INTRODUCTION

INTRODUCTION TO EMBRYOTOXICITY AND TERATOLOGY

The process of development in humans and most other animals is well characterized. Beginning with fertilization, normal morphologic development proceeds through blastocyst formation, gastrulation, histogenesis, organogenesis and functional maturation, a process that is not complete in some organs until puberty. Morphogenesis includes several developmental processes, such as morphogenetic movements, cell contacts, changes in cell shape, tissue interactions (induction), and cell differentiation. This is accomplished through a complex program of gene expressions which are turned on and off as the zygote begins to divide and grow. The genes and maternal products stored in each of the embryo's cells are transcribed into mRNA and translated into proteins which can perform physiological functions or regulate the expression of other genes. Thus, the embryo is a complex dynamical system where a precisely orchestrated interplay of information and materials takes place to generate an organism with a well-defined shape and functions.

However, this intricate mechanism of development, at times, gets deviated and may lead to various kinds of anomalies. As per **Wilson (1973, 1977)**, the four manifestations of deviant development are death, malformations, growth retardation and functional deficit. They may include various types of birth defects or congenital anomalies. A congenital anomaly may be viewed as a physical, metabolic, or anatomic deviation from the normal pattern of development that is apparent at birth or detected during the early phase of life. More recently, the view of teratology has extended to include adverse effects of exposure to stresses in the womb that are manifested much later in life (**Hamdoun and Epel, 2007**).

By far the largest category of malformations, about 65% falls into the group of those with an unknown cause(s), and are referred to as sporadic. Purely genetic causes of malformations (autosomal and cytogenetic) are estimated to produce around 20 to 25% of all human malformations and comprise the largest group of congenital malformations with known aetiology. Although environmental causes of human malformations account for about 10% or fewer of the total malformations, most of these environmentally induced malformations are

related to maternal disease states. Less than 1% of all human malformations are related to drug exposure, chemicals, or radiation. However, studies of environmentally induced malformations are important because they may teach us how to predict and test for teratogenicity, understand the mechanisms of teratogenesis from all etiologies, and provide a means by which human malformations can be prevented **(Brent and Beckman, 1990)**.

Since the middle to late nineteenth century, teratology, the study of how congenital malformations arise, has been the subject of scientific explorations **(Warkany, 1971)**. Embryologists used chemicals to alter development so as to understand the processes of normal development. However, with increasing foetal exposure to environmental contaminants, drugs, and other xenobiotics during pregnancy, the importance of understanding the processes involved in both normal and abnormal development was recognized. In the recent decades, more concern has been placed on developmental effects, in addition to malformations or the study of teratology, spawning the field of developmental toxicology, as well as developmental neurotoxicology, immunotoxicology, etc.

An extensively accepted notion by the developmental toxicologists is that "living things during early developmental stages are more sensitive than at any other time in their life cycle to adverse influences in the environment" **(Wilson, 1973)**. The developing embryo is most sensitive to the induction of malformations, growth retardation, and death during the period of major organogenesis, inferred from the fact that it usually requires a smaller dose during this time to produce effects than either earlier or later. The derailed development induced by the teratogen often includes defeating embryonic defences, interfering with the hormones that control the developmental decisions and maladaptive developmental responses **(Hamdoun and Epel, 2007)**.

TYPES OF EMBRYOTOXICANTS AND TERATOGENS

Decades ago, the congenital malformations were believed to have a genetic cause, until the disastrous thalidomide tragedy took place. Thalidomide a sleeping pill and tranquilizer, was given to pregnant women and was subsequently found to be responsible for severe developmental malformations in thousands of children. This drug disaster was – and remains to be a very impressive warning to the pharmaceutical industry, to academic and practicing physicians, to legislators and last but not least, to layman. Following this disaster, the whole field of human teratology including toxicology, pharmacology and pharmacokinetics as well

as pre-natal pathology has been explored with enormous increased interest (**Burgio, 1981**). As a result of this catastrophe, regulatory agencies began to require toxicity testing specifically addressing the possible adverse impact of any new chemical on the developing embryo and foetus (**Costa, 2003**).

A large body of evidences explains the susceptibility factor of developing embryo to the toxic substances. At a stage when the detoxification mechanisms are yet to be developed, life is building up in a sequential cascade of events, there are huge number of sensitive points which on slightest disturbance fail to reach their proper endpoint. There has been growing awareness that certain chemicals at levels below those associated with overt toxicity can modulate the developmental mechanisms. The period of prenatal development, mainly during the phase of embryogenesis and at the beginning of organogenesis, is delicate. This rapidly differentiating and growing system may be disturbed or changed from normal to anomalous by the introduction of toxic substances (**Sahu and Ghatak, 2002**).

As per **Brent and Beckman, (1990)** the aetiology of teratogenesis falls under two broad categories:

(i) **Genetic Factor:** the pathological nature of this process is determined before conception, or at least before differentiation, because of the presence of inherited genes or newly acquired genetic abnormalities like gene deficiency, gene abnormatilty, chromosomal rearrangement, chromosomal excess or chromosomal deficiency.

(ii) **Environmental factors:** the embryopathy is produced when these factors interact with the embryo during the developmental process. The outcome of these exposures can be stage specific and dose depended. The following are examples of environmental agents which are sighted to be embryotoxicants or teratogens.

a) Drugs: Accutane, aminopterine, thalidomide, androgenic hormones, penicillamine, tetracyclines, diethylstilbestrol, nitrazepam, etc.

b) Ionizing radiations: radiation therapy, radioiodine, atomic weapons.

c) Maternal infection: Cytomegalovirus, herpes, rubella, syphilis, toxoplasmosis, etc.

d) Metabolic imbalances: alcoholism, diabetes, folic acid deficiency, hyperthermia, phenylketonuria, etc.

e) Environmental chemical: heavy metals (e.g. lead, mercury), ethidium bromide, certain pesticides, narcotics (e.g. heroin, methadone, opiates, cocaine).

And many other compounds are under varying degree of debate.

