryo. The outer layer, ectoderm, gives rise to cells of epidermis and the nervous system. The inner layer, the endoderm forms the lining of digestive tube and its associated organs. The middle layer, the mesoderm, sculpts circulatory and urinogenital system. The connective tissues like bone, cartilage, lymph, and blood, muscles etc, are also mesodermal in origin. Followed by this, the organs finally attain functional maturity **(Gilbert, 2003)**.

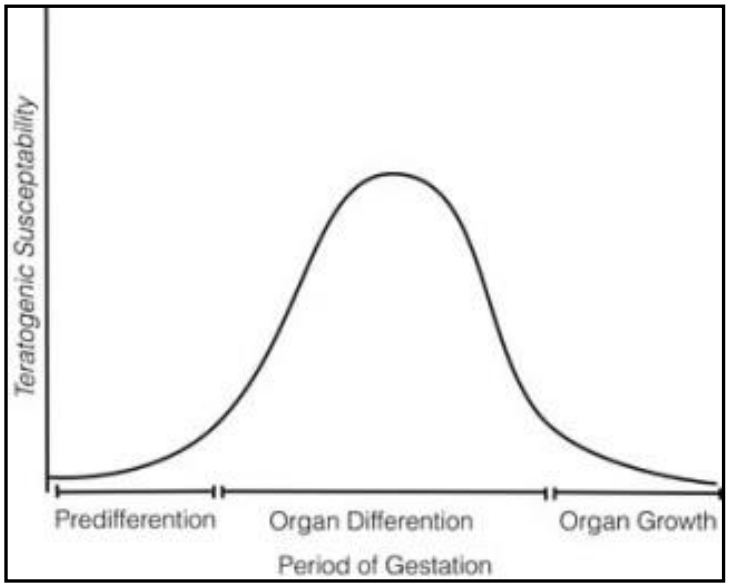

Figure C: Embryonic periods of differential susceptibility to a teratogen **(Simpson, 1976)**.

There is often a period of vulnerability to the effects of these toxic chemicals during foetal development and early childhood. This vulnerability occurs during the period of development of various organ systems (period of organogenesis) wherein permanent structural birth defects or permanent functional changes may occur **(Arbuckel and Server, 1998)**. A large teratogenic insult may kill the embryo, but the surviving embryo may not manifest any organ specific abnormality. During organogenesis, the overall growth of the embryo is affected (**Figure C**).

The embryo is most susceptible to teratogenic exposure as they act in an organ-specific manner; affecting the organ system at their respective stages of development. The precise time at which the insult occurs, thus determines not only occurrence of malformation but also the specific spectrum of anomalies.

EFFECT OF MIXTURE OF CHLORPYRIFOS AND CYPERMETHRIN

To observe the impact of such teratogens on the developing embryo we selected a widely used a combination insecticide which is popular amongst the farmers. The frequently used pesticides in the market belong to the class organophosphates (OP) and synthetic pyrethroids (PYR), which are offered in many formulations. It is also reported that when applied together, the organophosphates enhance pyrethroids toxicity **(Ray and Forshaw, 2000; Hernandez *et al.*, 2013)**.

In the current study, the focus was made on the combination of two insecticides namely chlorpyrifos and cypermethrin, representing the groups mentioned earlier. A commercial formulation Anaconda 505™: a combination of Chlorpyrifos (50%) and Cypermethrin (5%) as emulsifiable concentrate (EC) was selected for the study. Previous work from our laboratory has shown that an exposure of this combination insecticide (Ci) caused a variety of birth defects like distorted cephalization, umbilical hernia, ectopic viscera, spina bifida and anophthalmia at sub-lethal doses in the developing chick embryo **(Uggini *et al.*, 2012)**. Teratogenic potential of this Ci was also reported to be passed onto the next generations even though the hatchlings of which did not receive any direct pesticide treatment **(Khan *et al.*, 2015)**.

Chlorpyrifos

Chlorpyrifos is one of the most widely used pesticide at different places ranging from spraying on the agricultural fields to the residential usage in the form of containerized baits. The IUPAC nomenclature of Chlorpyrifos is O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate and is commercially manufactured by reacting 3,5,6-trichloro-2-pyridinol with diethylthiophosphoryl chloride. The empirical formula of chlorpyrifos is $C_9H_{11}Cl_3NO_3PS$ and the chemical structure is O,O- diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate **(Kidd and James, 1991; Muller, 2000)**.

Effects of Chlorpyrifos

Chlorpyrifos is a non-systemic and broad-spectrum insecticide which kills the pests by binding to the cholinesterase (ChE) enzyme and thereby affecting the nervous system **(USEPA 1999; Smegal, 2000; Lotti and Moretto, 2005; Pung *et al.*, 2006)**. By binding to the active site of the cholinesterase enzyme, this insecticide prevents the breakdown of the acetylcholine, a neurotransmitter **(Karanth and Pope, 2000).** Though the mode of action remains the same in

non-targeted organisms, the toxic effects varies species to species. Parenteral routes of chlorpyrifos exposure have reported to bemoderately toxic to humans; moderately to very highly toxic to birds and very highly toxic to freshwater fish and marine organisms **(Kamrin, 1997; Reigart and Roberts, 1999; Lotti and Moretto, 2005)**.

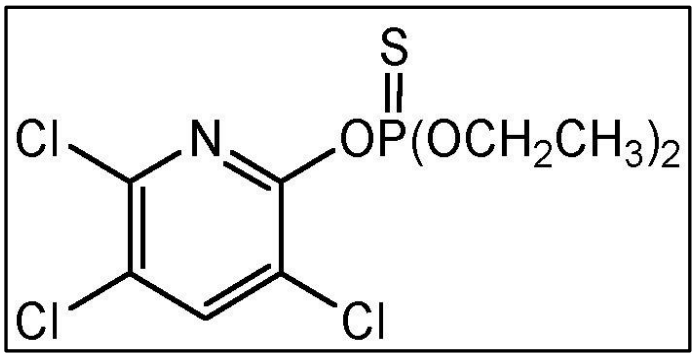

Figure D: Structural formula of Chlorpyrifos

Reproductive and developmental defects occurring due to chlorpyrifos exposure have been studied in various animal models. In adult rats, acute and chronic exposure of chlorpyrifos have reported to cause oxidative stress, developmental disorders, immunotoxicity and embryotoxicity **(Yin *et al.*,2009, Sandhu *et al.*,2013)**. In addition, rat neonates and young ones were found to be more susceptible to chlorpyrifos even at the concentration below causing ChE inhibition **(Smegal, 2000; Zheng *et al.*, 2000)**. Morphological defects in brain, reduction in viable offspring number, decreased body weight were some of the adverse effects of chlorpyrifos exposure. Even in the avian model, low level exposure 60-125 ppm induced reduction in egg production, thinning of egg shells and increased death. All of these shortcomings might be an accumulation of impaired DNA, RNA and protein synthesis, increased programmed cell death, altered gene expression and cell differentiation processes **(Whitney *et al.*,1995; Roy *et al.*, 1998; Dam *et al.*, 1999; Crumpton *et al.*, 2000; Ricceri *et al.*,2003)**.

Cypermethrin

Cypermethrin is from a class of synthetic pyrethroids which are chemically similar to pyrethrins (extract from chrysanthemum plants). These are designed to have long lasting effects compared to natural pyrethrins. Cypermethrin is a neurotoxic agent most probably acting through the central nervous system to cause repetitive nerve activity. It is readily absorbed from the gastrointestinal tract and minimally through intact skin **(Jin and Webster, 1989)**. It was first

synthesized in 1974 **(WHO, 1989)** and is widely used against pests to cotton, fruits, vegetables, for public health and in animal husbandry. Molecular formula of cypermethrin is $C_{22}H_{19}C_{l2}NO_3$ and the Chemical structure of Cypermethrin is [(±)-α-Cyano-(3-phenoxyphenyl) methyl (±)-cis/trans-3-(2,2-dichlorovinyl)-2,2 dimethylcyclopropane carboxylate].

