GENERAL CONSIDERATIONS

Every living organism starts its existence as a single cell and during its embryonic growth and development, this totipotent cell undergoes division to produce many different, though genetically identical, cells in a final design of spectacular complexity and accuracy culminating in the formation of an organism with unique morphology (Bianconi *et al.*, 2013). For a normal embryonic development and growth the cells undergo four required processes which are cell proliferation, polarity specification, cell movement and cell interactions and turn to a multicellular functional organism (Segalen and Bellaiche, 2009). During these phases of embryonic development sometimes there may arise some internal genetic errors like mutations (Kops *et al.*, 2005) or multipolar mitosis (Kalatova *et al.*, 2015) within the cells, which hinders the normal development and growth of an embryo. Sometimes external forces, for instance, exposure to a teratogen tends to result in various kinds of anomalies leading to malformations, growth retardation, functional deficits and even death as described by Wilson, (1973) in his watershed treatise "Environment and birth defect".

A teratogen can be a drug like chloroquine (Van Allen *et al.*, 1995), Nicotine (Chung *et al.* 2000), Lysergic acid diethylamide (Zellweger and McDonald, 1967), radiation (Brent 1985), infectious agent (Hall, 1990), abnormal maternal metabolic states (Sadler *et al.*, 1993), thermodisruption (Smith *et al.*, 1978; Jones *et al.*,1995), toxic metal (Wang *et al.*, 2009b), pesticide (Tebouri *et al.*, 2011) or a mechanical factor which interacts with an embryo during its developmental period and leads to some kind of congenital malformation. Among these, pesticides are fast becoming major factors widely affecting human health. Their increased and even uncontrolled use, especially in agriculture, is leading to widespread exposure of humans and livestock to these hazardous chemicals.

In essence a pesticide is designed and intended to control a pest. The fundamental requirement of a pesticide therefore is to be toxic in nature makes it a high risk entity for the environment. Although they are designed to kill target organisms, they are often found to adversely affect non-target organisms such as plants, animals and humans as well (Tebouri *et al.*, 2011). Because of the cost effectiveness, the most popular classes of pesticides currently in use are

organophosphates (OP) and synthetic pyrethroids (PYR), which are available in many formulations. Though available as individual pesticides, because of its augmented toxicity due to synergism, OP and PYR are often marketed as a combination insecticide foe better pest management. In this study, focus was made on two insecticides representing both these groups, utilized as single compounds or as a mixture of both – cypermethrin and chlorpyrifos. Nurelle D 505 EC, a mixture of Chlorpyrifos (50% EC) and Cypermethrin (5% EC) manufactured by Dow AgroSciences was selected in the study.

Combination insecticide was diluted in corn oil and three sub-lethal doses namely low, mid and high were made as described in the previous study from our lab (Uggini *et al*., 2012). A single dose of 0.01, 0.05 and 0.1 µg/egg of Ci diluted in corn oil was administered to the airspaces of fertilized eggs (Rhode Island Red fowl *Gallus domesticus*) from low, mid and high group respectively on day zero of incubation. The eggs from the control received corn oil in the similar manner. The injected portion of each egg was sealed by molten paraffin wax. The whole experiment was done in a sterile environment under a laminar air flow. The dose volume of Ci and corn oil was maintained at 25µl/egg.

Immediately after treatment, both the treated and control eggs were kept in the incubator (temperature of 37.5 ± 0.5^{o}C, 75-80% relative humidity) with proper marking for 18 days. On 18th day the egg candling was done and the viable eggs were transferred to hatcher till the day of hatch. On day 21 after hatching, the hatchling groups (F1 generation) from the treated and control eggs were tagged with wing bands with respect to their dosage treatment and were housed in separate pens with immediate supply of water. After three hours starter mash feed was provided to the chicks. To promote the survival of the chick, assisted hatching was involved in case of those chicks who failed to progress normally at hatching stage due to deformities or weakness caused by Ci.