PESTICIDES AS EMBRYOTOXICANTS AND TERATOGENS

Agricultural practices as well as the household maintenance today have a serious addiction to the use of pesticides. The world-wide annual consumption of pesticides is about two million tons, of which 24% is consumed in United States alone, 45% in Europe, and 25% in the rest of the world. The usage of pesticides in India accounts for more than 500 pesticide formulations, with an annual consumption of 164,080 tons of active ingredients, which average for $0.5 kgha^{-1}$. Globally, herbicides are the leading category of agrochemical used, followed by insecticides and fungicides. Conversely, in India, insecticides account for 80% of the total pesticides used, and the herbicide usage is insignificant (**Gupta, 2004; FAO, 2005; Abhilash and Singh, 2009**). Till the recent past, organochlorine insecticides were among the most commonly used pesticides in the developing countries in Asia. However, the concerns of environmental persistence and bioaccumulation of the organochlorines resulted in a change in the preference toward more environmentally safer pesticides like organophosphates, carbamates and pyrethroids (**Abhilash and Singh, 2009**).

Rachel Carson (1962) in her book *Silent Spring* documented the dangers of pesticides and the long lasting presence of toxic chemicals in water and on land and the presence of DDT even in the milk. The book has backed credits for being most influential event in sparking the environmentalists' movement. The new generation pesticides are designed such that they are short lived in the environment and do not accumulate in the human and animal tissues. However, owing to their very nature, that is, to disable and/or kill, they still pose a threat to the non-target species. Several pesticides used as herbicides, insecticides, and fungicides are known to be endocrine-disrupting chemicals. Mixture of carbamates, organophosphates, phenoxy acids, pyrethroids, and other pesticides in a study showed to induce hormonal imbalances even when exposed within the reference values (**Straube *et al.*, 1999**). Adult exposure to these chemicals is certainly an important factor; however, the concern is compounded when the exposure gets associated with the developing organisms, because they

are extremely sensitive to perturbations by chemicals with hormone like activity. The protective mechanisms that are available to the adult such as DNA repair mechanisms, a competent immune system, detoxifying enzymes, liver metabolism, and the blood/ brain barrier are not fully functional in the foetus or newborn (**Newbold *et al.*, 2007**). Even a brief exposure during critical windows of reproductive development can cause permanent adverse effects. Several reports (**Landrigan *et al.*, 1999; Slotkin, 1999, 2004; Rice and Barone, 2000; Landrigan, 2001; Weiss *et al.*, 2004**) demonstrate that certain insecticides have detrimental effects on the development of the living organisms at far lower exposures than those that elicit signs of systemic intoxication. Hence, unwarranted effects on the foetal body structure could occur in absence of any obvious recognition that exposure has taken place. Several studies in children (**Adgate *et al.*, 2001; Shalat *et al.*, 2003; Barr *et al.*, 2004**), pregnant women (**Whyatt *et al.*, 2002; Berkowitz *et al.*, 2003; Bradman *et al.*, 2005**), and foetuses (**Whyatt and Barr, 2001; Bradman *et al.*, 2003**) using urine, blood, amniotic fluid, and/or meconium samples demonstrated detectable pesticide levels in the majority of the cases. Adverse outcomes of these exposures during the preconceptional or developmental period may be observed immediately, or they may be expressed as latent effects that are not evident until later in life (**Selevan *et al.*, 2000; WHO, 2007**). Nevertheless, pesticide usage is validated by its many important contributions to the society, but the fact that the non-target species become vulnerable to its deleterious effects is a matter of grave concern. Therefore, understanding the consequences of exposure of insecticides and the causative levels of exposure, during the critical periods of embryonic development, is of prime significance.

Various studies demonstrated that the inert ingredients in the pesticide formulations enhance the toxicities of active ingredients and suggested that pesticide registration and their environmental monitoring should include full assessment of formulations (**Cox and Surgan, 2006; Mansour *et al.*, 2008a**). Therefore, two different commercial formulations were chosen as the test chemicals for this study.

(i) *Anaconda 505™*: combination of chlorpyrifos (50%) and cypermethrin (5%) as emulsifiable concentrate (EC).

(ii) *Tracer:* Spinosad 45% SC

ANACONDA 505™

Chlorpyrifos and cypermethrin belong to organophosphorus and pyrethroid insecticides, respectively.

Chlorpyrifos

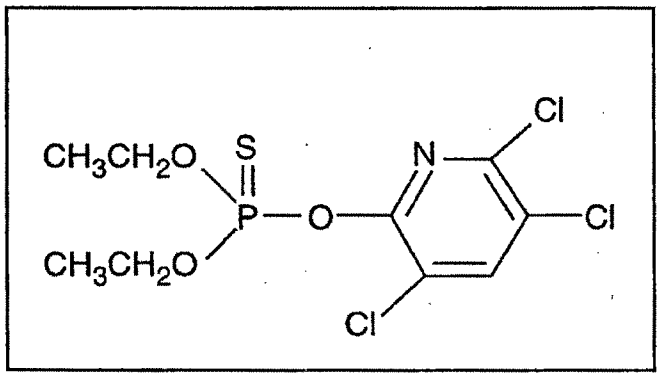
Chlorpyrifos is one of the most widely used organophosphorus insecticides in the world. Chlorpyrifos was introduced into the market in 1965, and was formerly used in both agricultural and non-agricultural environments **(ATSDR, 1997).** Chlorpyrifos is manufactured by reacting 3,5,6-trichloro-2-pyridinol with diethylthiophosphoryl chloride. Owing to the high toxicity it could cause to the non target species, in 1997, chlorpyrifos was voluntarily withdrawn from most indoor and pet uses by the manufacturer, Dow Elanco.

Chlorpyrifos-Physical and Chemical Properties

The compound is a colourless to white crystalline solid, has a mild mercaptan-like odour. The melting point of chlorpyrifos is 41.5-42.5^{0}C. Chlorpyrifos is stable in neutral and acidic aqueous solutions; however, stability decreases with increasing pH. Chlorpyrifos is practically insoluble in water, but is soluble in most organic solvents (i.e. acetone, xylene and methylene chloride). Chlorpyrifos is not particularly volatile based on its low vapour pressure of 1.87x10^{-5}mm Hg at 20EC (**Merck Index, 11th Edition**). Its maximum attainable vapour concentration is 25 ppb at 25EC.