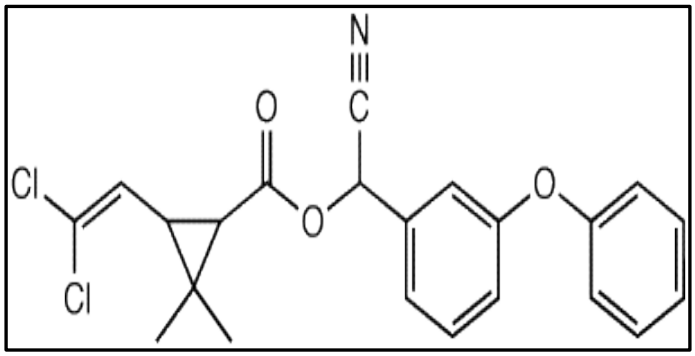

Figure E: Structural formula of Cypermethrin

Effects of Cypermethrin

Cypermethrin is a synthetic pyrethroid which is classified as 'moderately hazardous" by **WHO (1989)**. It is a neurotoxic agent affecting the central nervous system. It majorly delays the sodium ion channel closure causing prolonged sodium ion influx and as a result continuous stimulation of the nerves **(Vijverberg and Bercken, 1982; Chinn and Narahashi, 1986)**.

Pyrethroids have been widely used because of their low toxicity to birds and mammals. However, aquatic life forms and especially fishes have been reported to be more susceptible to cypermethrin **(Stephenson.,1982)**. **El-Toukhy**with his colleagues **(1993)** and **Siegfried (1993)** have reported cypermethrin to inhibit ATPase and GABA receptors in fishes and aquatic insects respectively. A dose-dependent apoptotis in the CNS of the tadpoles of the toad *Bufo arenarum* **(Casco *et al.*,2006)** and mice **(Singh *et al.*,2011)** has also been reported.

There is not much data available on reproductive abilities of the organisms but **Cantalamessa (1993)** observed developmental defects particularly in rat neonates. Incomplete development of the enzymes which metabolize cypermethrin can be considered as a critical factor in case of young ones. Several reports state occurrence of developmental delays in the rat young ones, when the females were exposed during their pregnancy period **(Amer *et al.*,1993; Tateno *et al.*, 1993; Shi *et al.*, 2011)**. Chronic exposure to male rats resulted in increase in abnormal sperms. Abnormalities in bone marrow and spleen cells have also been reported **(Amer and**

Aboul-ela.,1985). Moreover, cypermethrin exposure is known to have teratogenic effects on chick embryo **(Anwar, 2003).**

MODEL FOR THE STUDY

The chick embryo has been widely used in the field of developmental biology throughout the history **(Stern, 2005)**. It has been considered as a promising model for the screening of embryotoxic and teratogenic agents from many decades **(Wilson, 1978; Jelinek, 1982; Drake *et al.*, 2006)**. The chick embryo justifies all the minimal standards required for being a test system for teratological studies. There are several whys and wherefores for the usage of chick embryos as a model organism i.e. the availability of fertilized eggs, the rapid growth of the embryo, the ease in manipulation of the embryo, possibility of experimenting on a large scale for statistically valid results and considerably less expensive model system than the live animals. During the period of development, when the egg progresses from a single cell to a hatched one, all the complexities of embryological development can be observed and analysed

In-ovo chick model delivers a major advantage over other in-vitro systems that employ embryonic tissues with limited survival. It also eliminates the maternal influences such as biotransformation which may alter the concentration of the injected compound. Individual compounds, mixtures of compounds can be easily administered and tested **(Kotwani, 1998)**.

Ci, a known toxicant having a teratogenic propensity can induce a myriad of teratological malformations in the developing chick embryo. Here we therefore intend to make a reasonableanalysis of certain molecular signals which were waivered off from their regular developmental milestones.

Objective of the study

The overall objective of the current study was to unearth the mechanisms through which the commercial formulation of chlorpyrifos (50%) and cypermethrin (5%) combination induces various teratological defects in the regions of head and torso. The said objective would be accomplished through the following:

1) A comprehensive morpho-histological detail of the teratological defects presented by the chick embryos in the head and torso regions, after an early exposure to the sub-lethal dose of Ci.
2) Analysis of changes in the gene expression of certain important regulatory and/or pattern forming genes which play a vital role in the early embryonic development.

Materials and Methods

Test article

The test substance used for this study is a combination of chlorpyrifos and cypermethrin. It is manufactured by AIMCO Pesticides Limited, located at Santa Cruz, Mumbai, India. This insecticide is marketed as Anaconda505^{TM}, constituting of chlorpyrifos (50%) and cypermethrin (5%). The details of the product are given in the table.

Commercial Name	ANACONDA 505^{TM}
Manufactured by	AIMCO Pesticide Pvt. Ltd; Mumbai, India
Chemical name **a) Chlorpyrifos** **b) Cypermethrin**	O,O-Diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate [(±)-a-Cyano-(3-phenoxyphenyl) methyl (±)cis/trans-3-(2,2dichlorovinyl)-2,2 dimethylcyclopropanecarboxylate]
Registration No.	CIR-26,393/97/Chlorpyrifos + Cypermethrin (EC)-7
Manufacture License No.	0403/0001/M/D/ Date; 10/07/1981
Manufacturing Date	10/04/2014
Expiry Date	09/04/2017
Stability	Stable under normal storage conditions. Unstable at elevated temperatures.
Physical and Chemical Properties	
Appearance	pale yellow, viscous liquid
Melting Point	<5°C, insoluble in water
Specific Gravity	(Liq.) 1.398 at 43.5°C
Density	1.1g/ml at 20°C
Odour	Xylene

Test system

The study is focussed on studying the developmental toxicity on chick embryos. For this, eggs of Rhode Island Red breed of domestic chicken were chosen to study the developmental stages. The breed is predominantly known for its egg laying capacity and its easy availability. To avoid discrepancy with the developmental stages, only freshly laid fertile eggs were selected and were procured from Intensive Poultry Development Unit, Vadodara. Only the eggs which weighed approximately 55 ± 5 grams were taken for further study. These eggs were then wiped clean with 0.5% povidone iodine solution to remove any external contamination and were kept at 4°c until prior use.

Insecticide injections

The eggs were randomly allotted to the control group (which received olive oil) and the treatment group (which was administered with 0.05µg per egg of Combination insecticide in olive oil as vehicle). But prior to dosing, the air sac in the eggs were marked by a pencil (occurs mostly on the blunt end side) with the help of a candler. Eggs were then dosed in a sterile environment in the laminar air hood. The concentration of sub-lethal dose was decided from the pioneering work done in the lab **(Uggini et al., 2012)**. The injection volume was kept 50µl/egg. After dosing, the point of injection on the eggs were sealed by molten paraffin wax. The eggs were dosed by air sac method as devised by **Blankenship *et al*., 2003**, on day "0" of incubation as given below.

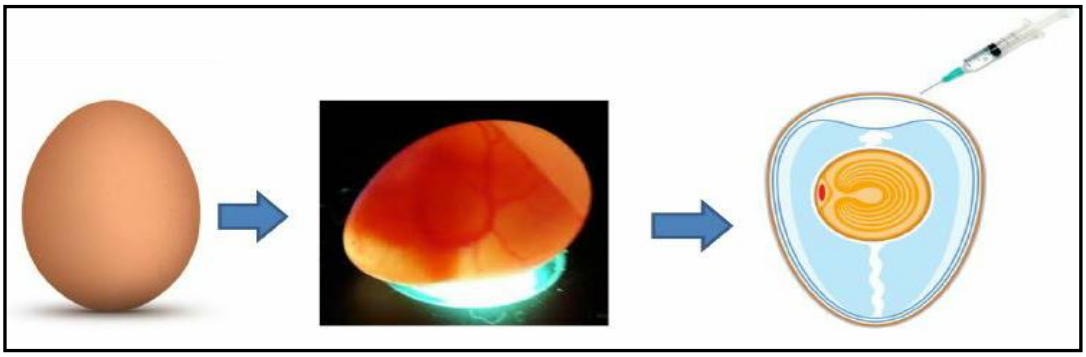

Figure F: Diagrammatic representation of candling and injection of test article into the air cell of the egg.

Incubation

Automated incubator (Scientific equipment works, New Delhi) was set at a temperature of 37.5°c ± 0.5 and relative humidity of 70–75% for incubation. The eggs were kept with their

broad ends facing upwards and were turned automatically every 1 hour. The eggs were candled every two days and the dead ones/unfertilized ones were scrapped out.

Sample collection

After incubation, the embryos that had reached Hamilton-Hamburger stages 13 (Day 2), 24 (Day 4), and 36 (Day 10) were collected.