The F1 generation chicks when reached 25 weeks old were randomized within the respective treatment groups and the similar weighed ones were selected for breeding. Twenty hens and two roosters from each of the four groups were selected and pen mated for a week. The eggs collected from the F1 generation were marked as per the treatment groups of F1 parental generation and incubated. On hatch out the F2 generation chicks were wing banded with respect to their parent′s group and reared on standard diet for 4 weeks. The haematological

and biochemical analyses were performed on 25 week old parents and 4 week old second generation chicks. All the series of experiments, formulations, evaluations conducted, utilized and applied in this study are described in detail in the materials and methods section. All the protocols followed herein were approved by IAEC as per CPCSEA.

As a summary of the results obtained during this study, the following are worth mentioning. The pesticide induced embryolethality and abnormal survivorship was seen to be increasing with increase in concentrations of combination insecticide in treated chick embryos as compared to control embryos in F1 generation as well as its F2 generation (Chapter 1). The toxicity of the Ci leads to various kinds of morphological anomalies like deformed beak, abnormal eyes, hematomas, wry neck, narrow neck, exencephaly, ectopic viscera, limb deformities, growth retardation, etc. The effect of toxicity of the Ci was observed to be transferred from one generation to another generation as various anomalies were observed in the second generation as well (Chapter 1). The morphological malformations marked in this study were similar to some earlier investigations made by Friedberg and Gartner (1990), Indyk (1999), Khalil (2000), Sahu and Ghatak (2002), Petrovova *et al.* (2009), Mobarak and Al-Asmari (2011), Pinakin *et al.* (2011) Nitu *et al.* (2012) and Uggini *et al*. (2012). The reason behind the malformations due to the pesticide exposure could likely be pesticide induced oxidative stress (Hodgson and Levi, 1987; Paskova *et al.*, 2011) or gene mutation (Giri *et al.*, 2003). Abnormally developed structures appeared in the treated chicks of the F1 and F2 generations like deformed beak, limb or neck. These could very well be due to the binding of Ci to Calmodulin, which decreases the Ca^{2+} entry across the cell membrane and hinders the process of normal osteogenesis as explained by Rashatwar and Matsumura (1985).

Further growth retardation results (Chapter 1) observed in Ci treated F1 and F2 animals could be possible due to pesticide induced deviant metabolism (Garg *et al.*, 2004), preventing gluconeogenesis (Pushpanjali *et al.*, 2005) or interrupting retinoid signalling pathway and hence the *Hox* expression pattern during development (Lemaire *et al.*, 2005). Hence, the results in present investigation depicts that the combination insecticide can induce morphological malformations, can lead to abnormal survivorship. Moreover, the toxicity can get transferred from F1 generation to its F2 generation, though the mechanism behind such a transfer remains to be elucidated, which is testament to the potent embryotoxic and teratogenic capabilities of the pesticide.

The next parameter evaluated was hematological alteration caused by Ci in F1 and its respective progeny (F2) generation (Chapter 2). The haematological analysis was performed on 25 week old animals of the F1 generation and 4 week old chicks of the F2 generation. The evaluated haematological parameters included total erythrocyte (RBC) count, haemoglobin (Hb) concentration, packed cell volume (PCV), total leukocyte (WBC) count and differential leukocyte counts. Complementary indices of the erythrocytic component, including mean corpuscular haemoglobin concentration–MCHC, mean corpuscular haemoglobin – MCH, and mean corpuscular volume-MCV, were also computed. The results showed a decrease in PCV, Hb and RBC blood parameters in Ci treated animal of F1 and F2 generation, which could be due to hyperactivity of bone marrow caused by pesticide toxicity as opined by Tung and coworkers (1975) and also due to Ci induced oxidative stress which is known to hinder Hb formation and hence, shorten the life of the RBC (Betrosian *et al.*, 1995; Jyotsna *et al.*, 2003) and thrombocytopenia (Araujo *et al.*, 2008). Studies have been performed independently for Chlorpyrifos (Ambali *et al.*, 2011) and cypermethrin (Kale *et al.*, 1999) to analyze the induction of oxidative stress and have reported RBC lysis and anemia. The hyperactivity of bone marrow could be the reason for which the values of MCV were heightened, while the values for MCHC were lowered from the control group, indicating macrocytic and hypochromic anemia (Barger, 2003). Further, in this study leucopenia has been recorded in F1 generation (Chapter 2) which is in agreement with the earlier investigations of blood alterations induced by fenvalerate (Garg *et al.*, 2004), chorpyrifos and methidathion (Ojezele and Abatan, 2009). Leukocytosis in F2 generation can be due to resistance of the chicks to the inflammation (Benjamin, 1978) or severe haemorrhages in lungs and liver (Latimer *et al.*, 2004) caused by transferred toxicity. Similar observations for leukocytosis were made by Sharaf *et al.* (2010) in cypermethrin treated chick and chlorpyrifos treated Wistar rats (Ambali *et al.*, 2010a).