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 (Fig.1.1). The molecular weight is 350.6.

Figure 1.1. Chemical Structure of Chlorpyrifos



Chlorpyrifos - Mode of Action

Chlorpyrifos is an acetylcholinesterase inhibitor, and its insecticidal activity is due to the overstimulation of cholinergic receptors by excess acetylcholine. In addition, it is transformed inside animals to chlorpyrifos-oxon (**Sultatos, 1991; Chambers and Carr, 1993**) which is about 3000 times as potent against the nervous system as chlorpyrifos itself (**Chambers *et al.*, 1989**). They inhibit an enzyme, acetylcholinesterase (AChE) that breaks down acetylcholine, a chemical involved in transmitting nerve impulses across the junctions between nerves. Without functioning AChE, acetylcholine accumulates, producing rapid twitching of involuntary muscles, convulsions, paralysis, and ultimately death (**Thirugnanam and Forgash, 1977**). Other than AChE, it impedes respiration (production of energy within a cell) in the livers of laboratory animals. This results from the effect of chlorpyrifos on the activity of ATPase, an enzyme important in cellular respiration (**Holcombe *et al.*, 1982**). Chlorpyrifos-oxon inhibits the enzyme cholesterol ester hydrolase and inhibition of this enzyme in rats eliminates one of their normal reactions to stress (**Jarvinen *et al.*, 1988**).

Chlorpyrifos – Usage

In the home, chlorpyrifos has been used to control cockroaches, fleas, and termites; it has also been an active ingredient in some flea and tick collars of pets. On the farm, it is used to control ticks on cattle and as a spray to control crop pests. It is applied as 1.12-3.36 kg a.i/ha. Growers use chlorpyrifos insecticide to defend more than 50 different crops- virtually every crop presently under cultivation globally- against damage caused by insect pests. The crops with the most intense chlorpyrifos use are cotton, corn, almonds, and fruit trees including oranges and apples (**NASS Agricultural Chemical Database**).

Chlorpyrifos – Metabolic and Environmental Degradation

Chlorpyrifos is oxidized to its oxon form, chlorpyrifos-oxon which is generally regarded as the principal toxic metabolite, and is responsible for inhibition of cholinesterases. Chlorpyrifos-oxon is either enzymatically or spontaneously hydrolysed to form the diethylphosphate and 3,5,6-trichloro-2- pyridinol (TCPy). In addition to the formation of chlorpyrifos-oxon, chlorpyrifos is oxidized via cytochrome(s) P-450 to an unstable intermediate that spontaneously hydrolyses to form diethylthiophosphate and TCPy. These metabolites are excreted in the urine, or form glucuronide and sulphate conjugates, which are also excreted in the urine.

Following application to crops, chlorpyrifos quickly binds to soil and plants. Though it typically degrades rapidly in the environment, residual levels of chlorpyrifos can last for long periods of time. The vapour pressure of chlorpyrifos (1.87×10^{-5} mm Hg at 25°C) suggests that it will quickly volatilize into the atmosphere. Chlorpyrifos is poorly soluble in water and rapidly binds to particles in the soil or on plants, so very little enters any surrounding water sources. If chlorpyrifos enters a water system it will typically volatilize from the surface of the water. Chlorpyrifos and its metabolites are susceptible to photodegradation, with a half-life of approximately 3 days; in the presence of hydroxyl radicals in the atmosphere the half-life is lowered to about 6 h. Upon entering surface water, chlorpyrifos degradation is associated with abiotic hydrolysis or photosensitized oxidation. In soil, photodegradation plays a role in hydrolysis, dechlorination, and oxidation of chlorpyrifos. However, in indoor environments, chlorpyrifos can persist for several months because of the relative lack of sunlight, water, and/or soil microorganisms that contribute to its rapid degradation in the outdoor environment.

Chlorpyrifos-Toxicity Studies

The oral LD_{50} for chlorpyrifos in rats is 95 to 270 mg/kg (**Gallo and Lawryk, 1991; Kid and James, 1991**). The LD_{50} for chlorpyrifos is 60 mg/kg in mice, 1000 mg/kg in rabbits, 32 mg/kg in chickens, 500 to 504 mg/kg in guinea pigs, and 800 mg/kg in sheep (**Gosselin *et al.*, 1984; Gallo and Lawryk, 1991; Kid and James, 1991**). The dermal LD_{50} is greater than 2000 mg/kg in rats, and 1000 to 2000 mg/kg in rabbits (**Dow, 1986; Gallo and Lawryk, 1991; Kid and James, 1991**). The 4-hour inhalation LC_{50} for chlorpyrifos in rats is greater than 0.2 mg/L (**Dow Elanco, 1992**).

Chlorpyrifos-Embryotoxic and Teratogenic Studies

Several studies have been conducted in the past to demonstrate the developmental toxicity by chlorpyrifos exposure during the critical windows of development. Chlorpyrifos-induced neurobehavioral anomalies have been reported for exposures targeted to the neural tube stage (**Icenogle *et al.*, 2004**), in late gestation (**Levin *et al.*, 2002**), and through postnatal stages of terminal differentiation and axonogenesis (**Dam *et al.*, 2000; Carr *et al.*, 2001; Levin *et al.*, 2001; Tang *et al.*, 2003**). Developmental exposure of rats to chlorpyrifos lowers hemicholinium-3 binding in adolescence through adulthood in basically all regions possessing cholinergic projections (**Dam *et al.*, 1999; Qiao *et al.*, 2003, 2004; Richardson and Chambers, 2003, 2004**). In a number of reviews, chlorpyrifos has been identified as a