Rate of mortality

The eggs were regularly candled every 2 days till the 10th day of incubation to cull out the unfertile or dead embryos. The rate of mortality was calculated by the formula given below:

$$Embryo\ mortality\ rate\ (\%) = \frac{Number\ of\ dead\ embryos}{Total\ number\ of\ fertile\ eggs} \times 100$$

Rate of malformation

The chick embryos were checked for occurrences of any malformations after opening the eggs. Any malformation observed was photographed.

$$Chick\ malformation\ rate\ (\%) = \frac{Number\ of\ malformed\ chick}{Total\ number\ of\ fertile\ eggs} \times 100$$

RNA isolation and real time reverse transcription polymerase chain reaction

The chick embryos were isolated and then were dissected to collect the tissue from the abdomen region. From this sample, total RNA was isolated by TRIzol reagent (Applied Biosystems, USA) according to the recommended protocol. 1 µg of total RNA was reverse transcribed to cDNA using a one-step cDNA synthesis kit (Applied Biosystems, USA) as per manufacturer's protocol. Primer for all the genes used in the study were designed from the regions on mRNA of Gallus gallus, and were obtained from NCBI. The list of the primer sequences is given in the table 2.

Quantitative real-time PCR was performed using Lightcycler96 (Roche Diagnostics, Switzerland). After initial denaturation step of 3 min at 95°C, 42 cycles of amplification for each primer was carried out. Each cycle included a denaturation step for 10s at 95°C, an annealing step for 10s at 60°C and elongation for 10s at 72°C. Melt curve analysis was performed to confirm specific product formation. Data was represented as mean Cq values

Gene	Forward Primer	Reverse Primer
Shh	TGCTAGGGATCGGTGGATAG	ACAAGTCAGCCCAGAGGAGA
Pitx2	GCTACCCCGACATGTCCAC	TTCTTGAACCAAACCCGAACC
Gli3	TCTCGTAGCAGTTCGTCAGC	TCAGAGCAGGGCTTATTGCG
Gli2	GGGGATGGCTTTACGGAGAC	CAATGGAGGAGGCCCGTG
Sox9	GAACAAACCCCACGTGAAGC	TCATTCAGCAGCCTCCACAG
RunX2	CTGGTGCCTTTTGGGTTGTG	TCGACACGTCTTGCTTAGCG
Dlx5	GGAATGCGGATGGGGGATTT	CCACAGCTGAGCCGAAAAAC
Col10a1	CTTCACGGTTTAGCTTCACAAG	TTGTGGTCCTGCAACAGAGT
L1CAM	TTCCCCCGGAGTATGGTGC	CTGGGGAAGACCACGAGTTG
Hox10a	GGAGCCCGTAGGCAATTCAAA	ACGCTCACGAGTCAGGTACA
Hox11a	CAGCTCCAGTGGACAACGG	CAGCTCCAGTGGACAACGG
Pax6	AGCAAGGATACAGGTGTGGT	TGTGGGATCGGCTGGTAAAC
E-cadherin	GAAGACAGCCAAGGGCCTG	TCTGGTACCCCTACCCTCTTG
bmp2	ATGTTGGACCTCTATCGCCTG	CCAAAACTTCTTCGTGGTGG
bmp4	AGCCCACGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC
bmp7	ATCTGCCTACAAAATTGGTTCTC	TACTCACAGCGCATTCTCACTT
Caspase-3	AAAGATGGACCACGCTCAGG	TGACAGTCCGGTATCTCGGT
vimentin	GACCAGCTGACCAACGACAA	GAGGCATTGTCAACATCCTGTC
fgf2	ATCCGGGAGAAAAACGACCC	TTGGTCGTCTCGCTCCAAAC
fgf8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT
N-cadherin	AGCCCACGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC
bmp1	CCAGCAAAGTGTGTGTGTGG	GAGGCGCTTTTGATGTCGTC
pcna	TGTTCCTCTCGTTGTGGAGT	TCCCAGTGCAGTTAAGAGCC
Wnt11	GACCTGGGTATCGATGGGGA	GGCTTTCAAGACCTGTCTCC
Wnt1	AAGTCGGGAAGGAGAGGTGA	GAGCCATCTGAAACTGCCCT
Wnt5a	GACACTTGGCAGCACAATGG	CCCTAGAGACCACCAAGAGC
Wnt7a	TATCGTCATCGGGGAAGGGT	GCTGCTTCTCTGCTACCCAC
Wnt6	TTGGTCATGGACCCCAACAG	CCTCGCTGACGATTTCTGGT
Pxn	TCTGACTTTAAGTTCATGGCACAG	TCGCTACCCCCAGTTTGTTC
MyoD1	CGGAATCACCAAATGACCCAA	ATCTGGGCTCCACTGTCACT
AHR	ACCTGTGCAGAAAATAGTAAAGCC	GCTGAGCCTAAGCACAGACA
Cyp1A1	ACCACGACGAGAAGATCTGG	AGATCAGCACCTTGTCAGCC
Cyp3A4	AGTGCAATGGGACTCCTTCC	GGCCATATCCCATAGAGCACC
Cyp3A5	TGGGTATGAGCCCACCAGTA	CATACGTGAGCGGAGCCTTA
18S rRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGAAC

Table 1: Primer sequences obtained from NCBI.

normalized with internal control (endogenous control) levels and fold change in expression was calculated using the $2^{-\Delta\Delta Cq}$ method of **Livak and Schmittgen (1976)**.

Bone and cartilage staining

The control and treated embryos were collected on day 10 for studying the skeletal deformities if any. For this the samples were first isolated and then washed in chilled PBS solution to remove the debris. After cleansing, the samples were immediately stored in ethanol for 48 hours at 4°C followed by acetone for 2 hours. These samples were further processed in freshly prepared alcian blue and alizarin red stain, for differential staining for 4 hours at room temperature and in dark conditions. The stained specimens were rinsed in ethanol for 1hr, washed under running tap water and then cleared in 1% KOH solution until skeletons were visible. The destaining procedure was done in a graded sequence of glycerol and 1% KOH and finally stored in 100% glycerol. Alcian blue stains proteoglycan components of extracellular matrix associated with chondrocytes and those are used to identify cartilaginous tissues in embryos and in adults, whereas Alizarin red stains calcified matrix associated with bone **(Mcleod, 1980)**.

Biochemical Estimation of Hydroxyproline

Out of few proteins containing amino acid hydroxyproline, collagen is one. To estimate collagen content in given tissue, hydroxyproline assay was performed as described by **Edwards and O'Brien (1980)**. The tissues were excised and weighed. It was then homogenized with equal amounts of 6M HCL and kept at 120°C for an hour. Upon completion of hydrolysis, the hydrolysates were kept for drying in oven. Citrate buffer, Chloramine-T solution and DMAB solution were mixed and incubated for 20mins at room temperature. This mixture was then added to the samples and absorbance of the reaction was read at 560nm. Sample concentrations were determined from the standard curve.

Histological studies

Embryos were isolated, rinsed in PBS and then were fixed in 10% neutral buffer formalin. The tissue was further processed, and paraffin wax blocks of the tissue samples were prepared. Longitudinal sections and Cross sections of 5μm thickness of the embryos were taken and stained with Harri's haematoxylin and eosin. The histological structure of the tissues on the

slide was visualized using Leica DM2500 Microscope and pictures were captured using EC3 Camera (utilizing LAS EZ software).

Western blot

The tissue samples collected from the control and experimental group were homogenised in Tris-SDS lysis buffer with protease inhibitor (Sigma Aldrich, USA). 10% of the homogenates were assayed for total protein content, method devised by **Bradford (1976)**. Equal amount of total protein was loaded and separated by SDS-PAGE on 10% gels. Protein was transferred onto nitrocellulose membrane by semi-dry transfer at 100 mA for 30 mins. The membrane was probed overnight with appropriate antibodies followed by incubation with the respective secondary antibodies. Alkaline phosphatase system was used for staining the protein of interest.

Animal ethics

The experimental protocols were in full compliance with the guidelines of Drugs and Cosmetics rules 1945, Appendix-III animal care standard and were approved by the Institutional Animal Ethics Committee (IAEC No.84/08/2014-2) in accordance with the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Statistical analysis

The data was analysed by two-tailed Student's t-test using Prism v6.01 (GraphPad Software Inc., USA). Values were expressed as Mean ± SEM, and the differences between the control and treated groups were considered significant when the P value was less than or equal to 0.05.