In the next phase of the study (Chapter 3), biochemical parameters including Serum glucose, Serum albumin, Serum globulin, Serum protein, Urea, Blood Urea Nitrogen (BUN), Creatinine, Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and liver nucleic acid (DNA, RNA) content were evaluated for both F1 and F2 generations. A significant increase in the levels of blood sugar, serum urea, BUN, creatinine, ALT, AST, ALP, and LDH was observed in all the doses of pesticide contamination when compared to the control. The increase in blood glucose of the treated animals may be due to the damage caused by the pesticide to the liver, skeletal

muscle or cardiac muscle which leads to improper utilization of major metabolites. This triggers the stimulation alternate pathways of carbohydrate metabolism like gluconeogenic pathway and accelerated glycogenolysis in the treated group of birds resulting in hyperglycemia (Ceron *et al.*, 1997). The phenomenon of hyperglycemia induced by Ci is similar to the earlier studies of chlorpyrifos (Ambali, 2009) and cypermethrin (Rahman *et al.*, 1990; Chermaki et. al., 2013) toxicity.

It well documented that insecticidal stress results in decreased total serum proteins, serum albumin and globulin (Khan *et al.*, 2009). The observed increase in ALT, AST, ALP and LDH levels indicate hepatic injury or dysfunction as noted by many (Luskova *et al.*, 2002; Jyotsna *et al.*, 2003; Yousef *et al.*, 2006; Obaineh and Matthew, 2009). Further, it is opined that increase in plasma creatinine and urea could be an index of impaired renal function (Yousef *et al.*, 2003 and 2006; Ahmad *et al.*, 2011). Low levels of DNA and RNA in the liver tissue is observed in treated animals. As per Chitra (1999) the depletion of DNA could be due to slight hypotrophy of tissue under insecticidal stress and as per Arshad *et al.* (2007) the decrease in RNA level results could be due to the ability of Ci to arrest protein synthesis at translation stage (Chapter 3).

As per the discussion above we conclude that organophophorous and cypermethrin classes of pesticides are safe only to the point where they are used sparingly with proper guidance and knowledge. If they are used unaccountably, they will kill or diminish the target species, but also pose a high risk to non-target species including humans. An evaluation of the present results reveals that the combination insecticide can result in severe teratological manifestations and, more importantly, show the tendency to get transferred across generations. It allows us to assess the potential hazard posed by chemicals to avian embryos and the risk of embryotoxic and teratogenic potency of drugs. The treatment of combination insecticide results in neonatal mortality, morphological anomalies; histopathological, haematological and biochemical alterations; decline in hatchability and body weight. Thus the chicks feeding on grains from pesticide-treated crops in and around agricultural fields where pesticides are in practice, ingest pesticides at least in lower doses (at microgram level) which through the food chain might reach the human beings through chick eggs or flesh and thereby posing health hazards. Therefore, it can be safely concluded that farmers should be intercepted from using chemical pesticides for killing the pests and should be advised to

follow more biological control devices/mechanisms against the pests for betterment of society and fsssor keeping our environment a safe one to breathe in.