developmental neurotoxicant, which effects the brain development by a series of mechanisms other than just cholinesterase inhibition (**Pope, 1999; Slotkin, 1999; Barone *et al.*, 2000**). It is inferred that the neurotransmitters which serve to communicate information across a synapse in a developed animal, serve as trophic factors that control the fate of their respective target cells during the embryonic development. Chlorpyrifos also alters the expression and function of receptors for serotonin (**Aldridge *et al.*, 2003**), one of the essential neurotrophic factors in mammalian brain development (**Lauder, 1985; Hamon *et al.*, 1989; Whitaker-Azmitia, 1991, 2001; Weiss *et al.*, 1998; Azmitia, 2001**). Further downstream from the receptors, chlorpyrifos and its metabolites interact with signaling intermediates such as G proteins andadenylyl cyclase (mercaptan); as well as protein kinases (**Caughlan *et al.*, 2004; Izrael *et al.*, 2004**). Finally, chlorpyrifos may interact directly with cellular energetics or the nuclear transcription factors necessary for cell replication and differentiation (**Bagchi *et al.*, 1995, 1996; Whitney *et al.*, 1995; Song *et al.*, 1997, 1998; Dam *et al.*, 1998; Johnson *et al.*, 1998; Schuh *et al.*, 2002**), including the generation of oxidative stress (**Bagchi *et al.*, 1995; Jett and Navoa, 2000; Garcia *et al.*, 2001; Gupta, 2004**). Therefore, chlorpyrifos exposure during the developmental period is potentiated to induce a multitude of awry of developmental mechanisms and endpoints. Various other investigators also reported the embryotoxic and teratogenic effects of Chlorpyrifos in rats (**Muto, *et al.*, 1992; Roy, *et al.*, 1998; Amina, *et al.*, 2003**), mice (**Deacon, *et al.*, 1980; Tian *et al.*, 2005; Ahmad and Asmatullah, 2007**) and chick (**Slotkin, *et al.*, 2008**).

Cypermethrin

Cypermethrin is a pyrethroid insecticide. It was first synthesized in 1974 (**WHO, 1989**). Cypermethrin is a synthetic chemical similar to the pyrethrins in pyrethrum extract (which comes from the chrysanthemum plant). Pyrethroids, including cypermethrin were designed to be effective longer than pyrethrins (**WHO, 1989**). Cypermethrin acts as a stomach and contact insecticide (**Jin and Webster, 1998**). It has wide uses in cotton, cereals, vegetables and fruit, for food storage, in public health and in animal husbandry.

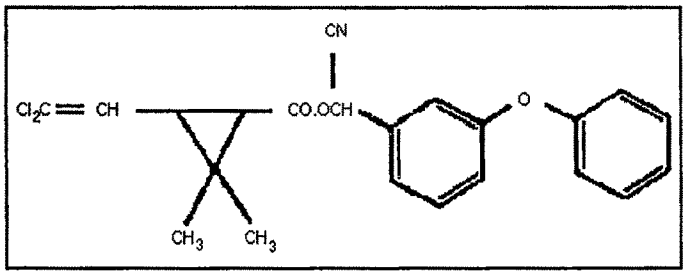
Cypermethrin–Physical and Chemical Properties

Technical cypermethrin varies from a viscous, yellow liquid to a semi-solid crystalline mass at ambient temperatures. Cypermethrin is highly stable to light and at temperatures below 220°C. It is resistant to acidic rather than alkaline media with an optimum stability at pH 4. Cypermethrin hydrolyses under alkaline conditions in a similar way to simple aliphatic esters.

Dilute aqueous solutions are subject to photolysis, which occurs at a moderate rate. The absorption and elimination of cypermethrin was rapid in the different mammalian species tested. The major metabolic reaction is cleavage of the ester bond followed by hydroxylation and conjugation of the cyclopropane and phenoxybenzyl moieties. The highest levels of cypermethrin are found in body fat, which is consistent with the lipophilic nature of the compound.

Cypermethrin is the racemic mixture of eight optical isomers with a cis:trans isomer ratio of approximately 40:60. Molecular formula of cypermethrin is $C_{22}H_{19}O_3NCl_2$ and chemical name is (+/-)alpha-cyano-(3-phenoxyphenyl)methyl(+)-cis,trans-3-(2,2-dichloroethylene)-2, 2-dimethyl cyclopropane carboxylate (Fig. 1.2). The molecular weight is 416.3.

Figure 1.2: Chemical Structure of Cypermethrin



Cypermethrin - Mode of Action

Cypermethrin is a synthetic pyrethroid and a permethrin analogue. This group of chemicals act primarily on the basal ganglia of the central nervous system, causing repetitive nerve action through prolongation of sodium permeability during the recovery phase of the action potential of neurons.

Pyrethroid intoxication results from their potent effects on nerve impulse generation within both the central and peripheral nervous systems. Under normal conditions, neurons possess a transmembrane voltage of about -60 mV on the inside. The nerve impulse or action potential consists of a transient depolarization (positive wave) whose upstroke is driven by an influx of Na^+ ions, followed by a down stroke from the efflux of K^+ ions. These ion fluxes occur due to the opening and closing of specific ion channel proteins embedded within the nerve membrane. The action potential is propagated down the axon until it reaches the nerve

terminal, where it stimulates the release of chemical transmitters. Pyrethroids modify the gating characteristics of voltage-sensitive sodium channels in mammalian and invertebrate neuronal membrane (**Eells *et al.*, 1992**) to delay their closure, which produces a protracted sodium influx. They are dissolved in the lipid phase of membrane (**Narahashi, 1996**) and bind to a receptor site on the alpha sub-unit of sodium channel (**Trainer *et al.*, 1997**) and cause modulation of the function of voltage-gated sodium channels and cause membrane depolarization leading to discharges from sensory neurons. This lowers the threshold of sensory nerve fibres for the activation of further action potentials, which may progress to hyperexcitation of the entire nervous system (**Narahashi *et al.*, 1995**). Delayed sodium channel closure thus increases cell membrane excitability. This results in multiple nerve impulses, causing the nerve to release the neurotransmitter acetylcholine and stimulates other nerves (**Eells *et al.*, 1992**). Cypermethrin also inhibits the gamma aminobutyric acid receptor, causing excitability and convulsions. Cypermethrin has been shown to inhibit ATPase enzymes involved in movement of ions against a concentration gradient which are regulated by active transport. This action is especially critical to fish and aquatic insects where ATP provide the energy necessary for active transport, and is very important at sites of oxygen exchange. ATPase inhibition and disruption of active transport, possibly affect ion movement and the ability to maintain ion balance, and disrupt respiratory surfaces, indicating that cypermethrin is inherently more toxic to aquatic organisms (**Siegfried, 1993**).