Results

A mechanistic study was undertaken to check the effect of a sub-lethal dose of combination insecticide on the developing chick embryo which resulted into abdominal wall and cranio-facial defects. To accomplish this objective, the pre-decided dose of the combination insecticide was taken and administered into the air sac of the fertile RIR egg on day zero of incubation **(Blankenship *et al*., 2003)**. Embryos were isolated on day 2, 4 and 10 and the rate of mortality was checked. Subsequently, the embryos were examined for gross morphological changes and the extent of damage caused by Ci were analysed by haematoxylin and eosin staining. Followed by the histopathology, the mRNA expression levels of the molecules involved in the closure of abdominal wall and the protein expression of the major regulators were taken into consideration. The details of the study are given hereunder.

1. Abdominal wall defects

Rate of Mortality and Malformation

The pesticide dosed embryos were opened for examination on day 2 (stage 13), day 4 (stage 24) and day 10 (stage 36). The stage-13 control embryos showed the standard features i.e. presence of twenty somites, broad curves in cranial and cervical flexures and well-established stalk. The embryos treated with 0.05µg of Ci after incubation of 48 hours showed significantly reduced number of somites. 33% of the embryos showed abnormal somite disposition **(Table 2)**. High rate of mortality and anomalies were observed in the treatment group. A steep rise ($p \leq 0.001$) was observed in the mortality of embryos in pesticide dosed groups as compared to the control and it continued to increase as the embryo developed to day 10 **(Table 3)**. As the study was focused on ventral body wall defects, the rate of occurrence of these malformations was checked. It was observed that 60.6% of the total live embryos observed on day 10 had defects in their abdominal wall closure **(Table 4)**.

Group	Day 2 (Stage-13)	
	Somite number	% of embryos with abnormal somites
Control	18 (17, 19)	6.0 ± 0.57
Treated	16 (15, 18) *	33 ± 1.73 **

Table 2: Reduction in somite numbers: Values are expressed as mode with range in parenthesis; n=3 with 30 eggs per group per experiment; *$p \leq 0.05$, **$p \leq 0.01$.

Group	Day 2	Day 4	Day 10
Control	3.66 ± 0.19	3 ± 0.28	3.66 ± 0.50
Treated	10.66 ± 0.35**	12.33 ± 0.57***	14.66 ± 0.58***

Table 3: Mortality on day 2, day 4 and day 10 for sub-lethal dose Combination insecticide: The values represent mean ± standard error of mean; n=3 with 30 eggs per group per experiment; **p ≤ 0.01, ***p ≤ 0.001.

Group	Number of live embryos of Day 10	% of embryos with malformations
Control	26.33 ± 0.52	7.6 ± 0.52
Treated	15.33 ± 0.58***	60.6 ± 1.18***

Table 4: Percent malformation on day 10 from live embryos: The values represent mean ± standard error of mean; n=3 with 30 eggs per group per experiment; ***p ≤ 0.001.

Teratological malformations

When the embryos were examined at stage 24 (96hr), the controls showed normal development with distinct eye pigmentation and limb buds **(Fig. 1A)**. However, defects like microphthalmia, and stunted growth were found in 96hr incubated treated embryos **(Fig. 1B)**. While the control embryos **(Fig. 1C)** examined on day 10 showed normal patterns of development of visceral organs and the abdominal wall, the treated embryos showed protrusion of heart, bowel and liver due to defect in midline fusion of abdominal wall **(Fig 1D)**. The other associated anomalies included anophthalmia, phocomelia, hematomas, and defect in ventral body wall closure in the thoracic region.

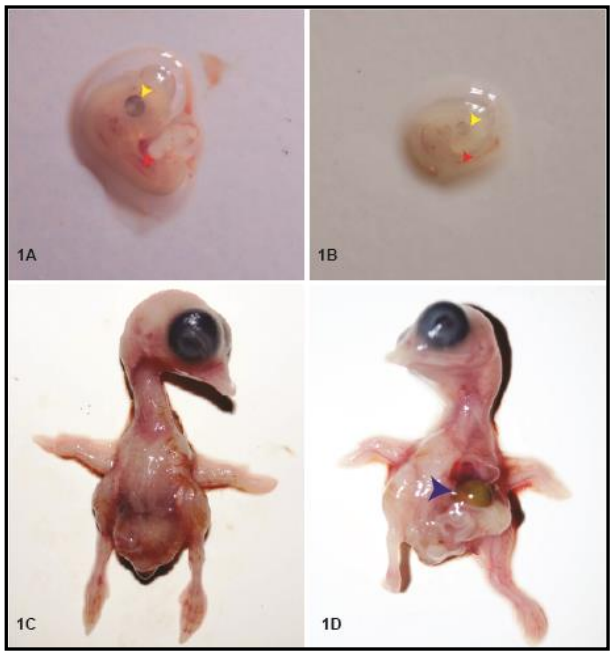

Figure 1: Ci induced structural anomalies: A) Control embryo of day4 with well-developed eye (yellow arrowhead) and limb buds (red arrowhead); B) Ci treated day 4 embryo with under developed eye (yellow arrowhead) and appendages (red arrowhead). C) and D) shows Day 10 - Control and Treated embryo respectively. The blue arrow head indicates protrusion of heart, liver and bowel without any covering sac in the treated embryo.

Histopathology

The extent of damage caused by the pesticide load in the tissue architecture was studied by differential staining in day 4 and day 10 chick embryos by using hematoxylin and eosin. The control embryos on day 4 showed properly arranged somites with well-developed myelencephalon and optic cup **(Fig. 2A).** The heart was well formed with clear demarcation between atrium and the ventricle. The treated embryo showed stunted growth, with a reduction in the somite numbers and distorted optic cup **(Fig. 2B).** The histological picture of day 10 treated embryos showed less developed musculature, and the internal organs were left uncovered. Absence of sternum in the treated embryo was a crucial observation **(Fig. 2C and 2D)**.

Relative mRNA expression levels

In day 4 embryos, the relative mRNA expression levels of *Shh* were found to be significantly lowered ($p \leq 0.001$) in the treatment groups when compared to the controls, the bmp4 ($p \leq 0.001$),

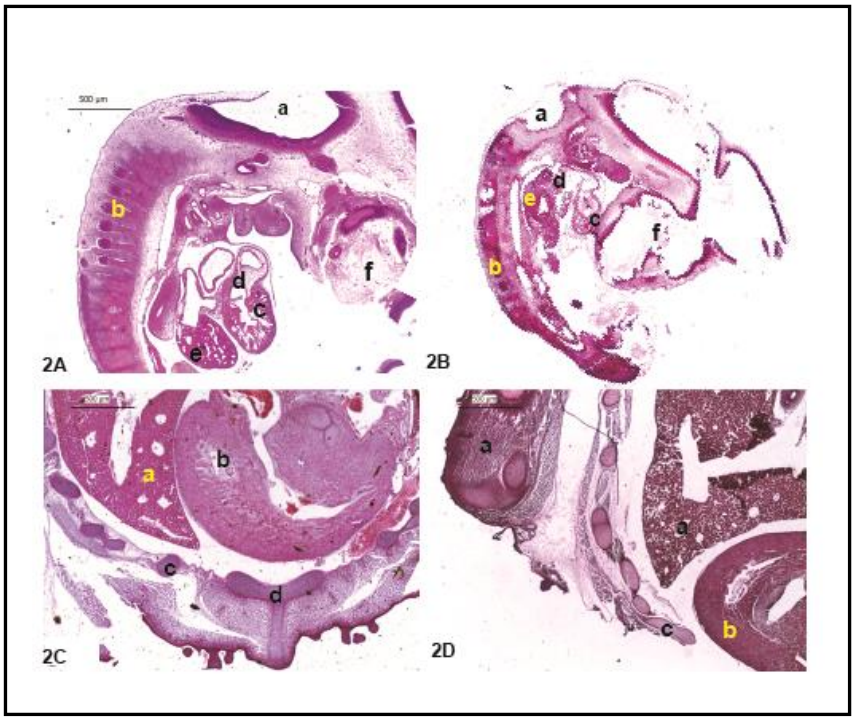

Figure 2: Photomicrograph of Day4 and Day 10 chick embryo: longitudinal sections of A) Day 4 Control with well patterned structures; B) Day 4 Treated with reduced somites and distorted optic cup. Where, a: Myelencephalon; b: Somite; c: ventricle; d: Atrium; e: Liver-prominence, f: optic cup C) Cross section (insight) of abdominal region from day 10 control embryo shows well-formed abdominal wall; Figure D) Cross section (insight) Day 10 Treated embryo showing improper closure of the ventral part of the body. Where, a: liver; b: stomach; c: Inter coastal muscles; d: Sternum.

Pitx2 ($p \leq 0.05$), E-cadherin ($p \leq 0.001$) and Caspase-3 ($p \leq 0.001$) were found to be significantly

increased in the treated group. No significant changes were seen in case of N-cadherin and fgf8 expression **(Fig. 3)**.

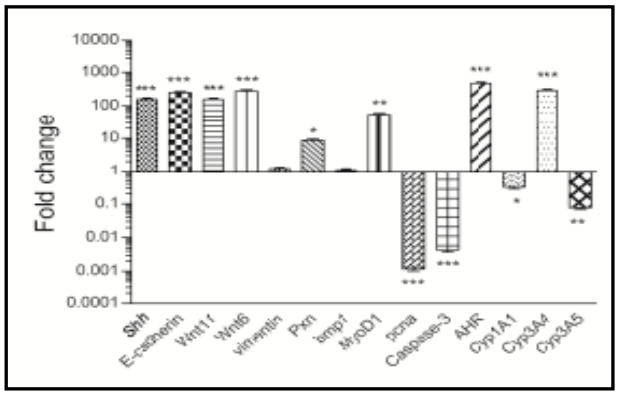

Figure 3: Transcript levels for Ci treated Day 4 embryo: Fold change expression values for the genes. Fold change values for control embryo is 1.0 for all the genes, n=10eggs/group.

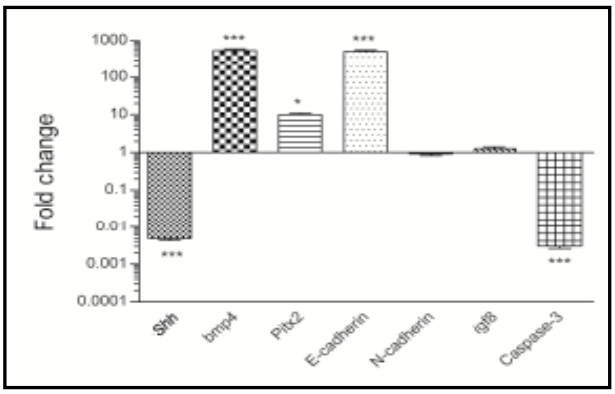

Figure 4: Transcript levels for Ci treated Day 10 embryo: Fold change expression values for the genes. Fold change values for control embryo is 1.0 for all the genes, n=10eggs /group.

The qRT-PCR analysis in day 10 embryos showed that along with *Shh*, the E-cadherin, Wnt11 and Wnt6 expressions were significantly upregulated ($p \leq 0.001$). The change in expression levels of vimentin were found statistically insignificant. Pxn ($p \leq 0.05$) was found to show an increased expression. While the bmp1 supposedly remained unchanged, the levels of MyoD1 have significantly gone high ($p \leq 0.01$) in the treated embryos. The PCNA and Caspase-3 showed a highly significant ($p \leq 0.001$) decrease and increase respectively. AHR ($p \leq 0.001$) and Cyp3A4 ($p \leq 0.05$) expression increased with significance, while Cyp1A1 ($p \leq 0.05$) and Cyp3A5 ($p \leq 0.01$) showed a down regulation **(Fig. 4).**

Western blot analysis

In order to reaffirm the results of qRT-PCR analysis few representative proteins were quantified through western blot. Immunoblot analysis revealed reduced expression of Shh in the treated embryos on day 4 compared to that of in the control. This confirms that sonic hedgehog levels were reduced at both transcription and translation levels by pesticide intoxication. On day 10, E-cadherin and caspase levels were found to be increased and hence, validated the RT-PCR results **(Fig. 5)**. β-actin was used as internal control.

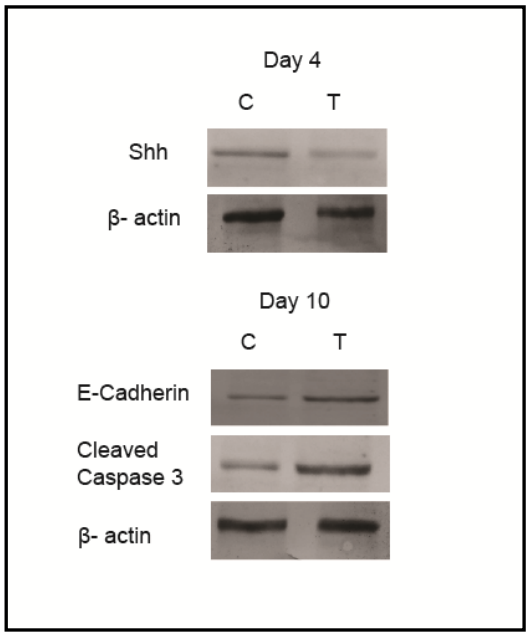

Figure 5: Western Blot images showing comparative expression of various proteins: Shh on Day 4; E-Cadherin and Cleaved caspase-3 on Day 10. β-actin was taken as loading control, n=10 eggs/group.

2. Craniofacial defects

Craniofacial defects are the shortcomings in the development of cranium and facial structure of the organism. These developmental anomalies could be the result of impaired pattern formation for the development of head and facial bones during early embryogenesis. When the Ci under study was injected into the embryos, several of these incongruities were observed. Some of these included anophthalmia (absence of one or both the eyes), acrania, distorted cephalisation, change in somatic dispersion pattern and crimped neural tube were observed.

Mortality rate

The embryos were dosed with the combination insecticide on day '0' of incubation and were opened on day 2 (H-H stage 13), 4 (H-H stage 24) and day 10(H-H stage 36). From the **Table 5**, it was evident that the pesticide was toxic to the embryo and therefore the mortality was seen to be heightened in case of dosed group when compared with the control group.

Group	Day 2	Day 4	Day 10
Control	4 ± 0.57	5 ± 0.88	4.66 ± 0.33
Treated	11.66 ± 0.88**	10.33 ± 0.57***	13.66 ± 0.88***

Table 5: Mortality on day 2, day 4 and day 10 for sub-lethal dose Combination insecticide: The values represent mean ± standard error of mean; n=3 with 30 eggs per group per experiment; **p ≤ 0.01, ***p ≤ 0.001.

Teratological observations

The eggs were opened on day 2 (Stage 13), day 4 (Stage 24) and day 10 (Stage 36). The control group embryos showed normal development with distinct eye formation and pigmentation when examined at stage 24 **(Fig 6A)**. However, the pesticide intoxicated group showed anomalies like microphthalmia, anophthalmia and partially developed head **(Fig 6B)**. The embryos on day 10 in control group showed proper development of the cranial structure **(Fig 6C)** when compared to the treated embryos with signs of toxicity such as acrania, anophthalmia, hematomas and short beak **(Fig 6D)**.

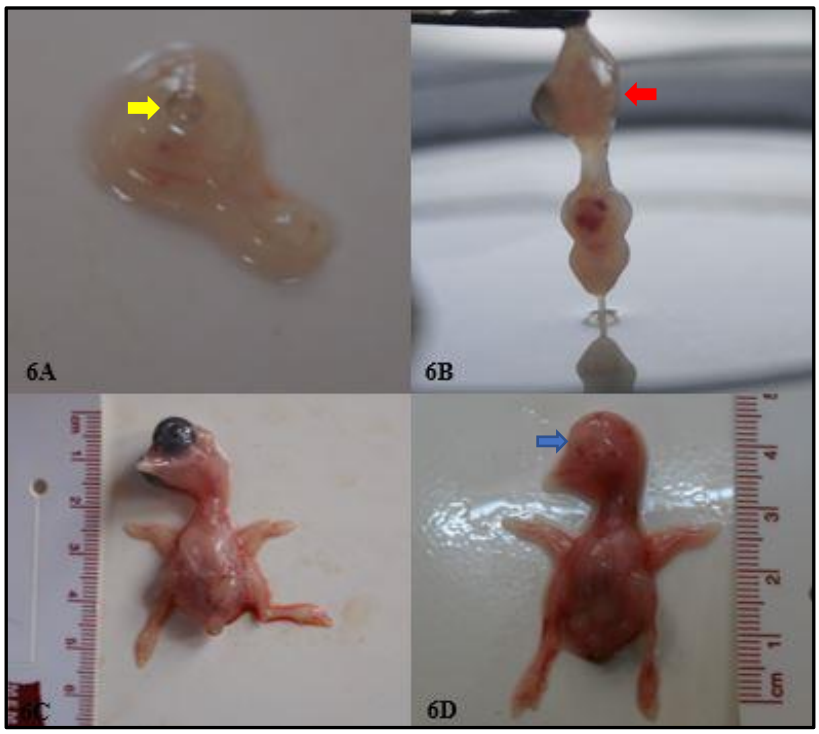

Figure 6: Ci induced structural anomalies: A) Control embryo of day4 with well-developed eye (yellow arrowhead); B) Ci treated day 4 embryo with under developed eye (red arrowhead). C) and D) shows Day 10 - Control and Treated embryo respectively. The blue arrow head indicates anophthalmia (absence of eyes).