Cypermethrin- Usage

It is most commonly used to control agricultural pests, primarily lepidopteran larvae known to attack fruit, vegetable and cotton plants. It is used in relatively low dose rate of 0.02-0.25kg a.i/ha. It is also used to treat ectoparasites on cattle and poultry. Because of its minimal environmental impact, low mammalian toxicity and effectiveness for pest prevention, cypermethrin is also commonly used as a barrier treatment in hospitals, schools and non-food areas of restaurants.

Cypermethrin-Metabolic and Environmental Degradation

Cypermethrin is metabolized through cytochrome P450 (**El-tawil and Abdel-Rahman, 2001**). Synthetic pyrethroids are generally metabolized in mammals through ester hydrolysis, oxidation, and conjugation (**WHO, 1989**). The major urinary metabolites of cypermethrin are a variety of conjugates of cis and trans isomers, 3-phenoxybenzoic acid and 3-(4'-hydroxyphenoxy) benzoic acid. Marked differences in the urinary metabolite profile by oral

and dermal routes in human volunteer studies suggest that cypermethrin could be significantly metabolized in the skin before systemic circulation occurs (**Woollen *et al.*, 1992**).

Cypermethrin has a very low vapour pressure and is not readily volatilized into the atmosphere. A low Henry's Law Constant (H), $2.5x10^{-7}$ atm $m^{-3}mol^{-1}$ at 20°C, indicates that cypermethrin has almost no tendency to volatilize from an aqueous solution. Hydrolysis and photolysis play major roles in the degradation of cypermethrin in soil. Hydrolysis of the ester linkage is the principal degradation route. Cypermethrin also photodegrades rapidly on soil surfaces into many by-products, with half-lives of 8-16 days (**Walker and Keith, 1992**). Many photoreactions are involved in photodegradation and the photodegradation rates are closely correlated with the organic matter content of the soil (**Takahashi *et al.*, 1985**). Under aerobic conditions, these metabolites may undergo further breakdown to CO_2 at a much slower rate (**Kaufman *et al.*, 1981; Bacci *et al.*, 1987**). Cypermethrin displays low water solubility, hence is hydrophobic. Cypermethrin is a non-polar pesticide and readily adsorbed onto the soil surface and bound there. According to **Kaufman *et al.* (1981)**, very little cypermethrin insecticide would move through the soil profile, although all of the degradation products are more mobile than the parent product.

Cypermethrin - Toxicity Studies

The oral LD_{50} for cypermethrin in rats is 250 mg/kg (in corn oil) or 4,123 mg/kg (in water) (**Meister, 1992**). EPA reports an oral LD_{50} of 187 to 326 mg/kg in male rats and 150 to 500 mg/kg in female rats (**US EPA, 1989**). The oral LD_{50} also varies from 367 to 2,000 mg/kg in female rats, and from 82 to 779 mg/kg in mice, depending on the ratio of cis/trans-isomers present (**Hays and Laws, 1990**). This wide variation in toxicity may reflect different mixtures of isomers in the materials tested. The oral LD_{50} reported in rabbits is 3,000 mg/kg (**Occupational Health Services, 1993**). The dermal LD_{50} in rats is 1,600 mg/kg (**Occupational Health Services, 1993**), and in rabbits is > 2,000 mg/kg (**Meister, 1992**) or > 4,800 mg/kg (**Hays and Laws, 1990**). The pyrethroids are widely used because of their general low toxicity to birds and mammals. However, they are highly toxic to aquatic organisms and fish as well as to bees - with the same mode of action in each organism. The LC_{50} values for small fish and other aquatic organisms typically lie below 1 µg/l, and the LD_{50} value for bees is 0.03 - 0.12 µg/kg. For use with conventional hydraulic sprayers, buffer

zones of 16-24 m are needed to reduce mortality of butterflies in the surroundings (**Davis *et al.*, 1993**).

Cypermethrin - Embryotoxic and Teratogenic Studies

There is limited data on the developmental effects of Cypermethrin. Cypermethrin was reported to have induced teratologic effects in chick embryos (**Anwar, 2003**) but was not teratogenic or embryotoxic to rats, mice and rabbits (**Gupta, 1990**). Cypermethrin was not teratogenic in either rats at 70 mg/kg/day or rabbits at 30 mg/kg/day. No effects on reproductive performance were seen in a 3-generation reproduction study on rats administered 10 mg cypermethrin/kg diet (**EPA, 1989**).

Combination of Chlorpyrifos and Cypermethrin

The organophosphates and pyrethroids both have ester bonds and hence are rapidly hydrolysed by animal esterases: phosphoric triester hydrolases, paraoxonase and carboxylesterases. Cypermethrin as an individual compound is quickly metabolized in mammals. The product of hydrolysis thus formed is nonactive and rapidly excreted out of the body (**Wielgomas and Krechniak, 2007**). With the concurrent exposure of cypermethrin and organophasphate, the later causes a nonreversible inhibition of esterases, which leads to slowing down of enzyme activity responsible for cleavage of ester bonds in pyrethroid molecules (**Gaughan *et al.*, 1980; Latuszynska *et al.*, 2001**). Thus, when applied together, the organophosphates enhance pyrethroids toxicity (**Ray and Forshaw, 2000**) by blocking its hydrolysis. Therefore, combination of these two insecticides was introduced in the agricultural market for the reason that together they show a synergistic effect and also could effectively control insects that developed resistance to either of the pesticides in isolation (**Tiwari *et al.*, 2008**). A study reported by **Wielgomas and Krechniak (2007)** showed that rats on co-exposure to cypermethrin and chlorpyrifos inhibited the hydrolysis of cypermethrin, which, in turn, caused an increase in cypermethrin content in the tissues. Earlier reports by **Deacon *et al.* (1980), Gupta (1990), Muto *et al.* (1992), Roy *et al.* (1998), Tian *et al.* (2005), Farag *et al.* (2007), Ahmad and Asmatullah, (2007), and Slotkin *et al.* (2008)** highlighted the teratogenic potential of chlorpyrifos or cypermethrin individually. But the teratogenic and embryotoxic potential of the combination of these two insecticides has not been studied so far with the avian embryonic model. Moreover, with regard to the fact that, in nature, the food chain is often contaminated by more than a single type of these

toxicants due to their variable utility in agricultural fields and household, it was felt crucial to select combination pesticides for the study.

TRACER (SPINOSAD 45% SC)

The other test chemical chosen was Spinosad available in the market as 45% suspendable concentrate (SC). Spinosad is a new insect control agent that is derived from a fermentation product of a naturally occurring soil actinomycete bacterium, *Saccharopolyspora spinosa*. It comprises a mixture of spinosyns A and D and is the common name of the active ingredient that is present in Tracer Naturalyte (**Mertz and Yao, 1990**). It is effective against controlling a variety of insect pests (**Sparks *et al.*, 2001**) by excitation of nervous system consistent with activation of nicotinic acetylcholine receptors, along with effects on γ- amino butyric acid receptor function (**Hanley *et al.*, 2002**). The successful introduction of Spinosad into the agricultural market place represents an important milestone in the use of natural products for commercial pest.

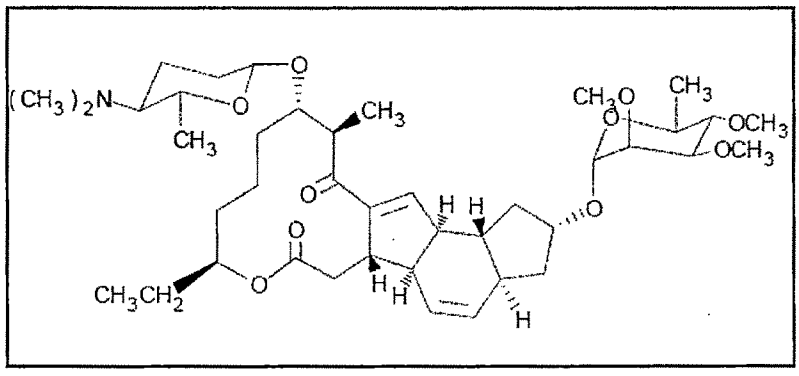
Spinosad – Physical and Chemical Properties

Chemically, spinosyns are macrocyclic lactones with two sugars attached one to the lactone ring and the other to a complex 3-ring structure. Spinosyn D has one more methyl group than Spinosyn A. The name Spinosad is derived from combining the characters from spinosyn A and spinosyn D. The material is a mixture of about 85% Spinosyn A and 15% Spinosyn D with other spinosyns as minor impurities.

Spinosyn A is: 2-[(6-deoxy-2,3,4-tri-O-methyl-*alpha*-L-mannopyranosyl)oxy)-13-[(5-dimethylamino)tetrahydro-6-methyl-2H-pyran-2-yl)oxy)-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a, 16btetradecahydro-14- methyl-1H-as-indaceno(3,2-d)oxacyclododecin-7,15-dione.

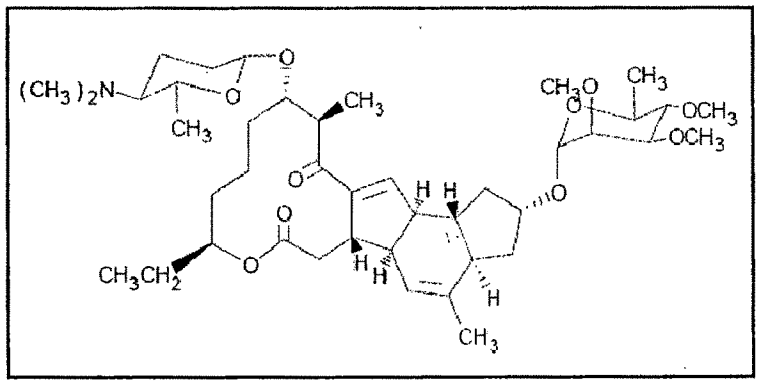
Spinosyn D is 2-((6-deoxy-2,3,4-tri-o-methyl-*alpha*-L-mannopyranosyl)oxy)-13-((5-(dimethylamino) tetrahydro-6-methyl-2H-pyran-2-yl)oxy)-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16 btetradecahydro-4,14-dimethyl-1H-as-indaceno (3,2-d) oxacyclododecin-7,15-dione (**Dow 1997; Jacheta 2001**). Technical Spinosad contains 90% spinosyns and about 10% residual materials from the fermentation broth.

Figure 1.3. Chemical Structure of Spinosyn A



Spinosyn A: R= H, Empirical Formula $C_{41}H_{65}NO_{10}$; Molecular weight - 731.98

Figure 1.4. Chemical Structure of Spinosyn D



Spinosyn D: R= CH_3, Empirical Formula $C_{42}H_{67}NO_{10}$, Molecular weight - 745.99

Spinosad - Mode of Action

Spinosad demonstrates both rapid contact and ingestion activity in insects, but especially by ingestion, which is unusual for a biological product. It is not a plant systemic, but will penetrate the leaves. Thus, it is active against leafminers and has activity against flies and thrips. The addition of a penetrating surfactant increases translaminar movement and activity on pests that mine leaves **(Larson, 1997)**.

The mode of action of Spinosad (although not fully elucidated) is characterized by excitation of the insect nervous system, leading to involuntary muscle contractions, prostration with tremors, and paralysis. These effects are consistent with the activation of nicotinic acetylcholine receptors and prolongation of acetylcholine responses through a novel mechanism **(Dow AgroSciences, 2001).** Apart from altering the nicotinic currents, electrophysiological evidences indicate that spinosyns can disrupt the function of γ-aminobutyric acid (GABA) receptors of small neurons from the central nervous system **(Sparks *et al.*, 2001)**. The loss of GABA receptor function coincides with the initiation of relatively small amplitude of chloride conductance. However, spinosyns were not shown to affect the binding of either nicotinic or GABA receptor radioligands **(Salgado *et al.*, 1997)**. These results suggest that the spinosyns can affect the nicotinic/GABA receptors through an undetermined mechanism that may underlie their insecticidal property.