Relative quantification of transcripts

In day 2 embryos, the relative mRNA expression levels of Shh, fgf8, Wnt7a, L1CAM, bmp7, gli3, Hox10a, Hox11a and Pax6 were found to be significantly lowered (p≤0.001) in the treatment groups when compared to the controls. Caspase-3 (p≤0.001) was found to be significantly increased in the treated group. The Wnt5a (p≤0.01), Wnt1 (p≤0.01), E-Cadherin

(p≤0.01), N-Cadherin (p≤0.01) and Proliferating Cell Nuclear Antigen (p≤0.05) showed downregulated mRNA expression levels **(Fig. 7)**.

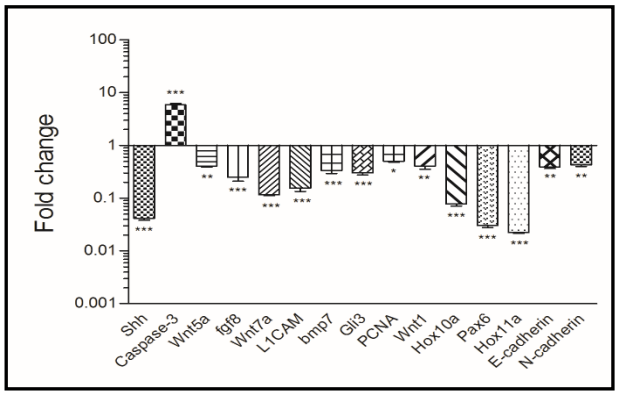

Figure 7: Transcript levels for Ci treated Day 2 embryo: Fold change expression values for the genes. Fold change values for control embryo is 1.0 for all the genes, n=10eggs/group.

The day 4 embryos showed downregulation in mRNA expression levels of Shh, Wnt5a, fgf8, L1CAM, gli3, Wnt1, Hox10a, Hox11a, Pax6, E-cadherin and N-Cadherin with a significance of (p≤0.001) in the treatment groups when compared to the controls. Highly significant increase in Caspase-3 (p≤0.001) levels was noted in treatment group. The Wnt7a and Proliferating Cell Nuclear Antigen mRNA levels were found to be lessened with a significance of (p≤0.01). No significant changes were seen in the levels of bmp7 **(Fig. 8)**.

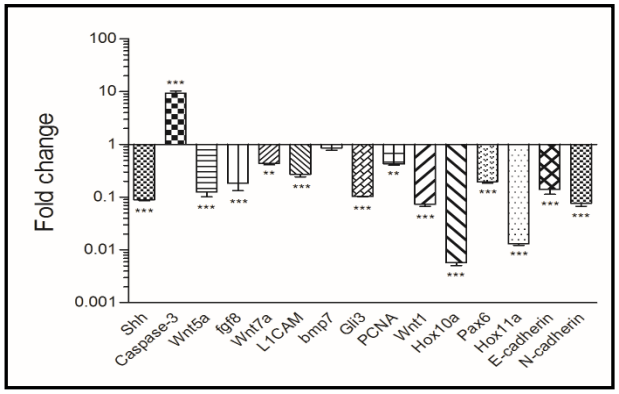

Figure 8: Transcript levels for Ci treated Day 4 embryo: Fold change expression values for the genes. Fold change values for control embryo is 1.0 for all the genes, n=10eggs/group.

On investigation of day 10 embryos, it was noted that along with Shh, the Sox9 and Col10a1 expressions were significantly downregulated (p≤0.001). An increase in mRNA levels of Caspase-3 (p≤0.001) and fgf8 (p≤0.001) was found when compared to controls. Expression levels of Wnt5(p≤0.05), RunX2 (p≤0.05), bmp2 (p≤0.01) and bmp7 (p≤0.01) were significantly increased in treated groups. The change in

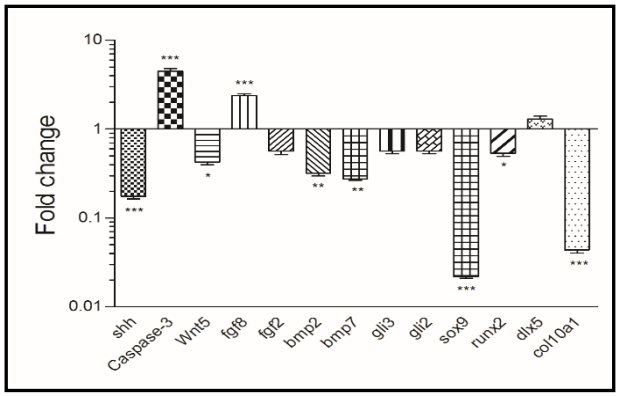

Figure 9: Transcript levels for Ci treated Day 10 embryo: Fold change expression values for the genes. Fold change values for control embryo is 1.0 for all the genes, n=10eggs/group.

expression levels of fgf2, gli3, gli2 and Dlx5 were found to be statistically insignificant **(Fig. 9)**.

Skeletal staining

The defect in chondrogenesis caused by Ci, day 10 embryos were stained with differential stain Alcian Blue and Alizarin Red **(Fig. 10)**. The intact stained heads showed delay in bone mineralization in treated than in control. The head where eye balls were removed before staining, showed hampered chondrogenesis in optic sinus region of treated embryos.

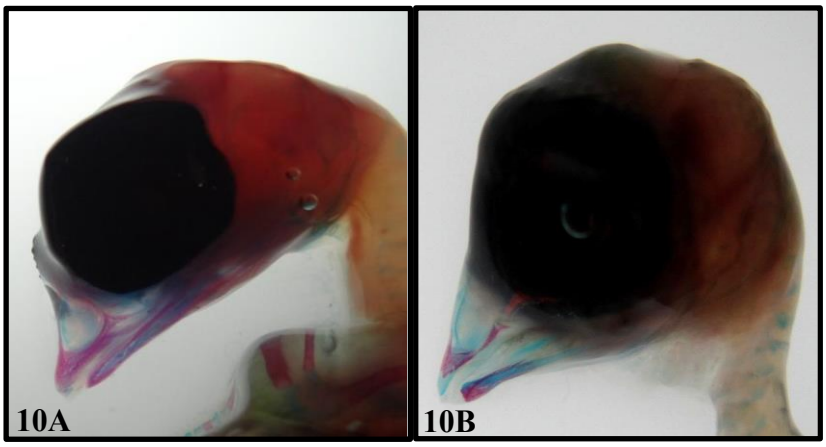

Figure 10: Ci induced delay in chondrogenesis: A) Control embryo of day10 with well-stained beak (yellow arrowhead); B) Ci treated day 10 embryo with less mineralized beak; n=10eggs/group.

Hydroxyproline content

Estimation of collagen supported the morphological anomalies' report. As hydroxyproline is a built-in amino acid of collagen, its content was estimated as a measure of collagen by biochemical assay. Significant drop in the levels of hydroxyproline was observed in Ci treated embryos when compared to control **(Fig. 11)**.

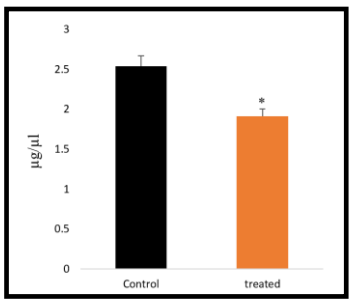

Figure 11: Hydroxyproline is a chief constituent of the protein collagen and plays a crucial role in collagen stability. Pesticide treated group demonstrated significant reduction in Hydroxyproline levels (*p < 0.0 5, Student's t-test) compared to control group; n=10eggs/group.

Discussion

New generation insecticides are designed such that they are short lived in the environment and do not accumulate in human and animal tissue. However, their very nature to kill or disable organisms still poses a threat to many non-target species. The ominous danger of insecticide intoxication during development is even more worrisome since a brief exposure during the critical window of embryonic development can cause morphological and anatomical anomalies.

These compounds are known to induce deformities through disruption in several mechanisms of cellular signalling, i.e. altering the expression pattern of genes, which will hinder the normal development **(Hernández-Díaz *et al*., 2012)**. The present study was taken up to explicate the direct or indirect relation between the patterning genes, compromised chondrogenesis, somitogenesis and pesticide (chlorpyrifos plus cypermethrin) intoxication in chick embryo. To simplify the work, it was focused on two major anomalies namely 1) Ventral body wall defects: Improper closure of the lateral body wall folds.; 2) Craniofacial defects: Malformations arising due to improper development of head and facial bones

Ventral body wall defects

Having known that toxicants like Ci are capable of inducing teratological manifestations in the developing embryos, the current study was designed to look for the underlying changes in the molecular mechanisms. The visual and microscopic observations of the treated embryos on day 2, 4 and 10 showed several teratological and histomorphological aberrations, amongst which the ventral body wall defects were chosen for an elaborate study. The treated embryos which showed the VBD had an exposed viscera protruding out of ventral abdominal wall due to a failed midline closure.

The VBW formation can be traced back to its embryonic origin from the mesoderm formed during the gastrulation. Several studies were conducted in the past on different embryonic models, to understand the formation of the ventral body wall. The contribution of lateral plate mesoderm and paraxial or somatic mesoderm towards the formation of ventral body wall was discussed earlier by **Sadler and Feldkamp (2008)**. The etiologies of failure in the formation of VBW were also investigated. These studies discussed about the embryonic dysplasia, vascular disruption, mechanical disruption, and also a disruption in the crosstalk between the epithelium and mesenchyme of the gastrulating embryos as the possible candidates leading to

defects in VBW. The role of various pattern forming genes like Wnt, Pitx2, bmps, and cell adhesion molecules too were elaborated **(Kitamura et al., 1999, Brewer and Williams, 2004, Sadler and Feldkamp, 2008; Matsumaru et al., 2011; Snowball et al., 2015)**.

Shh, one of the early expressed pattern forming genes plays a significant role in most of the above discussed cellular processes. *Shh* and its synchronised activity with BMP4, regulates the patterning of the somites in developing embryos. Also, *Shh* controls the temporo-spatial expression of bmp4 signal by inhibiting it, and holding the LPM, from extending medially and ventrally **(Gilbert, 2003)**. The BMP is known to have a dual role, while expression of BMP4 in dorsal neural tube promotes muscle differentiation, its ectopic expression in the paraxial mesoderm results in inhibition of 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)**. In the present study, 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 disregulation in the expression of *Shh* and bmp4 must have created the initial disturbances in the paraxial mesoderm, thereby disrupting the somite patterning and subsequently 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 exposed embryo indicates a disparity. Contrary to our observations with regard to Pitx2, according to **Kitamura et al. (1999)** deficiency of Pitx2 caused a failure of ventral body wall closure in mice, and they have also observed that the Pitx2 deficiency was influenced by a different genetic cascade other than fgf8-derived. 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 are reports **(Tao et al., 2016)** indicating that an elevated Pitx2 ropes in the 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. To further understand this response, we tried to look into some of the indicators of xenobiotic metabolizing mechanisms. The aryl hydrocarbon receptor (AHR), which is a ligand associated transcription factor acts like a

xenosensor and has a role in upregulation of the cytochromes which subsequently work on metabolizing the xenobiotics **(Stevens et al., 2009)**. Here, we observed a very significantly upregulated AHR and Cyp3A4 expressions in day 10 embryos, which is a clear indication of combat to overcome the stress laid by the Ci. 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 both day 4 and day 10 embryos which can be correlated to the large-scale apoptosis occurring in the Ci treated embryos. At the same time the levels of pcna were found to be downregulated indicating that the cell proliferation was hampered. 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, we have observed an upregulated expression of the *Shh* in the day 10 Ci treated embryos relative to the controls.

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 complex set of signals. The differentiating new cell types depend upon a finely balanced and coordinated regulation of gene expression and precise interaction amongst their neighbours. We therefore also sought to identify few such molecular signals that regulate the said processes. The expression of E-cadherins during embryonic development happens quite early where they play a role in adhesion and compaction of the blastomeres. It also signals the controlled epithelial-to-mesenchymal conversion and regulates developmental processes like cell migration and proliferation **(Barth et al., 1997; Kalluri and Weinberg 2010; Bahm et al., 2017)**. The migration of mesodermal cell is favoured by downregulation of the E-cadherin and loss of cell adhesion **(Ciruna and Rossant, 2001, Basson, 2012)**. Our results have shown that levels of E-cadherins were found to be upregulated in both the day 4 as well as day 10 Ci treated embryos, giving clear evidence that the cell migration was delayed and/or hampered. Further, the E – cadherin is known to be downstream target of *Shh* **(Xiao et al., 2010)**, which means that an inhibition of *Shh* would result in decreased E-cadherin expression. However, our results contrastingly have shown that the *Shh* was downregulated while E-cadherin was upregulated in the 4 day treated embryos, while in day 10 treated embryos, both were found to be upregulated. This indicates that E-cadherin though regulated by *Shh*, might as well be under the control of

some other upstream signal. A negative correlation between the *Shh* and E-cadherin 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 also elevated levels of Pxn are 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. Also, 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 dorsomedial and ventro-lateral lips of dermomyotome and a deficit 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)**.

A review by Wang et al., discusses the role of BMP4 in early developmental process, where, an absence of it leads to failure of mesoderm formation. And at a later stage, BMP1 deficiency would lead to the defects in ventral body wall closure. MyoD expression is a marker to the early myoblasts **(Zhang *et al.*, 2014)**. A relatively higher expression of the MyoD1 in the treated embryos could be an indication that the myoblasts remained so and failed to undergo further differentiation in the intoxicated embryos. Visual clues to this failure of muscle differentiation could be drawn from the histological sections of the embryos (Fig. 2B and 2D). Sonic hedgehog regulates MyoD expression to enhance the embryonic skeletal myogenesis **(Voronova*et al.*, 2013)**. Here an upregulation of MyoD1 would have been subsequent result of *Shh* upregulation, discussed earlier.

Craniofacial anomalies

Neural crest cells are destined to form the neurons and glia. Signaling molecules such as Wnt, BMP and FGF are known for neural crest cell induction. Wnt genes encode an important class of signaling proteins that control many aspects of animal development and adult homeostasis, and that are conserved from worms to man **(Logan and Nusse, 2004)**. One of the studies has shown the role of Wnt signals in the vertebrate nervous system in the developing axonal guidance to synaptic region **(Salinas, 2005)**. In the developing vertebrate brain, there are some Wnts such as Wnt1, Wnt2b, Wnt3, Wnt3a, Wnt5a, Wnt7a, Wnt7b, Wnt8b, which are expressed with an overlapping spatio-temporal pattern. During brain morphogenesis in early embryos, alteration in the Wnt signaling pathway leads to cranio-facial dysmorphism **(Brault et al., 2001)**. Over and above this, the role of sonic hedgehog has been observed in the survival of cranial neural crest, cranial facial morphogenesis, proliferation/differentiation of sympathetic cells **(Helms et al., 1997; Ahlgren and Bronner-Fraser 1999; and Williams et al., 2000; Garg et al., 2001)**. Also, plasticity of *Hox* genes expression has been observed in the hindbrain and cranial neural crest of chick, mouse and zebrafish embryos **(Trainor and Krumlauf, 2000)**. With this information on hand, Wnt7a was selected as the major candidate gene for studying the effect of combination insecticide (Ci) on the developing chick embryo with neural crest development as the point of focus.

The hypothesis was based on the cross-talk see between Wnt7a and Wnt5a **(Mericskay et al., 2004)**, which induces cell proliferation. Wnt7a also directly regulates the patterning genes Hoxa10 & Hoxa11 and additionally induces cell proliferation by inducing sonic hedgehog.