Spinosad - usage

Spinosad is applied to plants at a concentration of about 540g/ha **(Jachetta, 2001)**. Spinosad is approved for use on more than 100 crops, including apples, almonds, citrus, eggplant, tomatoes, and cotton against Pests - Bollworms and Budworms, cotton bollworms and budworms have developed resistance to many standard commercial insecticides **(Kirk *et al.*, 1998; Dow AgroSciences, 2001).** These insects infest over 75 percent of the cotton crop in United States.

Cotton treated with Spinosad showed mild damage by bollworm and budworm than plots treated with the other pesticides **(Dow AgroSciences, 1997; Kirk *et al.*, 1998)**. Larvae found on cotton treated with standard insecticides were more mature, suggesting that Spinosad prevented small larvae to grow into larger and causing more damage. Findings also suggest that some larvae were probably resistant to the standard insecticides. Experiments established that Spinosad is non-toxic to most beneficial insects. Many lady beetles and pirate bugs were found on cotton treated with Spinosad than with the standard insecticides **(Dow AgroSciences, 1997)**.

Use of Spinosad in conventional agriculture started with applications of the Tracer formulation on cotton in 1997. It was applied for caterpillars in cotton, especially in situations where the caterpillars were resistant to pyrethroids or other broadspectrum insecticides **(Brett**

et al., **1997)**. Due to its low mammalian toxicity and perceived low impact on the environment, EPA registered Spinosad as a reduced-risk material **(EPA, 1997; Dow, 2001; Jachetta, 2001)**.

Studies conducted show that it exhibited little tendency for detrimental actions such as foliar sprays of fertilizer, administration of sulphur, and other agronomic interactions **(Jachetta, 2001)**. However, adverse impacts against beneficial organisms are a potential concern. Fresh sprays could kill honeybees, trichograma and other parasitoids **(Brett *et al.*, 1997; Suh *et al.*, 2000; Tillman and Mulrooney, 2000)**.

Spinosad is especially effective for caterpillars in cotton, loopers in cabbage, leafminers in various crops, leafrollers on apples, thrips in citrus, etc. It does have activity for thrips, flies, and the larval forms of some beetles that eat lots of foliage. Spinosad has been found to provide effective control of pests in the insect orders Lepidoptera, especially the tobacco bollworm (*Heliothis virescens*), the cotton bollworm (*Helicoverpa zea*), American bollworm (*H. armigera*) and armyworms (*Spodoptera spp.*).

Spinosad - Metabolic and Environmental Degradation

Investigations on *Heliothis virescens* larvae (tobacco budworm) showed that spinosyn A is one of the slowest in penetration speed during the first few hours after application. However, once inside the body, it is not readily metabolized. This apparent lack of metabolism may, in part, compensate for slow rate of penetration and, thereby, contribute to the high level of activity **(Sparks *et al.*, 2001)**. Animal metabolism studies by oral dosing of Spinosad in milking goats and laying hens showed that a considerable amount of residue, 45% for spinosyn A and 20% spinosyn D was found in tissues and milk, predominantly in fat. The metabolites were characterized without being fully identified. Metabolites Met A-Li-3, Met A-Li-4 and Met D-Li-3 were shown to be hydroxylated in the macrolide ring between C9 and C14 **(Rainey, 1996)**.

Spinosad presents a favourable environmental profile. It does not leach bioaccumulate, volatilize, or persist in the environment. The degradation of Spinosad in the environment occurs through a combination of routes, primarily photodegradation and microbial degradation to its natural components of carbon, hydrogen, oxygen and nitrogen. Spinosad will degrade photochemically when exposed to light after application. The half-life of

Spinosad degraded by soil photolysis is 9-10 days. It is less than one day for aqueous photolysis and leaf surface photolysis results in a half- life of 1.6 to 16 days. The half life of Spinosad degraded by aerobic soil metabolism in the absence of light is 9-17 days. Hydrolysis does not contribute significantly to degradation as Spinosad is relatively stable in water at a pH of 5-7 and has a half life of at least 200 days at a pH of 9.

Soil microbes demethylate both spinosyn A and spinosyn D, giving these compounds half-lives of about 9-17 days. Spinosyn A is converted to spinosyn B, which is then hydroxylated. Spinosyn D is converted to N-demethylated spinosyn D, which is hydroxylated. Although spinosyns A and D degrade quickly, spinosyn B produced from the degradation of spinosyn A can persist 4 months later under certain field conditions **(Hale and Portwood, 1996).**

Spinosyn B is almost as insecticidal as spinosyn A **(Hale and Portwood, 1996; Crouse *et al.*, 2001)**. About half of the spinosyn D remains as demethylated metabolite up to 4 months. A maximum of 20% of spinosyn A totally degrades to CO_2 after one year **(Hale and Portwood, 1996)**. Soil microbes degrade Spinosad into other spinosyns that are more persistent and are biologically active. Repeated applications could lead to some build-up of spinosyns in soil, though the original material is rather quickly degraded.

Spinosyn A is more water soluble than the other component of Spinosad. Spinosyn A and its soil metabolites bind to soil and have low soil mobility. No degradation products were found in soil below 24 inches **(Saunders and Brett, 1997).**

Spinosad is quickly converted to degradation products by sunlight on leaf surfaces. Half-lives for spinosyn A were 1.6 to 16 days depending on the amount of sunlight received **(Saunders and Brett, 1997)**. When Spinosad is applied to water, very little hydrolysis occurs, and the substance can be persistent. In the absence of sunlight, half lives of spinosyn A and D are at least 200 days. In water exposed to sunlight, photodegradation occurs **(Saunders and Brett, 1997).**

Spinosad-Toxicity Studies

Spinosad has low acute toxicity in rats. The oral LD_{50} in male rats is 3,738 mg/kg. The oral LD_{50} in female rats is >5,000 mg/kg. **(Dow, 1997; EPA, 1997; Jachetta, 2001)** and, rat inhalation LC_{50} is 5.18 mg/l air. Spinosad is rapidly absorbed and extensively metabolized in

a rat. Within 48 hours of dosing, 60-80% of Spinosad or its metabolites are excreted through urine or faeces **(Dow, 1997; EPA, 1997).** The rabbit dermal LD_{50} is >5000 mg/kg. It does not cause significant dermal or ocular irritation in rabbits, and is not a guinea pig skin sensitizer. It is metabolized and excreted fairly quickly by mammals. Within 48 hours of dosing, 60-80% of Spinosad or its metabolites are excreted through urine or faeces **(EPA, 1997; Dow, 1997).** However it does show a tendency to accumulate in fat.