Real time quantitative analysis gives the supporting proof by showing that there is change in the patterning genes expression which possibly culminates from the disruption of the graded Wnt signaling in the neural crest cells (Figure 7, 8).

Subsequently, we also observed severely compromised chondrogenesis, especially in the craniofacial region of the chicks. It is well documented that chondrogenesis is regulated by BMP signaling**(Retting *et* al., 2009)**. Therefore, it was suspected that the Ci treatment might have hampered the BMP signaling and hence, hindered the process of cartilage condensation. In order to test the above notion a mechanistic study primarily focusing on BMP expression pattern on 10^{th} day of development was envisaged.

In higher vertebrates BMP signaling is shown to regulate bone and cartilage formation. To get insight into the extent of bone and cartilage on day 10 embryos, head region of control and

treated groups were stained with Alcian Blue and Alizarin Red. Alcian Blue is cartilage specific whereas Alizarin Red stains the bone (Figure 10) **(Mcleod, 1980)**. The pesticide in question delayed the bone formation in the dosed group as compared to control. Further, to check the same biochemically, estimation for hydroxyproline was undertaken (Figure 11). The treated group showed diminished level of hydroxyproline as compared to control indicating the toxic manifestation of Ci.

The teratological and staining observations were supplemented by mRNA expression analysis and the molecular aspect of BMP signaling specifically BMP2 and BMP7 in bone and cartilage formation was also examined. SMAD dependent BMP signaling triggers activate and regulate expression of transcriptional regulators RunX2, Sox9, col10a1 **(Smith *et al*., 2006)**. The results vividly expressed that pesticide treatment induced down regulation 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)**. The analysis of the result showed a definite reduction in the level of expression of BMP2 and BMP7 (Figure 9). Moreover, it has been documented that the up regulated 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 signaling in treated groups.

In the light of the current study it could be construed that combination insecticide on one side hampered Bmp signaling and on the other side altered the early pattern forming genes which has resulted in widespread congenital malformations like craniofacial dysmorphism, unilateral phocomelia and amelia, twisted phalange, wry neck and omphalocele in the developing chicks.

Conclusion

The present investigation proved beyond doubt that the sub-lethal dose of 0.05µg which was administered *in-ovo* induced malformations by disturbing key signalling pathways. Visual and microscopic observations and the mRNA profiling of the molecular signals regulating the processes like epithelial-to-mesenchymal transition, cell proliferation, migration, chondrogenesis and survival or death were sought for, in the Ci treated chick embryos. We observed that the fate of these embryonic cells, which constantly rely on interpretation of the signals from their neighbours and surroundings, show a larger erroneous display in not just a single pathway, but in the intricate regulatory networks. The combination insecticide was

observed to affect the mesodermal derivatives which in turn influenced the organogenesis process in the developing chick embryo.

The alterations came up in a way more complicated than do a single gene knockout studies that lead to the ventral body wall defects and craniofacial defects. The developing embryonic mechanisms while metabolizing the toxin and clearing the oxidative burden, might face a grave consequence due to changes in signal strength and might have ultimately lost their potency to achieve the developmental milestones. Hence, the present study brought our attention to review the current pesticides that are in use and to focus on developing them as more target organism specific. The study also warrants the necessity to include in-silico screening for genes involved in developmental malformation along with the traditional pre-clinical toxicological evaluation as per the current regulatory guidelines.

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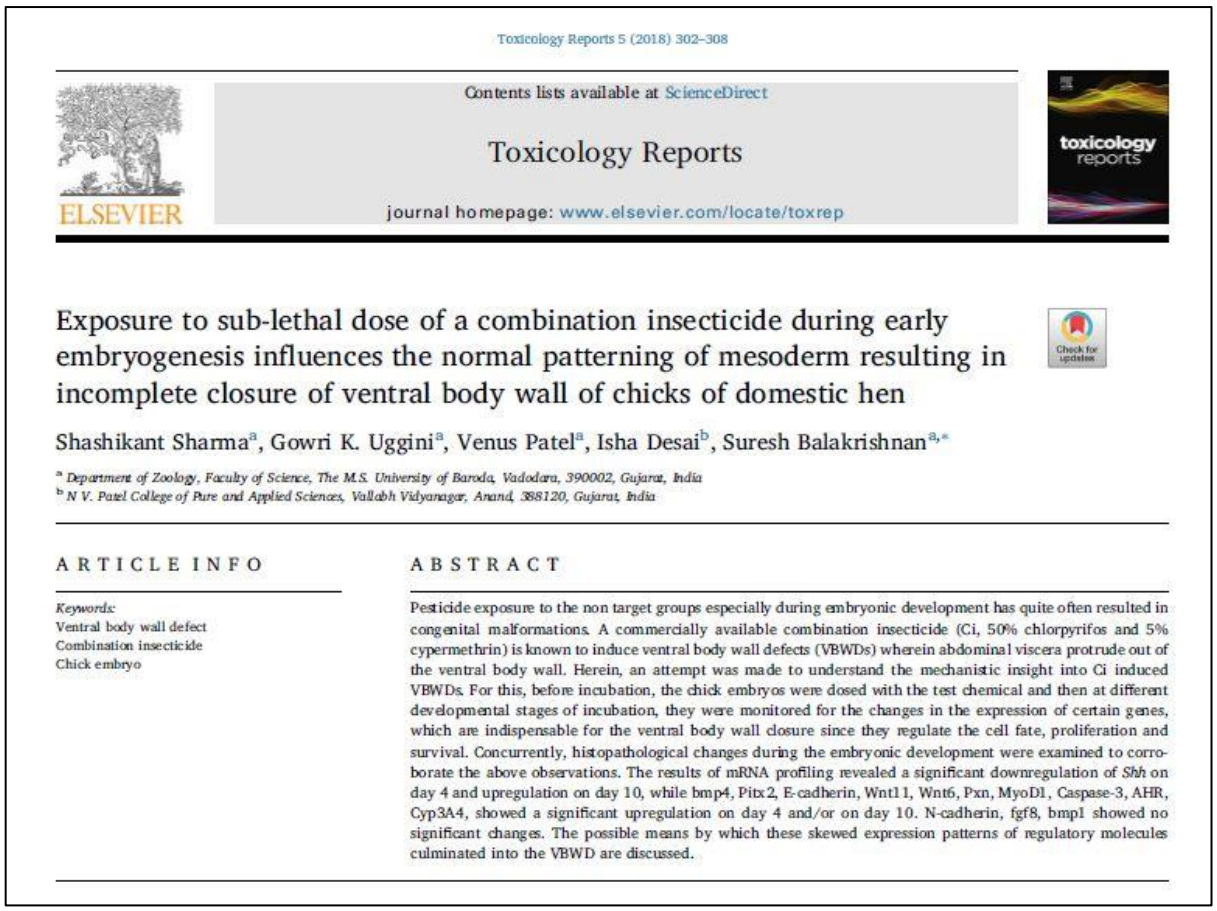

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Exposure to sub-lethal dose of a combination insecticide during early embryogenesis influences the normal patterning of mesoderm resulting in incomplete closure of ventral body wall of chicks of domestic hen

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ABSTRACT

Pesticide exposure to the non target groups especially during embryonic development has quite often resulted in congenital malformations. A commercially available combination insecticide (Ci, 50% chlorpyrifos and 5% cypermethrin) is known to induce ventral body wall defects (VBWDs) wherein abdominal viscera protrude out of the ventral body wall. Herein, an attempt was made to understand the mechanistic insight into Ci induced VBWDs. For this, before incubation, the chick embryos were dosed with the test chemical and then at different developmental stages of incubation, they were monitored for the changes in the expression of certain genes, which are indispensable for the ventral body wall closure since they regulate the cell fate, proliferation and survival. Concurrently, histopathological changes during the embryonic development were examined to corroborate the above observations. The results of mRNA profiling revealed a significant downregulation of *Shh* on day 4 and upregulation on day 10, while bmp4, Pitx2, E-cadherin, Wnt11, Wnt6, Pxn, MyoD1, Caspase-3, AHR, Cyp3A4, showed a significant upregulation on day 4 and/or on day 10. N-cadherin, fgf8, bmp1 showed no significant changes. The possible means by which these skewed expression patterns of regulatory molecules culminated into the VBWD are discussed.

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