Spinosad shows slight toxicity to birds, moderate toxicity to fish and slight to moderate toxicity to aquatic invertebrates. It is highly toxic to bees and Oysters and other marine Molluscs **(EPA, 1997; DOW, 2001).** Spinosad demonstrated safety for earthworms. The LD_{50} for earthworms was greater than 970 mg/kg **(Jachetta, 2001).**

Spinosad - Embryotoxic and Teratogenic Studies

Dietary administration of Spinosad to rats at a dosage of 100 mg/kg/day over two generations produced parental toxicity and effects on the offspring **(Hanley Jr. *et al.*, 2002; Thompson *et al.*, 2000).** However, developmental toxicity studies with Spinosad in rats and rabbits showed no evidence of developmental effects even at dosages that produced maternal toxicity **(Breslin *et al.*, 2000).**

Considering the fact that the xenobiotics at their lowest level of exposure during critical windows of development may induce developmental defects and that studies on Spinosad testing its embryotoxicity are very meagre, a necessity was felt to evaluate the same.

CHICK EMBRYO AS A MODEL FOR EMBRYOTOXIC AND TERATOGENIC STUDIES

The chick embryos have been a standard animal model for embryonic development, for close to a century. Comprehensive descriptions of chick development are widely available **(Patten, 1948; Hamburger and Hamilton, 1951; Lillie, 1952).** For more than half of the past century chick embryo has been consistently used as a major model for understanding mechanisms underlying nervous system development. In the past few decades several investigators began using chick embryos as a model for toxicant-induced teratogenic studies. **McLaughlin *et al.* (1963)** published a toxicological survey of chemical compounds that can affect the chick embryonic development. Several other studies have been conducted to evaluate the chemical

induced teratogenicity in chick embryos **(Hoffmann and Campbell, 1978, Jelinek and Marhan, 1994).**

The method is advantageous over the *in vivo* system by offering an elimination of the maternal influences such as biotransformation of the compound, which may alter the injected parent compound before it reaches the embryo. Moreover, the developing embryo in the egg carries a complete set of developing morphogenetic system and manifests an advantage over in vitro systems, which have limited survival **(Kotwani, 1998).**

Chick embryos were chosen to conduct teratogenicity studies because they are easily obtainable relatively inexpensive when compared to other vertebrate and/ mammalian models. It is easy to expose, incubate, and handle; each of the embryo can be counted individually as a test unit rather than in a group such as a litter; and each of them can be dosed individually, making it easier to control the delivered dose.

They are fairly non-threatening to the animal rights community because of the widespread use of chickens in agriculture.

In short, the chick embryo model provides a convenient and inexpensive system in which one can apply modern experimental tools to ascertain how a teratogen interferes with specific mechanisms that underlie organogenesis and morphogenesis.

OBJECTIVE OF THE STUDY

Ever since the introduction and usage of a multiple variety of chemicals for manifold purposes, there also happened an indiscreet accumulation in the environment and the food chain as well. This condition has gradually peaked up causing undue consequences towards the health and sustenance of humans as well as the other forms of life. The situation future worsens where the effects are silently passed on to the succeeding generations. Often the embryonic development goes vulnerable to the toxic effects of these pollutants at far lower levels, which do not manifest any systemic toxicity in the parent. During very early embryogenesis, cells multiply at a rapid rate and are relatively undifferentiated; exposure to a variety of agents during this time tends to result in death or compensation and continued normal development. Exposure to a variety of agents during this period has been shown to cause major structural defects, as well as death, growth retardation, or postnatal functional

changes. So understanding the outcomes of these early exposures is very important. Hence the present study was focused towards evaluating the embryotoxic and teratologic manifestations of two diverse pesticides using chick embryos as the model of study. The first pesticide is a combination of chlorpyrifos and cypermethrin. The rationale behind the selection of the present test article is that, chlorpyrifos and cypermethrin are amongst the most frequently used insecticides; they are used as individual products as well as combination. There are possibilities that the food chain be contaminated by more than a single type of pesticide. Therefore, a commercially available combination of these two most frequently used insecticides was chosen for the study. The second class of insecticide is a naturalyte product and amongst the most recently introduced insect control agents. WHO has classified Spinosad as a reduced risk agent. In spite of the fact that Spinosad is a reduced risk insecticide, it is a neurotoxicant by nature and has not been tested for embryotoxicity to chick embryos. Therefore, Spinosad was chosen for the study.

In an effort to evaluate the possible embryotoxic and teratologic effects of the concerned insecticides, the following parameters were assessed and presented in the following chapters:

Chapter 1: Dose range-finding studies and study of morphological and skeletal malformations: as a preliminary investigation the range study of the insecticides was performed, the LD_{50} values of the two test chemicals was calculated and the growth and development at different stages of incubation and post hatch were monitored among the controls and treated embryos and chicks.

Chapter 2: In-ovo exposure to insecticide formulations: Signs of toxicity during embryonic development - marking the changes in Chapter 2, to understand the early changes happening in the embryos, the embryos at various stages of development were examined for cell death, neural tube defects, the neural crest cell movement and *sonic hedgehog* (*shh*) protein expression.

Chapter 3: Haematological and biochemical changes in pesticide intoxicated day old hatchlings and early embryos - the day old hatchlings and eight day embryos were evaluated for certain haematological and biochemical parameters.

Chapter 4: Evaluation of pesticide induced developmental immunotoxicity in RIR chicks – a week after hatching, the chicks were examined to test their innate immunity.

Chapter 5: Evaluation of genotoxic potential using comet assay and micronucleus test - to examine if the concerned insecticides lead to any damage at the genetic level, the Micro Nucleus Test and COMET assay were performed.